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Genetic and Pharmacological Approaches to Preventing Neurodegeneration

Marco Boccitto

University of Pennsylvania, mboccitto@yahoo.com

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Genetic and Pharmacological Approaches to Preventing Neurodegeneration

Abstract
The Insulin/Insulin-like Growth Factor 1 Signaling (IIS) pathway was first identified as a major modifier of aging in C.elegans. It has since become clear that the ability of this pathway to modify aging is phylogenetically conserved. Aging is a major risk factor for a variety of neurodegenerative diseases including the motor neuron disease, Amyotrophic Lateral Sclerosis (ALS). This raises the possibility that the IIS pathway might have therapeutic potential to modify the disease progression of ALS. In a C. elegans model of ALS we found that decreased IIS had a beneficial effect on ALS pathology in this model. This beneficial effect was dependent on activation of the transcription factor daf-16. To further validate IIS as a potential therapeutic target for treatment of ALS, manipulations of IIS in mammalian cells were investigated for neuroprotective activity. Genetic manipulations that increase the activity of the mammalian ortholog of daf-16, FOXO3, were found to be neuroprotective in a series of in vitro models of ALS toxicity.

The small molecule Psammaplysene A (PA) is known to increase the nuclear abundance of FOXO3. PA was also found to be protective in mammalian in vitro models of ALS toxicity as well as a fly and worm model of neurodegeneration. Due to the wide variety of neurodegenerative diseases that share aging as a risk factor, a small molecule modifier of FOXO/daf-16 such as PA could hold great therapeutic potential. Most clinically viable drugs have certain physico-chemical properties that fall within a well-defined set of values, which unfortunately PA does not share. Due to its poor "drug-likeness", an investigation into the mechanism of action of PA was undertaken in order to potentially identify more "drug-like" compounds with similar activities.

This investigation revealed the heterogeneous nuclear ribonucleoprotein K (HNRNPK) is a direct physical target of PA. PA modifies the ability of HNRNPK to stabilize rRNA but does not affect many of HNRNPK's other functions. How changes in rRNA stability modify IIS or whether these changes definitively underlie PA's neuroprotective mechanism remains to be determined.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Neuroscience

First Advisor
Robert G. Kalb

Subject Categories
Neuroscience and Neurobiology

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/494
GENETIC AND PHARMACOLOGICAL APPROACHES TO PREVENTING NEURODEGENERATION

Marco Boccitto

A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2012

Supervisor of Dissertation

____________________
Robert Gordon Kalb, MD
Professor of Neurology

Graduate Group Chairperson

____________________
Joshua I. Gold, PhD
Associate Professor of Neuroscience

Dissertation Committee

Todd Lamitina, PhD  Assistant Professor of Physiology
Amita Sehgal, PhD  John Herr Musser Professor of Neuroscience
Harry Ischiropoulos, PhD  Research Professor of Pediatrics
Diane E. Merry, PhD  Associate Professor of Biochemistry and Molecular Biology
GENETIC AND PHARMACOLOGICAL APPROACHES TO PREVENTING NEURODEGENERATION

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To

My wife, Baley for her loving support

And

My Parents for supporting my academic ambitions
ABSTRACT:
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Marco Bocitto
Robert G. Kalb

The Insulin/Insulin-like Growth Factor 1 Signaling (IIS) pathway was first identified as a major modifier of aging in C.elegans. It has since become clear that the ability of this pathway to modify aging is phylogenetically conserved. Aging is a major risk factor for a variety of neurodegenerative diseases including the motor neuron disease, Amyotrophic Lateral Sclerosis (ALS). This raises the possibility that the IIS pathway might have therapeutic potential to modify the disease progression of ALS. In a C. elegans model of ALS we found that decreased IIS had a beneficial effect on ALS pathology in this model. This beneficial effect was dependent on activation of the transcription factor daf-16. To further validate IIS as a potential therapeutic target for treatment of ALS, manipulations of IIS in mammalian cells were investigated for neuroprotective activity. Genetic manipulations that increase the activity of the mammalian ortholog of daf-16, FOXO3, were found to be neuroprotective in a series of in vitro models of ALS toxicity.

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CHAPTER 1

Background

Introduction

Aging is the greatest risk factor for the development of a variety of neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS). Although numerous mutations have been identified which invariably lead to neurodegenerative disease, the onset of disease pathology typically does not occur until mid to late life. This suggests that there is an innate capacity for cells/organisms to cope with the stresses of mutant proteins associated with disease, but that these systems lose their ability to cope with toxic proteins as they age. Therefore, manipulations that prevent the changes associated with cellular/organismal aging represent potential therapeutic interventions for neurodegenerative diseases.

**Insulin Signaling Pathways:**

One of the most well characterized modifiers of longevity and aging is the Insulin/Insulin-like Growth Factor Signaling (IIS) pathway. IIS was first implicated as a modifier of longevity when it was found that *C. elegans* with a hypomorphic allele of the insulin receptor, *daf-2*, had increased activation of the downstream transcription factor, *daf-16*, and lived roughly twice as long as wild type worms\(^1\). Since this original characterization, a more complete understanding of the role of the IIS pathway in aging has emerged. Upon ligand binding, a series of downstream phosphorylation events occur which conclude in phosphorylation of *daf-16*. Phosphorylated *daf-16* is retained in the cytoplasm and is unable to execute its pro-survival
transcriptional output. The downstream kinase age-1, which is activated by daf-2, acts to promote phosphorylation of daf-16 and exclude it from the nucleus. Mutants which are null for age-1 are extremely long lived and have a lifespan approximately ten times greater than wild type worms. Nuclear localization of daf-16, although necessary, is not sufficient to promote longevity, suggesting that other signaling events need to be integrated in order for daf-16 to execute its beneficial transcriptional profile. The transcription factor hsf-1 is thought to be an important cofactor for expression of a subset of daf-16 longevity promoting genes, with overexpression of hsf-1 leading to a daf-16 dependent increase in longevity. There is also a level of cross talk between stress induced signaling pathways and IIS. The c-Jun N-terminal Kinase (JNK) which is a stress responsive kinase, directly interacts with daf-16 and leads to its nuclear accumulation. TGFβ signaling is also known to influence IIS and modify longevity in adult worms. There are two canonical TGFβ signaling pathways in the worm. One functions to regulate reproductive lifespan independently of longevity while the other functions in a daf-16 dependent fashion to significantly enhance longevity. The family of Forkhead box O (FOXO) transcription factors represent the mammalian orthologs of daf-16. The FOXO family regulates cellular processes including metabolism, stress response, DNA damage repair and cell death and has also been implicated in lifespan extension in mammals. Many of the post-translational modifications that modify the function of daf-16 have also been found to regulate the function of the FOXO transcription factors. For a complete discussion of post-translational modifications that regulate FOXO function, see appendix 1.

Interestingly, mutations resulting in increased longevity in C. elegans often increase stress resistance as well. For example, alterations in the IIS pathway that lead to increased lifespan, such as mutations in daf-2 or age-1, also increase organismal resistance to a wide
variety of stresses such as heat shock, oxidative stress, heavy metal stress, UV damage and infection. Changes in IIS have also been found to increase resistance to proteotoxic insults such as expression of: polyglutamine expanded proteins, and mutant TDP-43. Decreased IIS signaling has also been shown to be protective against amyloid beta toxicity. A cooperative effect between daf-16 and hsf-1 has been described for protection against amyloid beta toxicity, with both playing a role in reducing amyloid beta toxicity in a model of Alzheimer’s disease when IIS is reduced. The transcriptional profile of daf-16 and hsf-1 helps to explain the robust increase in stress resistance that accompanies their activation when IIS is decreased. They initiate transcription of a number of chaperones, including members of the small heat shock protein family, reactive oxygen scavenging enzymes, and anti-microbial genes. A role for reduced IIS in protecting against mutant SOD1 toxicity in the worm had not previously been described, so we investigated the potential for mutations in daf-2 that promote daf-16 nuclear localization to protect against SOD1 toxicity. The ability of reduced IIS to broadly increase stress resistance and protect against such varied insults makes this pathway of particularly broad therapeutic interest. Our work, along with other findings in the worm, suggests that genetic or pharmacological manipulations that enhance the transcriptional activity of daf-16/FOXO could beneficially modify disease progression in numerous neurodegenerative diseases.

Lifespan extending alterations in IIS signaling are mediated cell nonautonomously. Mosaic analysis of worms with a hypomorphic allele of daf-2 revealed that reductions in IIS are not required throughout the whole organism to promote longevity. Rather, reduced IIS in specific cells leads to a signaling cascade which coordinates aging throughout the worm. A specific subset of neurons, as well as the intestine, were strongly longevity promoting when IIS
was reduced in these cells. Drosophila FOXO has also been shown to regulate the expression of insulin responsive genes in a non-cellautonomous fashion\textsuperscript{18,19}. While descriptions of cell nonautonomous effects of reduced IIS on longevity exist, similar studies have not been undertaken to describe cell autonomous/nonautonomous changes in stress resistance under conditions of reduced IIS. In order to explore whether reduced IIS signaling functions in a cell autonomous or cell nonautonomous fashion, we employed a worms strain with a genetic background that increases the effectiveness of feeding RNAi in the nervous system. Our work suggested that reduced IIS in the periphery had little or no effect on SOD1 toxicity the nervous system. A thorough description of cell autonomous/nonautonomous changes in stress resistance after reduced IIS may be of clinical importance. When designing therapeutics based on decreased IIS activity it will be important to know which cell types should be targeted in order to elicit the maximal increase in stress resistance.

Although the worm has become a favorite tool for the study of longevity and aging, due to its relatively short lifespan and ease of genetic manipulation, the IIS pathway is an evolutionarily conserved regulator of longevity. As in \textit{C. elegans}, mutations which lead to decreased IIS signaling, either through reduced activity of the drosophila insulin-like receptor InR or reduction of its ligand chico, result in lifespan extension\textsuperscript{20}. dFOXO, the drosophila ortholog of \textit{daf}-16 has also been shown to extend lifespan when over expressed in the adult pericerebral fat body\textsuperscript{19}.

Regulation of longevity by the IIS pathway is conserved in mammals as well. A number of transgenic mice have been generated that have reduced IIS, including: fat specific insulin receptor knockout, insulin receptor substrate -/-, IGF-1R +/- and klotho over expression (klotho
is a circulating factor which represses signaling from insulin and IGF receptors), all of these mice have been found to have increased lifespan\textsuperscript{21-24}. Another interesting line of support for IIS regulating aging in mice comes from four different naturally occurring long-lived mouse strains. The Ames dwarf, Snell dwarf, Laron dwarf and Little dwarf mice all have significantly increased lifespans and all have very low levels of circulating IGF-1\textsuperscript{25}. Correlations between reduced IIS and longevity have even been observed in human cohorts. An association between longevity and decreased IGF-1R activity was reported in a cohort of Ashkenazi Jewish centenarians\textsuperscript{26}. Similarly, mutations in the FOXO1 and FOXO3 transcription factors have been reported to correlate with longevity in other long lived human cohorts\textsuperscript{27-30}. Taken together these data suggest that the IIS pathway is an evolutionarily conserved regulator of longevity and aging. The conserved nature of the IIS pathway suggests that findings from \textit{C. elegans} regarding effects of IIS on SOD1 toxicity may be of therapeutic interest in humans as well.

Figure 1.
Figure 1. The DAF-16/FOXO transcription factors act as an integrator of signals mediated by IIS, stress responsive pathways, germline signaling and changes in metabolism in order to coordinate control of longevity and aging.

**Amyotrophic Lateral Sclerosis**

Amyotrophic Lateral Sclerosis (ALS) is a progressive degenerative disorder of upper and lower motor neurons that results in progressive paralysis and finally death from respiratory failure. The incidence of ALS is approximately 2 per 100,000 individuals with more men affected than women. Average age of onset is between 55-60 years and after the onset of symptoms average survival is only 3 years. While the earliest and most overt symptoms of ALS affect motor neurons, it is becoming apparent that ALS is not a pure motor neuron disease. Recent
findings have demonstrated involvement of sensory neurons as well as spino-cerebellar pathways in ALS pathology, particularly in certain subsets of patients with frontotemporal dementia\textsuperscript{31,32}. Approximately 10% of ALS cases are familial (fALS) and mutations in 15 genes, as well as a hexa-nucleotide repeat expansion of an intron in C9orf72, have been associated with fALS\textsuperscript{33}. Despite the identification of numerous fALS associated genes, no clear pathogenic mechanism has yet been identified.

**Potential Mechanisms of Toxicity in ALS**

**Damage from Reactive Oxygen Species**

One potential cause of toxicity in ALS is ROS. ROS became a prime candidate for toxicity in ALS when the first genetic linkages to fALS were identified as mutations in SOD1, a Cu/Zn superoxide dismutase\textsuperscript{34}. SOD1 acts to convert harmful ROS into H\textsubscript{2}O\textsubscript{2}, thus protecting the cell against ROS damage. Elevated levels of oxidative damage to lipids, proteins, DNA and RNA have all been reported either in post mortem tissue from ALS patients or in mouse models of ALS. Although ALS associated mutations in SOD1 have been found in all five SOD1 exons\textsuperscript{35} these mutations do not seem to cause toxicity through a loss of function mechanism. In mutant SOD1 mouse models of ALS, an fALS associated SOD1 mini-gene is inserted into the mouse genome. This results in mutant human SOD1 being expressed under the control of its endogenous promoter. These mice develop a sporadic, fatal, motor neuropathy despite having fully functional mouse SOD1\textsuperscript{35}. This argues strongly for a gain of function toxicity of mutant SOD1. Mice null for SOD1 develop normally and show no sporadic motor abnormalities, again arguing against a loss of function model. ROS scavenging enzymes are known targets of *daf-16/FOXO,*
and reduced ROS damage may represent one way that genetic and pharmacological manipulations which increase daf-16/FOXO activity protects against mutant SOD1 toxicity.

**Proteotoxic Aggregates**

Like all other neurodegenerative diseases, insoluble protein aggregation is a hallmark of ALS. In a study of 102 autopsies of sporadic ALS cases with various ages of onset and clinical presentation between 1962 and 2000, all 102 cases presented with ubiquitin positive skein-like and/or spherical inclusions\textsuperscript{36}. TDP-43, SOD1 and FUS have all been identified as prominent protein constituent of inclusions in sporadic and familial ALS\textsuperscript{37-39}. Two genes linked to fALS, Ubiquilin2 and valosin containing protein, are involved in facilitating the degradation of improperly folded proteins, further supporting a link between protein aggregation and toxicity in ALS. The exact mechanism of protein aggregate toxicity remains a mystery. Much debate exists about the exact nature of the toxic species in ALS and other neurodegenerative diseases. While protein aggregates are a hallmark of neurodegenerative disease, their abundance often doesn’t correlate well with toxicity. This has led to the suggestion that large aggregated inclusions may not be the toxic species. When disease associated proteins adopt non-native conformations, they can form aggregates ranging in size from soluble oligomers all the way up to macro-aggregates, which can be viewed by optical microscopy. It has been suggested that the large protein aggregates, which are easily observable in many neurodegenerative diseases, may represent a protective mechanism (i.e. sequestration), where smaller toxic oligomers are sequestered into large aggregates by the cell in order to diminish their toxicity. The mechanism of toxicity of aggregated proteins and the exact nature of the toxic species continues to be a contentious topic in the field of neurodegenerative disease\textsuperscript{40}. We explored the capacity of
reduced IIS to modulate SOD1 toxicity and found that decreased IIS leads to an increase in the ratio of soluble to insoluble SOD1. We also found that total SOD1 burden was not correlated with impaired movement but rather that the soluble to insoluble ratio of SOD1 was a better predictor of changes in motor function. Reduced IIS elicits a significant increase in protein solubility at the whole proteome level\textsuperscript{41}. This fact coupled with the ubiquity of protein aggregates in neurodegenerative disease suggests that activation of \textit{daf-16}/FOXO may be broadly therapeutic against many proteotoxic insults associated with neurodegeneration.

\textbf{Abnormal RNA Processing}

A number of genes associated with fALS are known to play a role in RNA processing. These include TDP-43, FUS and Senataxin\textsuperscript{42}. TDP-43 is an RNA binding protein involved in translational control, RNA splicing and miRNA processing. TDP-43 is a prominent component of ALS inclusions\textsuperscript{37}. While TDP-43 is usually present in both the cytoplasm and nucleus, once TDP-43 inclusions appear in the cytoplasm there appears to be a loss of nuclear TDP-43 with TDP-43 becoming predominantly cytoplasmic and entering stress granules\textsuperscript{43}. This potentially represents a scenario where the aggregation of mutant TDP-43 results in a loss of function of wild type TDP-43 through nuclear exclusion. TDP-43 has been found to interact with roughly 30% of the mouse transcriptome. Immunoprecipitation of TDP-43 followed by deep sequencing of associated RNAs has revealed that TDP-43 preferentially binds GU rich sequences\textsuperscript{44}. This binding can occur in exons, introns and 3'UTRs but a strong bias exists for binding to introns. More specifically, intronic bindings sites that are relatively far (>500bp) from the nearest exon-intron boundary make up 82% of TDP-43 binding sites identified in adult mouse brain. After TDP-43 knockdown, mRNAs with the greatest reduction in abundance have a mean intron size 6x
greater than unaffected messages. This has led to the hypothesis that TDP-43 acts to stabilize messages with exceptionally long introns\(^45\). Interestingly this class of messages is particularly abundant in the brain and could potentially explain the susceptibility of neurons to mutations in a ubiquitously expressed gene such as TDP-43. Loss of TDP-43 also significantly effects alternative splicing, with knockdown resulting in changes in 203 alternative splicing events in the mouse brain\(^45\). While TDP-43 toxicity may be entirely dependent on changes in its own activity, TDP-43 is also known to regulate the stability and splicing of other genes associated with ALS and neurodegeneration including FUS, SMN2, progranulin, parkin and huntingtin\(^45,46\).

Fused in Sarcoma (FUS), another fALS associated gene involved in RNA processing, regulates mRNA transport, translation, and miRNA processing. Similarly to TDP-43, ALS associated mutations in FUS result in cytoplasmic localization of FUS in stress granules and the formation of FUS containing aggregates. This seems to be a result of a defect in nuclear transport caused by mutations in the C-terminal region\(^47,48\).

Hexanucleotide expansions of the first intron of C9orf72 were recently discovered to represent the most common genetic cause of ALS\(^49\). Patients with this expansion present with ubiquitin and p62 positive inclusions that are TDP-43 negative. One possibility is that like mutations of TDP-43 and FUS C9orf72 expansion causes foci of aggregated RNA binding proteins resulting in disrupted RNA processing\(^50\).

The potential involvement of abnormal RNA processing in ALS made the identification of HNRNPK as the target of Psammaplysene A (PA) all the more interesting. While our original interest in PA stemmed from the fact that it was able to activate FOXO transcription, we do not know definitively that this is its therapeutic mechanism. The fact that PA binds HNRNPK
suggested the possibility that PA is acting to correct toxicity associated with abnormal RNA processing in neurons. Alternatively it presented the possibility that there is a signaling pathway that links RNA processing with FOXO activity. Both are novel concepts that we wish to explore.

**Heterogeneous Nuclear Ribonucleoprotein K**

In our work identifying the target of PA we identify the Heterogeneous Nuclear Ribonucleoprotein K (HNRNPK) as a PA target. HNRNPK is a member of the family of heterogeneous nuclear ribonucleoproteins (hnRNP), historically characterized for their ability to bind and form a complex with heterogeneous RNA polymerase II transcripts in the nucleus. Further studies have revealed that HNRNPK is not only present in the nucleus but also functions in the cytoplasm and mitochondria. HNRNPK has a modular structure with three K homology (KH) RNA/DNA binding domains, a classical nuclear localization sequence (NLS), a K nuclear shuttling (KNS) domain which facilitates bi-directional shuttling across the nuclear membrane, and a highly unstructured protein-protein interaction domain (KI) (figure 2). It is believed that the highly unstructured nature of the KI domain allows this domain to take on various structures based on protein-protein interactions and accounts for the fact that HNRNPK has over 100 binding partners. HNRNPK is phosphorylated at numerous sites by a variety of kinases and has two distinct isoforms. HNRNPK binds tenaciously to poly-C stretches and has been shown to interact with both coding and non-coding RNAs. The ability of HNRNPK to interact with such a wide variety of proteins as well as RNA and DNA make it an excellent protein for integrating cellular signals and facilitating cross talk between environmental signals and RNA processing/transcription. HNRNPK’s role as an integrator of signaling pathways, including stress responses, made it an intriguing target of PA. Unfortunately, the broad range of HNRNPK
activities made identification of a specific PA dependent change in HNRNPK function quite challenging.

**Figure 2.**

![Figure 2](image-url)

**Activities of HNRNPK:**

**Transcriptional Control:** HNRNPK is capable of binding DNA as well as RNA and acts as a transcriptional regulator of various genes. While HNRNPK is thought to constitutively associate with a number of promoters, including c-myc, its recruitment to many sites is thought to be dependent on extracellular stimuli. For example, serum shock selectively recruits HNRNPK to the immediate early genes egr-1 and c-myc. HNRNPK plays a crucial role in execution of the p53 transcriptional profile after cellular stress. HNRNPK has been found to act as a transcriptional co-activator of p53 target genes. HNRNPK and p53 are both recruited to p53 target gene promoters in a p53 dependent manner and loss of HNRNPK is found to inhibit p53 target transcription. Interestingly, a number of genes are known to be downregulated upon cellular stress and induction of p53, but the mechanism by which p53 inhibited these genes was largely unknown. Recent findings indicate that p53 regulates the expression of a number of long intergenic non-coding RNAs (linc). Linc-p21 acts as a transcriptional repressor of genes which had previously been identified as being downregulated upon activation of p53. Interestingly, this transcriptional repression by linc-p21 is dependent upon linc-p21 interaction with HNRNPK. HNRNPK acts as a regulator of transcription and is involved in transcriptional responses to a variety of external stimuli.
**RNA Shuttling:** HNRNPK is an unusual member of the hnRNP family because unlike many other hnRNPs it is capable of shuttling in and out of the nucleus. Evidence suggests that an important function of this shuttling ability is to move mRNAs out of the nucleus and load them onto polysomes. Knockdown of HNRNPK in Xenopus embryos leads to terminally differentiated neurons with a severely disorganized axonal cytoskeleton. Immunoprecipitation of HNRNPK followed by microarray analysis of its associated mRNAs revealed that messages encoding many of the dysregulated cytoskeletal components are associated with HNRNPK. Analysis of nuclear versus cytoplasmic abundance of mRNAs revealed a specific reduction in export and polysome loading of some HNRNPK target mRNAs, suggesting a role of HNRNPK in mRNA nuclear export.

**Translational Repression:** One of the best characterized examples of HNRNPK translational control occurs in developing erythrocytes. These cells undergo a complex maturation that involves loss of both the nucleus and mitochondria. This requires them to synthesize all of the necessary mRNAs needed for their development before loss of nuclear structure. These messages then need to be controlled at the level of translational activation for the remainder of the developmental process. Loss of mitochondria is one of the last steps in erythrocyte development and occurs immediately before these cells take on a purely oxygen carrying function. 15-lipoxygenase (LOX) plays a key role in the maturational degradation of mitochondria. Temporal control of LOX translation is achieved through binding of HNRNPK to LOX mRNA. HNRNPK binds to the differentiation control element (DICE) sequence in the 3’ UTR of LOX mRNA and prevents assembly of the 80s ribosome by inhibiting joining of the 60s subunit to the 40s subunit. In a developmentally regulated fashion, phosphorylation of HNRNPK by c-src inhibits its binding to DICE and allows for translation of LOX.
**RNA Stability:** HNRNPK has recently been demonstrated to regulate the stability of rRNAs. Homeostasis of translational machinery is crucial for normal cellular function. Regulation of rRNA abundance is one mechanism by which cells are able to alter translational capacity. Degradation of rRNAs is an important cellular response to stress and dysregulation of rRNA degradation has been implicated in certain types of cancer. Under conditions of cellular stress the eukaryotic initiation factor 3 subunit f (eIF3f) interacts with HNRNPK causing HNRNPK to dissociate from rRNAs and allowing for their degradation. Loss of eIF3f or overexpression of HNRNPK leads to abnormal stabilization of rRNAs and may contribute to malignant transformation. In addition to non-coding rRNAs HNRNPK has also been found to bind and stabilize mRNAs such as collagen 1A1, 1A2 and 3A1.

**Alternative Splicing:** Many of the hnRNPs, including HNRNPK, have been found to influence alternative splicing. Analysis of the effects of 14 different hnRNPs on 56 alternative splicing events in genes associated with apoptosis identified HNRNPK and HNRNPC as having the broadest effects. Further investigation of the effects of HNRNPK on alternative splicing of apoptotic genes revealed that HNRNPK promotes the generation of the anti-apoptotic Bcl-x(L) isoform over the pro-apoptotic Bcl-x(S) isoform.

**Summary:** HNRNPK is a DNA/RNA binding protein that is capable of integrating cellular signals, particularly those involved in stress response, and effecting downstream nucleic acid based processes. Our identification of HNRNPK as the target of PA is particularly interesting because of the important role HNRNPK plays in response to stress stimuli. We monitored many of the above functions for changes after PA treatment in order identify which aspects of HNRNPK biology were being affected by PA. No previous link between HNRNPK function and FOXO
activity has been described. Previous work in the worm has shown that knockdown of ribosomal proteins can be lifespan extending in a daf-16 dependent manner. although the ability of HNRNPK to modulate translation initiation through regulation of rRNA stability suggests a potential link to aging and FOXO. No small molecules have previously been described to interact with HNRNPK.

CHAPTER 2


**Daf-2 Signaling Modifies Mutant SOD1 Toxicity in *C. elegans***

*Marco Boccitto*¹,², *Todd Lamitina*³, *Robert G. Kalb*¹,²,⁴

Author affiliations: ¹Department of Pediatrics, Division of Neurology, Children's Hospital of Philadelphia Research Institute, Philadelphia, Pennsylvania 19104, USA ²Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA, ³Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania, 19104 USA, ⁴Department of Neurology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA.

**Abstract:** The DAF-2 Insulin/IGF-1 signaling (IIS) pathway is a strong modifier of *Caenorhabditis elegans* longevity and healthspan. As aging is the greatest risk factor for developing neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), we were interested in determining if DAF-2 signaling modifies disease pathology in mutant superoxide dismutase 1 (SOD1) expressing *C. elegans*. Worms with pan-neuronal G85R SOD1 expression demonstrate significantly impaired locomotion as compared to WT SOD1 expressing controls and they develop insoluble SOD1 aggregates. Reductions in DAF-2 signaling, either through a hypomorphic allele or neuronally targeted RNAi, decreases the abundance of aggregated SOD1
and results in improved locomotion in a DAF-16 dependent manner. These results suggest that manipulation of the DAF-2 Insulin/IGF-1 signaling pathway may have therapeutic potential for the treatment of ALS.

**Introduction:** ALS is an adult onset neurodegenerative disease characterized by progressive weakness, due to dysfunction and eventual death of motor neurons. The majority of cases of ALS are sporadic but single gene mutations have been described that lead to inherited versions of the disease. These genes include SOD1, TAR DNA binding protein (TDP43), fused in sarcoma, progranulin, ubiquilin 2 and a hexanucleotide repeat expansion of a noncoding region in C9ORF72. Expression of some of these mutant proteins in model organisms has been used to successfully model ALS pathology.

Point mutations in SOD1 (e.g., G85R) are an example of a genetic cause of familial ALS that has been successfully modeled in transgenic mice and nematodes. The G85R point mutation causes a toxic gain-of-function, which in mice leads to ubiquitinated SOD1 aggregates and motor neuron death. *C. elegans* expressing human G85R SOD1 in the nervous system accumulate SOD1 aggregates and demonstrate reduced mobility compared to WT SOD1 expressing worms. The availability of numerous loss-of-function mutants affecting highly conserved signaling pathways make *C. elegans* an ideal system in which to explore the relationship between such pathways and SOD1 aggregation and toxicity in an *in vivo* setting.

Aging is the greatest risk factor for the development of ALS. The Insulin/IGF-1 signaling (IIS) pathway is a robust modifier of longevity and aging in *C. elegans*. Loss of function of the Insulin/Insulin-like growth factor receptor, DAF-2, promotes longevity via signaling cascades mediated by inhibition of the phosphoinositide 3-kinase (*age-1*) and activation of the forkhead
transcription factor DAF-16 via its nuclear localization. While nuclear localization of DAF-16 is required for it to execute its transcriptional activities, it is not sufficient to enhance longevity and stress resistance. Other pathways are known to interact with the IIS pathway and modulate stress resistance and/or aging by regulating transcriptional activity of DAF-16, without modifying its nuclear abundance. In addition to promoting longevity, loss of function alleles of daf-2 or age-1 protects the worm against exogenous stressors including heat shock, oxidative stress, heavy metal stress, UV damage and infection. The beneficial effects of reduced IIS rely, in part, on the ability of decreased IIS to activate the transcription factor DAF-16, leading to increased expression of numerous stress resistance genes, such as small heat shock proteins and reactive oxygen species scavenging enzymes. Additionally, reduced IIS also results in changes in protein glycosylation, metabolism, mitochondrial abundance and lipid biosynthesis, all of which are thought to contribute to the stress resistant phenotype of reduced IIS. Reduced DAF-2 signaling has been shown to have beneficial effects on other age related neurodegenerative diseases such as polyglutamine expansion proteinopathy and Alzheimer’s disease. The ability to easily control the IIS pathway both genetically and via RNA interference (RNAi) makes the worm an excellent system for studying the interactions between aging and the toxicity of mutant SOD1. In the present study we asked if manipulation of IIS pathway can improve the reduced mobility and insoluble protein aggregation seen in G85R SOD1 expressing worms.

Results:
Decreased crawling speed of G85R worms is ameliorated by decreased IIS signaling

We began by monitoring the average crawling speed of G85R, G85R;daf-2(e1370), G85R;daf-16(mgDf50), daf-2(e1370) and daf-16(mgDf50) worms on a bacterial lawn (OP50) at
96, 120, 144 and 168 hours after depositing eggs onto plates with bacteria (figure 1 A). Group differences were observed at 96, 120 and 144hrs post egg drop (96, 120, and 144hrs: $F_{(4,25)}=22.96$, $F_{(4,25)}=11.08$, $F_{(4,28)}=12.57$ respectively by single factor ANOVA $p<0.01$ at all timepoints). At 96, 120 and 144hrs after growth initiation, the $G85R;daf-2(e1370)$ worms crawled approximately twice as fast as $G85R$ worms ($p<0.05$ at 96, 120 and 144 hrs by Tukey’s post-hoc) while at the 168hr time point no significant difference was observed. Although not statistically significant, $G85R;daf-16(mgDf50)$ worms tended to perform worse than $G85R$ worms at all time points. These results suggest that reduced IIS ameliorates the toxic effects of mutant SOD1, and while this benefit is maintained for 144hrs of life, it can not be sustained after this point. The fact that $G85R$ worms that are also null for DAF-16 tend to perform worse than $G85R$ worms suggests that part of the worm’s endogenous response to proteotoxic insults, such as mutant SOD1, may include activation of DAF-16.

In order to control for any potential variation in locomotion and/or behavioral differences in worms with the $daf-2(e1370)$ or $daf-16(mgDf50)$ mutations, we examined the average crawling speed of worms carrying these mutations on a non-$SOD1$ background. There were no statistically significant differences between these two groups in locomotory activity at any time point observed (by Tukey’s post-hoc), suggesting that these mutations are modulating the toxicity of $G85R$ as opposed to altering other aspects of behavior or locomotion in general.

**Decreased swim speed of $G85R$ worms is ameliorated by decreased IIS signaling**

While the crawling assay allowed us to identify an improvement in the locomotory activity of $G85R$ worms when they were on the $daf-2(e1370)$ background, the inability to control for behavior during a given observation window made speed measurements variable. In order
to reduce variability we examined swimming worms, a context which elicits continual movement.

We compared the following worm strains in the swimming assay: WT SOD1, G85R, G85R;daf-2(e1370), G85R;daf-16(mgDf50), and G85R;daf-2(e1370);daf-16(mgDf50) (figure 1 B). Between group differences were detected at all timepoints by single factor ANOVA (72, 96, 120, 144, and 168hrs: $F_{(4,25)}=15.53$, $F_{(4,26)}=14.22$, $F_{(4,25)}=8.67$, $F_{(4,25)}=10.82$, $F_{(4,25)}=9.56$ p<0.01 at all timepoints). WT SOD1 worms were significantly faster than G85R worms at all timepoints (p<0.01 by Tukey’s post-hoc). G85R;daf-2(e1370) worms were also significantly faster than G85R worms at all timepoints (p<0.05 by Tukey’s post-hoc) and had mobility equivalent to WT SOD1 worms at all time points (no significant difference by Tukey’s post-hoc). Ablation of daf-16 in the G85R;daf-2(e1370) worms eliminated the observed rescue effect of daf-2(e1370) as no statistically significant difference was observed between G85R and G85R;daf-2(e1370);daf-16(mgDf50) worms (by Tukey’s post-hoc). These observations provide further data in support of the hypothesis that the daf-2(e1370) background is strongly protective against the toxicity of G85R SOD1 as assessed by locomotory function and that this protection is daf-16 dependent.

There was a trend for G85R;daf-16(mgDf50) worms to perform worse than either G85R or (G85R;daf-2(e1370);daf-16(mgDf50)) worms although it was only significant at 96hrs (p<0.05 by Tukey’s post-hoc). The observation that G85R;daf-16(mgDf50) worms tended to perform worse than G85R;daf-2(e1370);daf-16(mgDf50) worms suggests that part of the beneficial effect of daf-2(e1370) may be daf-16 independent. As in the crawling assay, the fact that G85R;daf-16(mgDf50) worms tended to perform worse than G85R worms also suggests a potential role of daf-16 in the worm’s endogenous response to proteotoxic insults. Like the crawling assay, the
swimming assay further supports the hypothesis that reduced IIS activity reduces \textit{G85R SOD} toxicity.

In order to determine the specificity of \textit{daf-2(e1370)} on SOD1 toxicity we tested the effects of \textit{daf-2(e1370)} on worms expressing \textit{TDP-43} in the nervous system (P\textit{snb-1::hTDP-43}) (Figure 1 C). Like the mutant SOD1 expressing worms, transgenic expression of TDP-43 in the \textit{C. elegans} nervous system causes locomotory defects and protein aggregation\textsuperscript{14}. In a comparison of swim speed of \textit{TDP-43} worms versus \textit{TDP-43;daf-2(e1370)} worms, we found \textit{daf-2(e1370)} improved \textit{TDP-43} induced swimming deficit (p<0.01 by t-test).
Improvement of the \textit{G85R} phenotype is dependent on decreased IIS in the nervous system.

Figure 1.

A) Videos of worms crawling on OP50 at the indicated times were taken and used to calculate worm speed using the parallel worm tracker software. B) Average swim speed normalized to size was calculated at the indicated times using the parallel worm tracker. C) Swim speed normalized to size was calculated for \textit{TDP43} and \textit{TDP43;daf-2(e1370)} worms 72hrs post egg drop.
*daf-2*(RNAi) has previously been described to mimic the longevity/healthspan promoting effects of the *daf-2(e1370)* hypomorphic allele and RNAi to *daf-16* has been shown to abrogate the benefits of loss of function of *daf-2* \(^{79}\). We next compared locomotion of *G85R* worms fed *daf-2, daf-16* or empty vector (EV) RNAi. Unexpectedly, feeding neither *daf-2* nor *daf-16* RNAi to *G85R* worms had a significant effect on locomotion (Figure 2 D,E). One possible explanation for these differences is the variable effectiveness of RNAi in the worm nervous system.

The resistance of worm neurons to RNAi can be mitigated by transgenic over expression of SID-1 in the nervous system \(^{80}\). SID-1 allows for passive cellular uptake of double stranded RNA, therefore increasing a cell's response to RNAi. SID-1 is not normally expressed in neurons, therefore one way to selectively increase the nervous systems response to RNAi is through transgenic expression of SID-1 in the nervous system of worms that are null for *sid-1* in peripheral tissues. In order to maximize the efficacy of RNAi in the nervous system of *G85R* worms we generated *Psnb-1::G85R::YFP; sid-1(pk3321)[Punc119::sid-1];Pmyo-6::mcherry* worms, hereafter referred to as *G85R;sid-1*. *G85R* and *G85R;sid-1* worms were fed *G85R-YFP* or empty vector (EV) RNAi. *G85R;sid-1* worms fed *G85R-YFP* RNAi showed decrease YFP intensity in the nervous system and a significant (p<0.05 by t-test) increase in locomotory activity compared to *G85R;sid-1* worms fed EV RNAi (Figure 2 A-B). Feeding *G85R* worms *G85R-YFP* RNAi had no effect on fluorescence intensity or locomotory activity (Figure 2 A and data not shown). These observations lead to two important conclusions 1) they confirm the increased efficacy of neuronal RNAi in the *sid-1* nervous system expressing *sid-1(pk3321)[Punc119::sid-1];Pmyo-6::mcherry* background and 2) demonstrate that the locomotory phenotype in these worms is likely to be due to mutant *G85R* SOD1 expression and not integration of the transgene into a critical locus.
To better understand the role of IIS in the nervous system on G85R toxicity we compared the following groups: RNAi to daf-2, daf-16 and EV fed to G85R and G85R;sid-1 worms. While G85R worms fed daf-2 RNAi showed no significant improvement in mobility compared to G85R worms fed EV, G85R;sid-1 worms fed daf-2 RNAi had significantly improved mobility compared to all other groups (ANOVA $F_{(3,16)}=13.66$ $p<0.01$ and $p<0.01$ by Tukey’s post-hoc) (Figure 2 E). This disparity between the effects on G85R versus G85R;sid1 worms suggests the need for decreased IIS activity in the nervous system in this model to rescue locomotory activity.

We also compared locomotion of G85R;daf-2(e1370) worms fed daf-16 RNAi or EV. Feeding G85R;daf-2(e1370) worms daf-16 RNAi abrogated a significant ($p<.05$ by t-test) amount of the daf-2(e1370) induced rescue of locomotory function as compared to feeding EV RNAi (figure 2 C). It is interesting that the daf-16 (RNAi) appears to diminish daf-2(e1370) rescue on a non- Punc119::sid1;sid1(pk3321) background suggesting it is working outside of the nervous system. This raises the possibility that although reduced IIS is required in the nervous system to rescue locomotory activity in this model, there are daf-16 dependent effects in non-nervous system tissues.
Figure 2.

A) YFP signal was imaged in G85R or G85R;unc119p::sid1;sid1(pk3321) worms fed empty vector (EV) or G85R:YFP RNAi in order to demonstrate the efficacy of RNAi in neurons on the unc119p::sid1;sid1(pk3321) background B-E) Average speed normalized to size of swimming worms fed bacteria expressing the indicated RNAi
IIS activity modulates the solubility of SOD1

Aging in the worm is known to be a major modifier of generalized protein solubility and aggregation, and decreased daf-2 signaling has been found to decrease the amount of aggregation that occurs\textsuperscript{41}. Since aggregated SOD1 generally correlates with toxicity in ALS we wanted to determine if the daf-2(e1370) background was decreasing aggregation in G85R worms. Zhang et. al. demonstrated that the daf-2(e1370) background significantly reduces the steady state abundance of insoluble TDP-43 in the worms we assessed in our swimming assay\textsuperscript{14}. To determine if IIS was modifying the solubility of SOD1 in this model, soluble versus insoluble fractions were prepared from G85R, G85R;daf-2(e1370) and G85R;daf-2(e1370);daf-16(mgDf50) and immunoblotted for SOD1 and actin (figure 3A). We found a significant amount of insoluble SOD1 in the G85R worms which was greatly diminished in the daf-2(e1370) background. Deletion of daf16 in the G85R;daf-2(e1370);daf-16(mgDf50) worms suppressed this effect. While the absolute values of soluble SOD1 were not constant between the three groups the ratio of insoluble to soluble SOD1 was significantly greater in the G85R;daf-2(e1370);daf-16(mgDf50) and G85R worms as compared to G85R;daf-2(e1370) (figure 3B) (p<.05 by single factor ANOVA, $F_{(2,6)}=7.719$ and p<0.05 for both comparisons by Tukey’s post-hoc). This suggested that daf-2(e1370) improves the solubility of SOD1, leading to an increased steady-state abundance of the soluble species. These data were collected from a mixed population of animals so we were interested in further characterizing SOD1 abundance at various ages.

Total SOD1 burden does not correlate with locomotor activity
While no differences in YFP intensity were evident by eye when observing the different worm strains, we wished to explore the total burden of SOD1 at various times more closely. In order to do this we used the COPAS worm sorter to measure YFP intensity and time-of-flight (TOF, i.e., length) in individual animals from a mixed population of approximately ten thousand worms from the following groups: G85R, G85R;daf-2(e1370) and G85R;daf-2(e1370);daf-16(mgDf50) (figure 3C). Using TOF as a proxy for age, we were unable to identify a direct correlation between total SOD1 burden and locomotory activity. The data were binned into TOF measurements of 100 units and significant differences between groups were observed in all bins by ANOVA (0-100 TOF $F_{(2,11369)}=881.69$, 101-200 TOF $F_{(2,7209)}=250.90$, 201-300 TOF $F_{(2,4880)}=48.1$, 301-400 TOF $F_{(2,3075)}=243.23$, 401-500 TOF $F_{(2,1435)}=411.05$, 501-600 TOF $F_{(2,708)}=277.65$). In young worms (0-200 TOF) SOD1 intensity was equivalent between G85R and G85R;daf-2(e1370) worms (no significant differences between groups by Scheffe’s post-hoc), yet significant locomotory differences were observed between these groups at all ages assessed using the swimming assay. Conversely, young G85R;daf-2(e1370);daf-16(mgDf50) worms have significantly less YFP intensity than G85R and G85R;daf-2(e1370) worms (p<0.05 by Scheffe’s post-hoc), yet they perform worse than G85R;daf-2(e1370) worms and equivalent to G85R worms in our locomotory assays. Looking at older worms (300-600 TOF), G85R worms have a total SOD1 burden that significantly exceeds that of G85R;daf-2(e1370);daf-16(mgDf50) worms (p<0.05 by Scheffe’s post-hoc in 301-400, 401-500 and 501-600 bins) yet they show similar locomotory activity in the swim test. This is unlikely to simply be a floor-type effect as G85R;daf-16(mgDf50) worms perform worse in the swimming assay than both G85R and G85R;daf-2(e1370);daf-16(mgDf50) worms, suggesting that there is room for decline in the locomotory phenotype. Taken together these results suggest that overall SOD1 burden is not responsible for the
locomotory differences observed between these groups, but rather suggest that the locomotory differences result from changes in how SOD1 toxicity is handled in these various backgrounds.

Figure 3
A) Representative western blot from three experiments looking at soluble vs insoluble SOD1 B) Quantification of SOD1 insoluble : soluble ratio C) COPAS data showing average YFP intensity for bins of various worm TOF

IIS effects on longevity in the SOD1 background
The toxicity of mutant SOD1 expression in the *C. elegans* nervous system was previously reported to have a negative effect on lifespan\(^7\). In order to determine whether decreased \( \text{daf-2} \) signaling in this background has a beneficial effect on lifespan, similar to its effect on locomotion, longevity was monitored in WT \( \text{SOD1} \), \( G85R;\text{daf-2(e1370)} \) and \( G85R;\text{daf-2(e1370)};\text{daf-16(mgDf50)} \) worms (figure 4). \( WT \text{SOD1} \) and \( G85R;\text{daf-2(e1370)};\text{daf-16(mgDf50)} \) worms had similar lifespans, while the \( G85R;\text{daf-2(e1370)} \) worms had a modest but statistically significant increase in lifespan (\( p<0.05 \) by Mann-Whitney analysis). Interestingly, there is no correlation between health and lifespan as assayed by locomotory activity.

**Figure 4**

Longevity analysis, percent alive per day

**Discussion:**
Aging is a common risk factor for many neurodegenerative diseases\(^8\). The IIS pathway is a well characterized genetic modifier of aging in *C. elegans*\(^1\) and we demonstrate here that alterations in this pathway can robustly modify the toxicity of mutant \( \text{SOD1} \) as assessed by...
mobility, protein aggregation and longevity. We find that reduced daf-2 activity has a beneficial effect on the toxicity of G85R SOD1 and that this beneficial effect is daf-16 dependent. While aging in the worm has previously been shown to be coordinated at the organismal level, we found a requirement for IIS to be reduced in the nervous system in order to modify SOD1 toxicity. Although the genetic modifiers of aging are less well characterized in humans, these data suggest that IIS and pathways which beneficially modify lifespan/healthspan in humans may be potential targets for therapeutic intervention in ALS.

Although their exact role in disease pathology is not entirely understood, aggregated proteins are associated with numerous neurodegenerative diseases, including ALS\textsuperscript{82,83}. As aging occurs, the total burden of aggregated proteins increases, suggesting a diminished capacity for proper folding and/or degradation of aggregation prone proteins with age\textsuperscript{84}. In this study we demonstrate that a LOF mutation of daf-2 diminishes the amount of insoluble SOD1; an effect that might be due to an increased capacity for clearance/folding in these worms. Similar observations have been made regarding the ability of modifications of IIS to modulate the solubility and toxicity of other disease related proteins\textsuperscript{4,12,14}. This is likely due to the ability of decreased IIS to induce expression of chaperones such as the HSP family of proteins\textsuperscript{16}. An RNAi screen performed on these worms identified chaperones as the most highly represented functional class of proteins found to negatively modify SOD1 aggregation in this model\textsuperscript{72}. Increased capacity for folding/clearance in worms with reduced IIS is likely to contribute to decreasing the toxicity of mutant SOD1 in this model. We can not rule out the contribution of other changes in metabolism, lipid biogenesis, and free radical scavenger expression which have also been linked to increased lifespan and stress resistance due to decreased IIS\textsuperscript{11,77,78}. 
Our data from the COPAS support both the concept that: 1) the daf-2(e1370) background can help reduce buildup of insoluble protein and 2) the ratio of soluble to insoluble G85R SOD1, rather than its total abundance, is associated with toxicity in this model. Using TOF as an approximation for age, it appears that G85R worms accumulate less G85R SOD1 on the daf-2(e1370) as opposed to wild type background over time. This suggests an increased capacity for clearance of SOD1 over time in the daf-2(e1370) background. The abundance of SOD1 in G85R;daf-2(e1370);daf-16(mgDf50) worms is less than both G85R and G85R;daf-2(e1370) in young animals yet their mobility is reduced compared to G85R;daf-2(e1370) and equivalent to G85R at all time points assessed. Although the relative abundance of SOD1 varies between these strains over time, their relative locomotory activity remains constant, suggesting that it is not total SOD1 abundance that dictates toxicity.

Previous work with a worm model of Aβ toxicity has demonstrated that the daf-2(e1370) allele is protective against Aβ_{1-42} aggregates in two distinct ways. First daf-2(e1370) led to activation of hsf-1 which resulted in breakdown of Aβ_{1-42} fibrils. Second daf-2(e1370) led to activation of daf-16 which increased the abundance of Aβ_{1-42} in high molecular weight aggregates. It is possible that both activities diminish Aβ_{1-42} toxicity by removing Aβ_{1-42} from the putatively toxic fibril pool\textsuperscript{15}. If these observations can be generalized, they could account for the lack of correlation between total SOD1 abundance and locomotory deficits in these worms. G85R;daf-2(e1370);daf-16(mgDf50) worms may not show the robust increase in SOD1 over time seen in the G85R worms because they are not accumulating the large aggregates of protein. These worms still suffer from SOD1 toxicity due to high levels of insoluble SOD1, but may never reach the same YFP intensity as G85R worms due to a lack of high molecular weight aggregation facilitated by daf-16. Activation of hsf-1 in the G85R;daf-2(e1370);daf-16(mgDf50) worms may
also explain their increased mobility compared to \textit{G85R;daf-16(mgDf50)} worms in the motility assay.

Using worms with neuronal expression of \textit{SID1}, we demonstrate that the beneficial effect of reduced \textit{daf-2} is likely to be mediated by decreased IIS signaling in the nervous system. This contrasts with its effects on longevity, where \textit{daf-2} RNAi (on a background lacking \textit{pk3321} to enhance neuronal RNAi) mimics the lifespan extending effects of the \textit{daf-2(e1370)} allele. In this setting, \textit{daf-2} RNAi does not influence gene expression in the nervous system\textsuperscript{17}. \textit{G85R;daf-2(e1370)} worms fed \textit{daf-16} RNAi should have normal levels of DAF-16 in their nervous system and it would be activated due to the \textit{daf-2(e1370)} background. If the beneficial effect of decreased \textit{daf-2} activity was completely mediated by the nervous system, then these worms should have comparable locomotory function to \textit{G85R;daf-2(e1370)} worms fed empty vector. We find these worms to have an intermediate phenotype with a significant reduction in locomotory activity as compared to worms on empty vector RNAi plates. This suggests that part of the beneficial effect of \textit{daf-2(e1370)} might be mediated through non-neuronal tissue(s).

Alternatively, \textit{daf-16(RNAi)} may partially, although not completely, reduce daf-16 expression in both neuronal and non-neuronal cells. Taken together, these findings suggest that although decreased IIS is required in the in the nervous system in order to have a beneficial effect on SOD1 toxicity, some of the downstream actions of reduced neuronal IIS may be functioning in the periphery.

Our results demonstrate the strong capacity of the IIS pathway to modulate G85R proteotoxicity. One possible mechanism of action for this beneficial effect is through the ability of this pathway to increase the cellular capacity to prevent toxic insoluble protein accumulation.
Interestingly this beneficial activity of IIS may not be completely cell autonomous but may be in part a manifestation of alterations in cellular aging coordinated at the organismal level. This pathway may represent a possible therapeutic target for proteotoxic diseases cause by insoluble proteins.

**Materials and Methods:**

Worm Strains and Handling: *C. elegans* were cultured under standard conditions at 20°C and fed the *E. coli* strain OP50. The following worm strains were used: *daf-16(mgDf50)* and *daf-2(e1370)* were obtained from the CGC. *Psnb-1::hTDP-43;Pmtl-2::GFP* and *daf-2(e1370);Psnb-1::hTDP-43;Pmtl-2::GFP* were a generous gift of Chris Link. *Psnb-1::G85R SOD1::YFP* and *Psnb-1::WT SOD1::YFP* were a generous gift from Arthur Horwich and Jiou Wang. *Punc119::sid1;sid1(pk3321)* was a generous gift from Martin Chalfie. Double and triple worms were generated by standard genetic crosses and verified by PCR or fluorescence expression.

Locomotory Assay: Video recordings of worms were made using the image acquisition tool in Matlab 2009b. These videos were then analyzed using the parallel worm tracker software (downloaded from the Goodman lab [http://wormsense.stanford.edu/tracker/]). For monitoring locomotion on OP50 a 1 minute video was recorded from each plate at the center of the lawn of OP50. At 50 worms were analyzed for each genotype at each timepoint. The number of worms was greater than 30 for each genotype at each timepoint. For the swimming assay worms were suspended in a pool of M9 and their swimming was recorded for 30 seconds. At least 30 worms were analyzed per group per timepoint. Statistics were performed using the average of each video as an n of 1 for speed or size/speed.
Feeding RNAi: HT115 bacteria containing the indicated genes in the L4440 vector were grown overnight at 37°C in 50ug/ml ampicillin. They were then seeded on to NGM plates supplemented with 12.5ug/ml tetracycline and 4mM IPTG and allowed to grow overnight at room temperature. Gravid adults were allowed to drop eggs on the RNAi plates for 2 hrs and were then removed. Plates were then kept at 20°C until they were assayed.

Imaging: Worms were immobilized in 25mM levamisole on agar pad slides and then coverslipped. Images were acquired at a constant intensity on a confocal microscope using a 40uM Zstack.

Soluble vs Insoluble Protein Assay: Approximately 100ul of packed worms were lysed via sonication in 300ul RIPA buffer (150mM NaCl, 50mM Tris pH 8.0, 1mM EGTA, 5mM EDTA, 1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS) with complete protease inhibitor cocktail. A soft spin of 800g for 5 min was performed to remove unlysed worms and large debris from the lysate. The supernatant was then spun at 99,000 x g for 30min @ 4°C. The supernatant was kept as the soluble fraction. The pellet was sonicated again in RIPA as a wash step to ensure removal of all soluble protein. It was centrifuged again at 99,000 x g for 30min @ 4°C. The pellet was then solublized in 50ul urea buffer (40mM Tris, 7M urea, 2M thiourea, 1% CHAPS). Equal volumes of sample were run on SDS-page gels under reducing conditions and probed with anti SOD1 antibody (Cell Signaling #2770) and anti actin (Sigma). Westerns were visualized using the Odyssey system and quantified using ImageJ.

COPAS: COPAS was used to sort and collect fluorescence intensity from 10,000 worms from each group as described in 86.
Longevity Assay: An egg drop was performed on NGM plates with OP50. The lifespan assay was carried out at 20°C and worms were transferred to fresh NGM OP50 plates as necessary in order to avoid starving the animals. Each day worms were counted. FUDR was not included in this assay so during the active reproductive period of the worms they were transferred to a new plate each day.

Acknowledgments: We would like to thank Dr. David Raizen and Maria Lim for their valuable comments and assistance with C. elegans techniques on this project. We would like to thank Martin Chalfie, Arthur Horwich, Chris Link, and Jiou Wang for generously sharing worms strains.

Author Contributions: Conceived and designed the experiments: MB BK TL. Performed the experiments: MB. Analyzed the data: MB. Contributed reagents/materials/analysis tools: TL. Wrote the paper: MB.
Chapter 3

The Small Molecule Psammaplysene A is Neuroprotective in Multiple Models of Motor Neuron Disease

Adapted from:

FOXO3a is broadly neuroprotective in vitro and in vivo against insults implicated in motor neuron diseases. *J Neurosci.*, 2009 Jun 24;29(25):8236-47. For complete article see Appendix 2

Abstract:

Aging is a risk factor for the development of adult-onset neuro-degenerative diseases. While some of the molecular pathways regulating longevity and stress resistance in lower organisms are defined (i.e., those activating the transcriptional regulators DAF-16 and HSF-1 in *C. elegans*), their relevance to mammals and disease susceptibility are unknown. Although the mammalian ortholog of *daf-16* has been implicated in regulating longevity in mammals varying effects have been reported regarding its ability to increase stress resistance and cell survival. Using a compound (Psammaplysene A, PA) that increases nuclear localization of FOXO3 we demonstrate that PA protects against insults associated with motor neuron disease.

Introduction:

Although motor neuron diseases due to single gene mutations are unusual (~10% of cases), the affected genes have been successfully used to model these diseases in experimental systems. Rare, genetic forms of motor neuron disease that arise from mutations in superoxide dismutase (SOD) or p150 glazed have a disease phenotype that strongly resembles sporadic ALS with lower motor neuron predominance. Another predominantly lower motor
neuron disease, called spinobulbar muscular atrophy (SBMA or Kennedy’s Disease) is due to a polyglutamine expansion in the androgen receptor. Despite identification of the “disease protein”, the underlying pathogenic mechanism(s) remain incompletely understood.

In most cases of sporadic ALS, motor neuron death is triggered by the interaction of a genetic pre-disposition and environmental factors. Genome-wide association studies have failed to reveal consistent susceptibility loci. Correlative evidence suggests that aging is a risk factor for the development of ALS as well as other adult-onset neurodegenerative disorders. Studies from a variety of experimental systems have provided insight into the genetic factors controlling aging, in particular, the insulin/insulin-like growth factor signaling pathway. In Caenorhabditis elegans, hypomorphic alleles of the daf-2 gene (mammalian homolog, insulin/insulin-like growth factor receptor) and the downstream signaling molecule age-1 (mammalian homolog, phosphotidylinositol-3′-kinase, PI3′K) promote longevity and lifespan extension. These effects require the activity of the DAF-16 transcription factor (mammalian homolog, FOXO3a).

DAF-16/FOXO3a shuttles between the cytoplasm (where it is inactive) and the nucleus in a process that is controlled by its phosphorylation state. Phosphorylation of DAF-16/FOXO3a by the PI3′K substrate kinases Akt and SGK leads to the 14-3-3 protein-dependent export of nuclear DAF-16/FOXO3a and re-entry into the nucleus requires dephosphorylation and release of 14-3-3. Within the nucleus, DAF-16/FOXO3a leads to the expression of a number of genes that have context-dependent effects on cellular physiology. Expression of a constitutively nuclear FOXO3a can promote the death of purified motor neurons and cerebellar granule cells and this has been linked to the expression of FasL. In contrast, active FOXO3a
protects a variety of quiescent cells against death evoked by oxidative stress or glucose deprivation and this has been linked to the expression of manganese superoxide dismutase (MnSOD) and catalase\textsuperscript{101,102}. Thymocyte survival and differentiation is FOXO3a-dependent\textsuperscript{103}. Differences in beneficial versus harmful effects of FOXO3a probably relate to cell-circumstance-specific level of activation and post-translational modifications\textsuperscript{104}. (For a review of post-translational modifications effecting the activity of FOXO transcription factors see Appendix 1)

Some of the stresses that contribute to motor neuron death in ALS include excitotoxicity, reactive oxygen species, accumulation of insoluble aggregates of neurofilaments, and defects in axonal transport\textsuperscript{87,105-107}. Since DAF-16/FOXO3a-dependent gene transcription, in some contexts, combats cellular stresses, we inquired whether manipulating FOXO3a nuclear abundance using the small molecule PA protected neurons from insults relevant to motor neuron diseases. We show that PA is neuroprotective across phyla and that its neuroprotective qualities are likely due to enhancement of DAF-16/FOXO3a activity.

Results:

A chemical-genetic screen reported the identification of a series of compounds that can inhibit FOXO1a nuclear export. Compounds fell into two classes: 1) inhibitors of general nuclear export machinery and 2) inhibitors specific to the FOXO1a pathway\textsuperscript{108}. We inquired whether compounds in the second class would also block the nuclear export of FOXO3a since they would be predicted (based on the results above) to display neuro-protective activity. We focused on Psammaplysene A (PA), which was isolated from a marine sponge, because it was the most
potent of the class 2 inhibitors (Figure 1A)\textsuperscript{109}. In addition PA was reported to have no effect on AKT phosphorylation suggesting it acted either downstream of AKT or in a synergistic pathway.

We began by looking at the effect of a synthetic sample of PA (for synthesis see \textsuperscript{110}) on the distribution of FOXO3a endogenously expressed by neurons. We biochemically isolated nuclei from spinal cord cultures treated with PA or vehicle (Figure 1B). Based on the distribution of the nuclear envelope protein lamin, it is clear that our subcellular fractionation procedure greatly enriched nuclei. There was an \( \sim 2.5 \) fold increase in the nuclear FOXO3a/nuclear lamin ratio in the PA treated cultures in comparison to vehicle-treated cultures. Total FOXO3a and lamin levels were unaffected by drug treatment. These observations indicate that PA promotes the sequestration of FOXO3a into nuclei.

We next tested the ability of PA to promote FOXO-dependent transcription. HEK 293 cells were transfected with a plasmid containing a minimal promoter containing 3x Fork Head Response Elements (3x FHRE) driving luciferase expression and then treated with various concentrations of PA or vehicle for 72 hours. As a control for a non-specific action of PA on transcription, a parallel set of cells were transfected with the parent plasmid (pGL3) that lacked the 3x FHRE. In all experiments, an internal control plasmid expressing \textit{Renilla} luciferase was cotransfected and all results are expressed as the ratio Luciferase/Renilla fluorescence. PA treatment led to a dose-dependent increase in luciferase signal in 3x FHRE, but not pGL3, -expressing cells (Figure 1C). As an added control for specificity we looked at the ability of PA to enhance transcription from a 3x retinoic acid response element (RARE) luciferase construct and found that PA had no effect on this promoter. These results indicate that PA can promote FOXO transcriptional activity.
To determine if PA had neuroprotective activity, spinal cord cultures were treated with the drug (10 nM) for two days and then subjected to an excitotoxic challenge (Figure 1D). The percent of KA-induced cell death was ~50% in vehicle-treated cultures (65 ± 2 versus 35 ± 3 vehicle versus KA, p < 0.01, Student’s t-test) and ~3% in PA treated cultures (67 ± 3 versus 65 ± 2 vehicle versus KA, p < 0.06, Student’s t-test) indicating that PA protected motor neurons from excitotoxic challenge. Next we looked at mutant AR proteotoxicity (Figure 1D). Significant differences between groups (F(2,6) = 18.84, by ANOVA) were found in the three-way comparison of 1) No DHT, 2) DHT + vehicle, and 3) DHT + PA). The post hoc analysis demonstrated a DHT-dependent ~25% loss of motor neurons in vehicle-treated cultures (p < 0.01) and neuroprotection in the PA treated cultures (P > 0.05 in the comparison of no DHT versus DHT + PA).

We followed up these observations by asking if PA blocked the proteotoxicity of SOD or p150\textsubscript{glued}. Spinal cord cultures were infected with HSV engineered to express the WT or mutant forms of SOD or the WT or mutant forms of p150\textsubscript{glued} and received PA (or vehicle) every other day for 4 days. The drug treatment had no effect on transgene expression (not shown). After 4 days, the cultures were fixed and motor neuron number was determined. ANOVA revealed statistically significant differences between groups in LacZ versus WT SOD versus mutant SOD (± PA) comparisons (F(5,12) = 18.41, p < 0.001) as well as LacZ versus WT p150\textsubscript{glued} versus mutant 150\textsubscript{glued} (± PA) comparisons (F(5,12) = 19.26, p < 0.001) (Figure 1E). The post hoc analysis revealed that statistically significant protection against the toxicity of mutant SOD or p150\textsubscript{glued} was conferred by PA treatment on motor neuron survival. PA had no adverse effect on survival of motor neurons expressing LacZ or wild type versions of SOD or p150\textsubscript{glued}. Thus PA is non-toxic.
on its own, but can protect against four different insults \textit{in vitro} that are directly relevant to motor neuron diseases.

\textbf{Figure 1}

In light of the neuroprotective effect of PA \textit{in vitro}, we examined the effects of the drug \textit{in vivo} in two model systems of neuronal degeneration. Expression of polyglutamine-expanded AR in the \textit{Drosophila} eye leads to DHT-dependent degeneration \cite{111,112}. We found that flies reared on food supplemented with 0.5 mM PA had a reduced degenerative phenotype when
compared with vehicle treated flies (Figure 2a, b). These in vivo results complement the observations made in spinal cord cultures from mice expressing polyglutamine-expanded AR wherein we found treatment with PA blunts DHT-dependent mutant AR toxicity.

We next asked if the neuroprotective action of PA depended on FOXO. To this end, we examined the efficacy of PA in flies that both expressed the polyglutamine expanded AR and were haploinsufficient for Drosophila Foxo (dFOXO) (Figure 2c, d). In a dFOXO-deficient background (foxo$^{BG01018}$ allele)$^{113}$, PA lost its ability to protect against DHT-dependent degeneration. To corroborate these observations, we studied two additional dFOXO loss-of-function alleles (foxo$^{21}$ and foxo$^{25}$) and one predicted loss-of-function allele (foxo$^{C01841}$)$^{114,115}$. As above, on all of these dFOXO-deficient backgrounds, PA lost its ability to protect against DHT-dependent degeneration. Using a quantitative rating score, we found that PA led to a statistically significant mitigation of polyglutamine-expanded AR degeneration but this was lost in the dFOXO haploinsufficient flies (Figure 2 bar graph). Variation in the baseline (no PA provided) level of neurodegeneration phenotype among the studied fly lines is likely due to differences in the background genotype. These observations indicate that PA confers protection against mutant AR proteotoxicity in a FOXO-dependent manner.
We developed a C. elegans model system of neurodegeneration by combining a null mutation in the glutamate transporter GLT-3 (Δglt-3) with a transgenic strain (nul5s -116) in which the glr-1 promoter drives expression of an activated form of G_{ςς} (abbreviated G_{ςς*}) and GFP in glutamatergic neurons 117. The Δglt-3; nul5s double mutants exhibit necrotic neuron
death at all stages of postembryonic development, with the strongest effect seen in
developmental stage L3. PA had a dose-dependent neuroprotective effect at the L3 stage, with
complete rescue from death using 10 nM PA (Figure 3A). This concentration of PA had no adverse effect on WT nematodes. We asked if the effect of PA is mediated by changing the
timing of neurodegeneration or by reducing it throughout development (Figure 3B). To examine this, we studied the effect of PA on the Δglt-3; nuls5 double mutants as a function of larval stage and we found neuronal death was reduced in all developmental stages, with the strongest effect observed in the developmental stages most prone to excitotoxicity. Neuron death was reduced in a statistically significant manner in larval stages L2 (3.4 ± 0.2 versus 2.5 ± 0.2 dying neurons/animals, n = 44 versus n = 49, vehicle versus PA, p = 0.006) and L3 (4.2 ± 0.2 versus 2.2 ± 0.1 dying neurons/animals, n = 63 versus n = 65, vehicle versus PA, p < 0.001) (Figure 3B).

Finally, while we showed that PA leads to accumulation of FOXO3a in mammalian neuronal nuclei, we wished to determine if the same was true in C. elegans. To this end, we studied nematodes in which a DAF-16::GFP fusion protein was expressed in body wall muscles. Addition of PA, but not vehicle, to the growth substrate led to nuclear localization of the fusion protein and quantification of the nucleus/cytoplasm ratio of DAF-16::GFP revealed a statistically significant effect of PA (1.46 ± 0.05 versus 3.44 ± 0.55, n = 36 versus n=27, vehicle versus PA, p < 0.0001) (Figure 3C). This result indicates that PA has an evolutionarily conserved capacity to promote nuclear localization of the DAF16/FOXO3a transcription factor and this is associated with resistance to necrotic neuron death.
Many post-translational modifications of FOXO3 have previously been described that modify FOXO3’s activity and subcellular localization (see appendix 1). In order to better understand how PA was influencing FOXO3 localization, we explored whether treatment with PA led to any significant changes in the activity level of some of the kinases known to phosphorylate FOXO3. In addition we monitored the abundance of 14-3-3, the protein responsible to shuttling FOXO3 into the nucleus. As seen in figure 4, no changes in phosphorylation of FOXO3 at Thr32 or Ser253 were identified. These residues play a key role in regulating FOXO3’s nuclear localization. Similarly no changes were seen in AKT or MAPK activity. Also no change in the abundance of 14-3-3 was observed. These finding suggest that PA is acting in a previously undefined manner to regulate the nuclear localization of FOXO3a.
Discussion:

Biochemical pathways that regulate longevity in yeast, C. elegans, Drosophila melanogaster and mice also play a fundamental role in resistance to stresses such as UV radiation, oxidative conditions, heat shock and misfolded and aggregation-prone proteins. Two transcription factors, Heat-shock factor 1 and DAF-16/FOXO3a, are essential mediators of this longevity/stress resistance program in nematodes. Here we show that treatment with PA enhances nuclear localization of FOXO3 and protects mammalian motor neurons in vitro from insults directly relevant to motor neuron diseases as well as abrogating neurodegeneration in two in vivo invertebrate model systems. The broad neuroprotective action of PA suggests it
acts on a phylogenetically conserved, core stress resistance pathway, most likely the *daf-16*/FOXO transcriptional pathway.

Our results suggest that the neuroprotective actions of PA are mediated, at least in part, by FOXO3a/daf-16. First, in both rat neuron cultures and *C. elegans*, application of PA promotes nuclear accumulation of FOXO3a or DAF-16. Second, PA evokes a dose-dependent increase in FOXO-dependent transcription. Third, PA-mediated protection against mutant AR-evoked degeneration is dFOXO-dependent. Even without knowledge of the direct molecular target of PA, this evidence favors the view that PA acts, at least in part, in a FOXO3a transcription-dependent manner.

The onset of many neurodegenerative diseases in adulthood raises the possibility that the causes of aging are specific contributors to disease pathogenesis. To the extent that this is true, applying our understanding of the molecular biology of longevity may lead to new types of therapy for neurodegenerative diseases. The present work on FOXO3a is an example of how manipulation of a phylogenetically conserved, longevity-promoting signaling pathway can effectively block neurodegeneration. This is particularly interesting in light of a recent population-based study of human longevity showing a strong association of FOXO3a and healthy aging. Agents such as PA which enhance the activity of daf-16/FOXO transcription factors may therefore act to repress disease progression in a wide variety of neurodegenerative diseases.

**Materials and Methods:**

Tissue Culture: Embryonic Sprague-dawley rat spinal cord neurons were grown on confluent monolayers of cortical astrocytes, as previously described. The substratum was acid washed glass coverslips when imaging was performed and Primaria tissue culture plasticware (Falcon,
Becton-Dickinson) when biochemistry was performed. Culture media consisted of astrocyte-conditioned media supplemented with 10 ng/ml CNTF, BDNF, NT 4, CT 1 and GDNF and 50% of media was replaced with fresh media every 3 days.

Male SBMA mice transgenic for the prion protein promoter-driven androgen receptor cDNA containing an expanded (112) CAG repeat tract were mated with non-transgenic C57Bl/6 female mice. Dissociated, mixed spinal cord cultures obtained from 13.5-day embryos were grown for 3 weeks in conditioned medium (conditioned on normal mouse astrocytes) containing charcoal-stripped serum to remove hormones. At 3 weeks, motor neurons were well differentiated and distinguishable from other neurons, including sensory neurons, by size and morphology. At this time, cultures were treated with indicated conditions for 7 days; cells were then fixed with 4% paraformaldehyde and immunostained using antibodies to neurofilament heavy chain (NF-H) (SMI32; Sternberger Monoclonal Inc.). Motor neurons were visualized using a Leica DMR fluorescence microscope, photographed, and analyzed using IP Labs software. Statistical analyses of results were carried out using Student's t-test (viral infections) or ANOVA (compound treatments; SigmaStat).

Recombinant HSV: cDNAs were cloned into the PrpUC amplicon plasmid to generate recombinant HSV as previously described. The titer of virus used in these studies was routinely 3-5 x 10^7 plaque-forming units/ml. The sources of constructs were: Michael Greenberg, Harvard University (HA-tagged wild type and triple mutant human FOXO3a), David Borchelt, University of Florida (WT and G85R mutant SOD), Erika Holzbaur, University of Pennsylvania (WT and mutant p150<sup>glued</sup>) and Anne Brunet (3xFHRE-luciferase).
Excitotoxicity Assay: After 14 days *in vitro* (DIV), culture media was removed (and saved) and cells were exposed to 100 µM kainic acid (KA) for 1 hour. Subsequently they were washed three times in Locke’s buffer not containing KA, the original media was replaced and incubated for another 24 hours at 37 °C in 5% CO₂ before fixation in 4% paraformaldehyde. Motor neurons were identified in mixed culture by immunostaining for nonphosphorylated neurofilaments and counting only labeled cells with cell body diameter of 25 µm or greater. We have previously validated this method as a means of specifically recognizing motor neurons (Figure 1 in 123). In experiments involving recombinant HSV, 1 µL of viral stock was added to 1mL of culture media 24 hours or more prior to the next manipulation. Tubes containing viruses were color coded so that the operator was blinded to the specific virus used.

Quantification of motor neurons: The # of immunostained cells were counted in 3 randomly selected fields/coverslip and the mean value obtained. In each experiment 3+ independent coverslips were used per condition and the results presented were obtained for 4+ independent cultures and experiments.

Immunocytochemistry: Tissue culture cells were fixed in freshly prepared 4% paraformaldehyde in 0.1 M pH 7.4 phosphate buffer for 30 minutes prior to extensive washing in phosphate buffered saline. Overnight incubation with primary antibody was performed at room temperature and after washing, coverslips were incubated with Alexafluor conjugated secondary antibody (2 – 4 hrs.). When double labeling experiments were performed, species-specific secondary antibodies with distinct emission spectra were employed. Coverslips were
washed prior to mounting in PermaFluor (Thermo Electron Corporation) and viewing on an Olympus FV300 Fluoview laser confocal microscope.

Western Blots and quantification: Cultures were lysed in NP-40 lysis buffer (1% NP-40, 40 mM Tris pH = 7.4, 0.15 M NaCl, 10% glycerol, 0.1% SDS, 0.1% deoxycholate + protease inhibitors and phosphatase inhibitors), sonicated, particulate matter removed by centrifugation and subjected to PAGE-SDS prior to transfer to nitrocellulose. Equal amounts of protein (determined using BCA reagents from Pierce) were loaded in each lane. After blocking in 5% milk in phosphate buffer saline, membranes were incubated in primary antibody overnight, washed, incubated with secondary antibody, washed and visualized to GE Healthcare (Buckinghamshire, U.K.) chemiluminescent substrate according to the manufacturers directions. Densitometric analysis of films was obtained using TINA (Isotopenmeßgeräte, GmbH) from 4+ independent experiments, the results averaged and mean values ± S.E. formed the basis of the statistical comparisons. Quantitative data on band intensity was expressed as the fold change in comparison with values of HSV-LacZ infected cultures. The displayed western blot data are representative of the results obtained from at least 4 independent cultures. In some experiments the LI-COR Odysseus system was used for visualizing and quantifying western blot bands. Secondary antibodies were from LI-COR Biosciences (IR 800 or 680 goat anti-mouse IgG or anti-rabbit IgG). The source of primary antibodies was: (Calbiochem, Oncogene Research Products, rabbit anti- MnSOD (Stressgen Bioreagents, Victoria, British Columbia, Canada), rabbit anti- FKHRL1/FOXO3a and anti-phospho FKHRL1/FOXO3a (T32) (Upstate, Lake Placid, NY), anti-phosphoFOXO3a (S253) (Abcam) and rabbit anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA).
Species-specific HRP-conjugated secondary antibodies were from Amersham (GE Healthcare, Buckinghamshire, U.K.) and Alexa 488-conjugated anti rabbit secondary antibody from Molecular Probes, Invitrogen (Eugene, OR).

Subcellular fractionation

Nuclear protein lysate was prepared using Sigma N-Xtract Tm kit. Briefly, cells collected from one 60 mm dish were suspended in 150 ul hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, and 10 mM KCl). After incubating on ice for 10 minutes, 10% IGEPA CA-630 was added to final concentration of 0.6%. Vortexed vigorously for 10 seconds and centrifuged immediately at 10,000-11,000g for 30 seconds. The supernatant is cytoplasmic fraction and the pellet is nuclear fraction.

Luciferase reporter assay

Human embryonic kidney 293 cells were grown to approximately 80% confluence and then transfected either 3x Fork Head Response Element Luciferase (FHRE), the parent vector lacking the FHREs (pGL3), or pGL3 vector containing 3x Retinoic Acid Response Element (RARE-luciferase from Addgene). In all cases, the Renilla luciferase reporter construct, driven by the thymidine kinase promoter (pRL-TK), was co-transfected as an internal control for transfection efficiency and cell death. After 24 hrs the cells were trypsinized and replated into media containing the appropriate concentration of PA or vehicle. After 48 hrs the cells were trypsinized again and replated into a 96 well plate in fresh media containing PA or vehicle (12
replicates per condition). Twenty four hours later the plates were analyzed using the Dual-Glo Luciferase Assay System (Promega) on the Xenogen In Vitro Imaging System. The ratio of Luciferase signal to Renilla signal for each well was calculated and the various treatment groups were compared to vehicle.

Study of Drosophila

*Drosophila* stocks were crossed on standard cornmeal agar media at 29°C. Food was supplemented with DHT (Steraloids) and Psammamypsene A once it had cooled to <50 °C, to final concentrations of 1 mM and 0.5 mM, respectively. Eye phenotypes of female flies of each condition were examined and blindly scored according to the criteria described with one scoring modification to increase sensitivity to differences in affected eye areas.

Flies haploinsufficient for dFOXO were generated by mating GMR-GAL4, AR52Q flies to the following fly stock lines: foxo$^{BG01018}$ (Bloomington stock 12530); foxo$^{21}$, foxo$^{25}$ and foxo$^{c01841}$ (Exelixis, Harvard).

Study of *C. elegans*

The following *C. elegans* strains were obtained from the *C. elegans* Genetic Center (biosci.umn.edu/CGC) or constructed by us: $\Delta$glt-3: ZB1096 glt-3(bz34) IV; nuls5: KP742[glr-1::gfp; glr-1::G_{ds}(Q227L) V; lin-15(+)]; $\Delta$glt-3; nuls5: ZB1102 glt-3(bz34) IV; nuls5 V. $P_{myo-3}:daf-16::gfp$ Strain ZB2283 was produced by Jian Xue and Carolina Ibanez-Ventoso by making transgenic animals carrying an extra-chromosomal array containing $P_{myo-3}:daf-16::gfp$. 

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For PA treatment, we soaked the diluted drug into standard NGM culture plates. We transferred freshly growing nematode cultures to these plates and allowed them to grow for 2 days before assessing the effect. To determine the ratio of nuclear vs. cytoplasmic DAF-16 levels we used a strain transgenic for $P_{myo-3}$::$daf$-$16$::$gfp$, where the DAF-16::GFP reporter is expressed in body wall muscle cells. We compared the intensity of GFP labeling in the nucleus and adjacent cytoplasm using NIH-Image. We monitored the effect of PA or age-$1$ on nematode excitotoxicity by measuring the extent of neurodegeneration in $\Delta glt$-$3$; $nuls5$ animals ± PA or ± age-$1$ mutation. We observed free-moving animals with an inverted scope under Nomarski DIC optics with no anesthetics. Swollen cells in the nerve-ring region were counted as head necrotic figures indicative of neurodegeneration.

Statistics. Pairwise comparisons employed two-tailed Students’s t-test and when three or more groups were compared we used Analysis of Variance (ANOVA) and post hoc analysis with significance set at $p<0.05$.

**Acknowledgments:**

We thank Dr. David Borchelt for the gift of the anti-human SOD rabbit serum and Ernst Hafen and Julia Lüdke (Institute of Molecular Systems Biology, Zurich) for the Foxo$^{21}$ and Foxo$^{25}$ fly lines. This work was supported by the U.S. Public Health Service (NS34435, MD, CA24487, JC; NS32214, DEM; NS053825, JPT and NS52325, RGK) and the ALS and Muscular Dystrophy Associations.
Author Contributions: Conceived and designed the experiments: JM-P, NN, MB, IM, SG, YL, JPT, MD, DM, RK. Performed the experiments: JM-P, NN, MB, IM, SG, WZ. Analyzed the data: NN, MB, IM, SG, WZ, JPT, RK. Contributed reagents/materials/analysis tools: JC, RN. Wrote the paper: MB, RK.

Chapter 4

Unpublished Manuscript

The Neuroprotective Drug Psammaplysene A Binds the RNA Binding Protein HNRNPK
Marco Bocchito, Nayoung Lee, Satoshi Sakamoto, Hiroshi Handa, John Clardy, Benoit Chabot, Steve Seeholzer, Robert Kalb

Abstract: We previously characterized the compound Psammaplysene A as having strong neuroprotective properties in a number of in vitro and in vivo models of neurodegeneration. Based on the physico-chemical properties of Psammaplysene A it is not considered to be drug-like. Therefore we attempted to identify the target of Psammaplysene A in order to determine its mechanism of action. Two separate methods were used in parallel to purify the protein target of Psammaplysene A from cell lysates. Mass spectrometry was employed to identify the Psammaplysene A target as the RNA binding protein HNRNPK. We investigated the effects of Psammaplysene A on a number of HNRNPK regulated processes. The most consistent observation is that treatment of cells with Psammaplysene A results in decreased abundance of rRNAs. Coupled with prior work indicating HNRNPK can stabilize rRNAs, we suggest that the ability of Psammaplysene A to decrease rRNA abundance may represent its mechanism of neuroprotection.
Introduction:

Psammaplysene A (PA) is a marine sponge derivative which was originally described in a chemical screen to identify agents that promote FOXO1 nuclear localization in PTEN deficient cancer cells\textsuperscript{109,110}. We became interested in the potential neuroprotective qualities of PA based on the role of the FOXO transcription factors as phylogenetically conserved regulators of longevity\textsuperscript{81}. Increased activity of the \textit{C. elegans} ortholog of FOXO, \textit{daf-16}, is associated with lifespan extension ranging from 2-10 fold in worms, and polymorphisms in FOXO1 and FOXO3 have been linked to longevity in human cohorts\textsuperscript{1,2,27,30}. Many long lived \textit{C. elegans} mutants also exhibit increased stress resistance, including protection against reactive oxygen species, heat stress, and proteotoxicity\textsuperscript{11,13,14}. Age is also a major risk factor for numerous neurodegenerative diseases. Based on these observations we investigated the potential of PA to act as a neuroprotective agent via activation of FOXO3. Indeed, we found that PA protects against neuronal death in numerous \textit{in vitro} and \textit{in vivo} models of neurodegeneration\textsuperscript{124}.

Unfortunately the physico-chemical properties of PA make it an unlikely candidate for further development as a therapeutic. Testing \textit{in vitro} and in small model organisms such as the worm and fly allows for the use of DMSO for drug solubalization and delivery. This allows for the characterization of compounds which would otherwise be inactive if delivered orally. Chris Lipinski and colleagues defined a set of four characteristics that suggest whether a molecule is “drug-like” based on its molecular weight (max 500 Da), Log \textit{P} (max 5), number of hydrogen bond donors (max 5) and number of hydrogen bond acceptors (max 10)\textsuperscript{125}. Each time a compound violates one of these criteria it is given a flag and the total number of flags are tallied giving a compound a score between 0 and 4. Compounds with a score greater than 1 are
considered to be of marginal value for further development\textsuperscript{126}. PA receives a score of 2 for having a molecular weight of 769 Da and a Log $P$ value of 6.57 suggesting it would be of marginal clinical relevance. Owing to PA’s powerful neuroprotective qualities that we wanted to characterize its mechanism of action, with the hope that more drug-like compounds could be found that share the same activity as PA.

Using two parallel purification methods, we generated a list of PA target candidates that consisted almost exclusively of RNA binding proteins. From this list we identified the target of PA as heterogeneous nuclear ribonucleoprotein K (HNRNPK) and confirmed saturable binding using surface plasmon resonance. HNRNPK is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. This family of proteins was historically identified for its ability to bind heterogeneous nuclear RNA produced by RNA polymerase II as part of the hnRNP complex\textsuperscript{127}. Although originally described as part of the hnRNP complex, it is now appreciated that HNRNPK is present in the cytoplasm and mitochondria, as well as the nucleus. HNRNPK participates in a wide variety of cellular functions including transcriptional control, translational control, RNA transport, splicing, chromatin remodeling and RNA stability\textsuperscript{128}. HNRNPK contains three RNA binding motifs and exhibits robust binding to poly-C regions\textsuperscript{129}. In addition to its ability to interact with RNA, HNRNPK interacts with a very large number of proteins and can receive numerous posttranslational modifications. More than 100 HNRNPK binding partners have been characterized residing in the cytoplasm, nucleus and mitochondria\textsuperscript{51}. Many of these interactions are thought to be mediated through the K protein interactive (KI) domain, which is a highly unstructured region within the protein thought to allow for a great diversity of binding partners. HNRNPK is also the target of numerous posttranslational modifications including phosphorylation and methylation which act to integrate numerous signaling pathways\textsuperscript{130-132}.
Taken together these characteristics make HNRNPK an excellent platform for integrating protein based signaling cascades with the regulation of DNA/RNA based processes, making it an intriguing target for a neuroprotective compound.

Results:

**HNRNPK is a target of Psammaplysene A based on two parallel target purification strategies:**

In order to characterize the mechanism of action of PA, we attempted to purify its target from cell lysates. Two separate but complementary approaches were used to purify the target of PA. The first approach involved a modified version of PA that allowed tagging with a biotin moiety or fluorescent probe. The second approach involved covalently linking PA to magnetic nanobeads using a flexible linker sequence.

Four different PA derivatives were generated (supplemental figure 1). We wanted to verify that the modifications made to PA did not alter its activity so we compared the activity of the four PA derivatives to PA in a forkhead response element (FHRE) luciferase assay (figure 1 A). We had previously characterized PA as significantly increasing FHRE luciferase activity with a 72hr treatment. We found that PA as well as all four PA derivatives elicited an increase in FHRE activity upon 72hr treatment compared to vehicle. Single factor ANOVA showed a significant effect of treatment on FHRE luciferase activity. Post hoc analysis showed vehicle treated cells to have significantly less FHRE activity compared to all other groups (Vehicle vs: PA, 1A, 1B, 2A, 2B $F_{(5,22)}=2.76$, $F_{(5,22)}=5.8$, $F_{(5,22)}=3.81$, $F_{(5,22)}=2.64$, $F_{(5,22)}=3.17$ respectively. $p<0.05$ when comparing vehicle versus all drug treated groups by Sheffe’s post hoc). Our original characterization of PA suggested that its neuroprotective properties were tied to its ability to...
influence FOXO activity. Since the derivative compounds retained the ability to alter FOXO activity we presumed that they were still capable of binding the target of PA. The photoactivatable crosslinker and azide groups on compound 2B provided a versatile platform to work with therefore we conducted all derivative based purification assays with compound 2B.

We determined that 2B was binding to a discrete target and that it could be displaced from that target using an excess of PA. We incubated HEK293 cell lysate and C. elegans lysate with 2B, 2B + 100x PA, or no drug. After a one hour incubation we UV illuminated the lysates to crosslink 2B to its target and then used click chemistry to label 2B containing complexes with carboxytetramethylrhodamine (TAMRA). After labeling the proteins were dissolved in loading buffer and resolved by SDS-PAGE. TAMRA labeled proteins were then visualized using in gel fluorescence scanning (figure 1 B). Three specific bands were identified in HEK293 lysates and two specific bands were identified in C. elegans lysates. Next, in order to identify the proteins in the HEK293 lysate, we incubated 2B with lysate, UV crosslinked and then used click chemistry to attach a biotin to crosslinked target-2B complexes. We then purified target-2B-biotin complexes using magnetic avidin beads. The purified proteins were digested off of the avidin beads and analyzed by LC MS/MS. We identified 37 candidate targets via this approach (figure 1 D and supplemental table 1).

As a second parallel approach we covalently coupled a PA derivative to feriteglycidyl methacrylate (FG) nano-beads. These beads are particularly well suited for this type of analysis because their small size and uniform structure creates a large surface area for small molecule presentation while minimizing non-specific interactions. FG beads were coupled to PA at two different concentrations of PA, 1X and 5X. We incubated HEK293 lysates with uncoupled FG
beads, 1X PA coupled FG beads and 5X PA coupled FG beads. Beads were washed, and then boiled in loading buffer to elute the associated proteins. Eluted proteins were run on a SDS-PAGE gel and visualized via coomassie brilliant blue staining (figure 1 C). We attempted to identify bona fide PA interacting proteins by looking for bands whose abundance increased in a dose-dependent manner. We were able to clearly identify two bands whose intensity was strongly dose dependent (black *’s figure 1 C). These bands were also of roughly equivalent molecular weight to two of the bands identified by the 2B approach. These bands were excised and submitted to LC MS/MS. One additional band was excised (red * figure 1 C) based on its molecular weight, as it roughly matched the molecular weight of the largest binding partner identified by the 2B approach. Since the PA-FG beads were not covalently linked to their target in this approach we were unable to wash as stringently as in the 2B approach. Subsequently we identified 175 proteins in the three bands that were excised (figure 1 D and supplemental table 1).
Figure 1. A) Normalized luciferase signal from Forkhead Response Element (FHRE) Luciferase after treatment with PA or PA derivative compounds. All PA derivatives retain the ability to induce FHRE activity. B) Identification of discrete protein targets of PA.
by 2B labeling (HEK lysates and *C. elegans* lysates) and C) pull down with PA-FG beads (HEK lysates). D) Representation of PA candidate list identified by two parallel approaches.

Based on our two parallel approaches we generated a list of 207 candidate targets of PA, with five proteins identified by both approaches. Although an overlap of five proteins was significantly less than we had expected, there were functional similarities in the proteins purified by the two approaches. A bioinformatics analysis of the purified proteins indicated that both approaches identified proteins which were highly enriched for RNA processing (i.e. stability, splicing, metabolism, posttranscriptional gene expression) (Table 1). This also potentially helped explain the large number of proteins that we purified. While the FG beads are designed to have very few non-specific interactions, if the target of PA is a member of a large ribonucleo protein (RNP) complex we may have pulled down a macromolecular complex of proteins interacting with the specific target of PA.

**Table 1.**

**Gene Ontology for 2B Purified Proteins**

<table>
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<th>Gene Ontology Term</th>
<th>Count</th>
<th>%</th>
<th>PValue</th>
<th>Fold Enrichment</th>
<th>FDR</th>
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<tr>
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<tr>
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<td>13</td>
<td>12.9</td>
<td>1.52E-10</td>
<td>12.74</td>
<td>2.05E-07</td>
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<tr>
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<td>9.9</td>
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<td>20.56</td>
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<tr>
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<td>10</td>
<td>9.9</td>
<td>7.75E-10</td>
<td>20.56</td>
<td>1.05E-06</td>
</tr>
</tbody>
</table>
We began screening candidates from our list with a specific focus on the overlapping proteins. Due to the large number of candidates, we adopted a multi-pronged approach to identifying the PA target. To screen candidates we obtained plasmids for recombinant expression, over expression or knockdown of candidate proteins and then screened lysates.
using the 2B TAMRA coupling assay. We screened 14 targets by this method (supplemental table 2) before identifying a positive hit. In a bacterial expression system, induction of HNRNPK expression with Isopropyl β-D-1 thiogalactopyranoside (IPTG) yielded a strong band which is not present in un-induced lysates (figure 2 A). This band has approximately the same molecular weight as the smallest band identified in HEK293 lysates.

The target purification with 2B and PA-FG beads had suggested some other characteristics of the target of PA which HNRNPK fit. HNRNPK is an RNA binding protein known to be involved in numerous aspects of RNA processing, including translation, mRNA stability, and splicing. This makes HNRNPK a good fit for the types of processes that we suspect PA is involved in based on our bioinformatics analysis of candidate proteins. Secondly HNRNPK is thought to be a “hub” protein, interacting with numerous protein partners and acting to integrate various signaling cascades. HNRNPK is known to have over 100 interacting proteins\textsuperscript{51}. This is thought to be facilitated by its highly unstructured KI domain. This breadth of protein-protein interactions might help explain the high number of candidates identified by our purification methods. An analysis of the literature suggested that of the 37 proteins identified by 2B purification followed by on bead digest 12 were previously described as HNRNPK binding partners.

**Characterization of the PA interaction with HNRNPK by Surface Plasmon Resonance**

In order to better characterize the interaction between PA and HNRNPK we attempted to demonstrate specificity and characterize the Kd of PA binding to HNRNPK by surface plasmon resonance. We began by comparing PA binding to GST-HNRNPK versus GST. We were unable to identify saturable binding in this paradigm (figure 2 B), indicative of a non-specific interaction. We speculated that because HNRNPK participates in such a wide variety of protein, RNA and
DNA binding events, many aspects of its native structure may not be recapitulated during recombinant expression and purification. This may be particularly true of domains like KI which are thought to derive their 3D structure from partner binding. Without these secondary structural features, a non-specific interaction may be occurring between PA and HNRNPK. Although it would be impractical to try and introduce all of the potential HNRNPK interacting proteins into the system to see if they allowed for specific PA binding to HNRNPK, it was feasible to introduce RNA.

We began by observing whether RNA could be stably bound to HNRNPK. We applied whole cell RNA extracts over the GST-HNRNPK and GST surfaces and found a saturable and stable binding of RNA to GST-HNRNPK but not GST alone (data not shown). We then repeated the PA binding experiment and were now able to generate a saturable binding curve (figure 2 C). In addition to the interaction now being saturable, concentrations of 50, 100 and 150uM PA elicited a significantly higher response when HNRNPK was pre-bound with RNA. This suggested that the structure of HNRNPK was modified by RNA binding in such a way that its affinity for PA was increased.

In order to control for the possibility that PA was interacting with RNA rather than RNA bound HNRNPK, and to further demonstrate specificity of PA for HNRNPK, we used a second RNA binding protein as a control. We compared PA binding to GST-HNRNPK versus GST-HNRNPI (PTBP1). We considered HNRNPI to be a good control based on the fact that it binds pyrimidine rich tracts. HNRNPK binds cytosine rich domains so there is potentially some overlap in the RNAs that the two proteins will bind. In this paradigm we were again able to show specific, saturable binding of PA to HNRNPK after saturating both proteins with RNA (figure 2 D). From
these measurements we were able to generate a binding curve and estimate the Kd of PA for HNRNPK at 166 uM. Based on our work with 2B, we suspect that this Kd is not truly indicative of PA binding to HNRNPK in vivo. We are able to visualize PA interactions with HNRNPK in bacterial lysates with concentrations as low as 100 nM and PA binding appears saturated at 5 uM in HEK293 lysates (data not shown). This discrepancy may be due to the absence of HNRNPK binding partners or lack of post-translational modifications to the GST-HNRNPK used in the surface plasmon resonance system. Never the less these data support a specific interaction of PA with HNRNPK.
Figure 2. A) 2B binding and TAMRA visualization of PA binding to HNRNPK in induced and un-induced bacteria expressing an IPTG inducible HNRNPK construct. B) Surface plasmon resonance analysis of PA binding to HNRNPK.
PA Increases the Abundance of a Disulfide Linked HNRNPK Complex

As a first step in characterizing the mechanism of action of PA we began by monitoring any changes in HNRNPK abundance after PA treatment. We were unable to identify any changes in the total abundance of HNRNPK or its nuclear versus cytoplasmic localization after PA treatment (figure 3 C). Interestingly, on non-reducing western blots, we found a high molecular weight species using anti-HNRNPK antibodies which was lost under reducing conditions (figure 3 A). The abundance of this complex did appear to be PA responsive (figure 3 B). In order to better characterize the composition of this putative HNRNPK containing complex we immunoprecipitated HNRNPK, then resolved it by SDS PAGE and stained with coomassie brilliant blue under reducing and non-reducing conditions. Using this approach, we were able to visualize the high molecular weight complex under non-reducing conditions and excise the complex as well as the corresponding area in the reduced lane and analyze them by LC MS/MS. Based on semi-quantitative analysis, the complex appeared to be made up of HNRNPK, DDX17, and Lamin B1 (supplemental table 2). The combined molecular weight of these three proteins is consistent with the molecular weight of the non-reduced HNRNPK complex. Immunoprecipitation of HNRNPK from HEK293 lysates confirmed a strong interaction between HNRNPK and DDX17 as well as Lamin B1 (figure 3 D). While PA appears to increase the abundance of this high molecular weight HNRNPK containing complex, it is unclear what role this complex plays in normal cellular function or what the functional consequences of increasing the abundance of this complex are.
Figure 3. A) Detection of a high molecular weight HNRNPK containing complex in non-reducing cell lysates B) PA treatment increases the abundance of this high molecular weight HNRNPK containing complex in a dose-dependent manner. C) PA treatment does not alter total abundance or subcellular distribution of HNRNPK. D) HNRNPK co-immunoprecipitates with DDX17 and Lamin B1

PA Has No Effect on Numerous Functions of HNRNPK Biology

HNRNPK plays a role in a multitude of biological processes. We attempted to focus our investigation on PA induced changes in HNRNPK biology based on the knowledge that PA is neuroprotective, and the observation that PA seems to increase the abundance of an HNRNPK-DDX17-LaminB1 disulfide linked complex.
HNRNPK is known to act on κB enhancer elements and affect their transcriptional activities\textsuperscript{134}. Abnormalities in NF-κB signaling have recently been implicated in forms of inherited ALS\textsuperscript{135,136} so we wanted to determine whether PA might be modifying NF-κB signaling. We used two separate NF-κB luciferase constructs to monitor possible interactions between PA treatment and NF-κB signaling. We monitored normalized luciferase activity in PA and vehicle treated cells in an IL-1 stimulated and un-stimulated state (figure 4 A). PA did not have a significant effect on either basal or stimulated NF-κB activity.

HNRNPK is known to play an important role in p53 induced apoptosis\textsuperscript{54}. Under conditions of p53 activation there is an increase in the abundance of the long intergenic non-coding (LINC) p21. LINCp21 then interacts with HNRNPK and facilitates the repression of numerous pro-survival genes, thus facilitating cell death\textsuperscript{55}. We hypothesized that PA could disrupt this interaction and thereby increase cell survival. To test this, we generated biotinylated LINCp21 \textit{in vitro}. Cells were treated with various doses of PA, lysed and then incubated with biotinylated LINCp21. Biotinylated LINCp21 was purified with avidin beads and then boiled in loading buffer to release associated proteins. We were able to clearly identify HNRNPK by western blot. All three HNRNPK bands were observed under non-reducing conditions, but there abundance was not influenced by PA treatment (figure 4 B).

DDX17 is part of the DROSHA microprocessor complex\textsuperscript{137}. Based on our observation that PA appears to increase the interaction between HNRNPK and DDX17, we wanted to know whether PA was modifying miRNA biogenesis. Interestingly other components of the microprocessor were identified in our PA target purification including: DROSHA, DDX1, DDX3X, DDX5, DHX15, HNRNPH1, HNRNU-like1 and FUS. The DROSHA microprocessor complex cleaves
pri-miRNAs into pre-miRNAs which are then exported into the cytoplasm and undergo a final
cleavage step to become mature miRNAs. As important regulators of gene expression, any
changes in miRNA abundance due to association/dissociation of HNRNPK with DDX17 as part of
the microprocessor complex could be an important function of PA. In order to explore this
possibility, cells were transfected with flag-DROSHA and treated with PA or vehicle. Cells were
then lysed and immunoprecipitated for flag tag to assess the abundance of HNRNPK and DDX17
associated with the microprocessor complex. No changes in the abundance of co-precipitated
HNRNPK or DDX17 with DROSHA were detected after treatment with PA (figure 4 C).

Both HNRNPK and DDX17 are known to be involved in alternative splicing. Venables et.
al. previously described an assay for monitoring multiple alternative splicing events of genes
involved in apoptosis and found a broad effect of HNRNPK knockdown which they were able to
replicate in multiple cell lines. We used this assay to monitor alternative splicing events under
6 conditions: 1) mock transfected with HNRNPK, 2) over expressing HNRNPK, 3) treated with PA,
4) treated with vehicle, 5) HNRNPK knockdown, or 6) HNRNPK knockdown with PA treatment.
While we identified splicing events which were influenced by HNRNPK knock down or
overexpression, PA treatment did not result in significant changes in any alternative splicing
events (figure 4 D for representative examples, see supplemental figure 2 for heatmap of entire
dataset). This suggested that PA was not acting to modulate alternative splicing.

While none of these assays is a definitive proof that the neuroprotective activity of PA is
not mediated by one of these processes, we found the lack of a robust effect in any of these
assays sufficiently compelling to suggest that the neuroprotective properties of PA are operating
through a different mechanism.
Figure 4. A) PA does not alter NFκB signaling. B) PA does not alter the association between HNRNPK and Linc p21. C) PA treatment does not alter the abundance of DDX17 or HNRNPK associated with DROSHA. D) PA treatment does not alter alternative splicing.

PA treatment decreases rRNA Abundance

A recent study in *Xenopus laevis* by Liu *et. al.* used a RIP-Chip approach to characterize RNA targets of HNRNPK in the developing *Xenopus* brain\(^56\). They identified numerous neuron
specific cytoskeletal components important for axonogenesis that interact with HNRNPK. In order to analyze whether PA was changing the abundance or distribution of any of these transcripts we treated cortical cultures with PA and then performed qPCR to monitor message abundance. We analyzed our data using the \( \Delta \Delta CT \) method for relative quantitation and found that when normalizing to 5.8s rRNA all other messages appeared to increase, while when normalizing to beta actin or GAPDH we found no change. This led us to suspect that there may be alterations in the abundance of rRNAs upon treatment with PA.

HNRNPK was recently described as playing an important role in stabilizing rRNAs against stress dependent degradation\(^6\). Another potential link between PA and rRNA abundance lies in the fact that the DEAD box RNA helicases are the largest family of rRNA processing proteins and DDX17 has been characterized as playing a role in rRNA biogenesis. We investigated this further by analyzing the abundance of 28s, 18s, 5.8s and 5s rRNAs in both cortical cultures and HEK293 cells after PA treatment (figure 5A). Interestingly we found a statistically significant interaction between message and PA treatment by two way ANOVA in both groups (p<0.01 in both groups), with a specific reduction in the 5.8s and 5s rRNAs in both cortical neurons and HEK293 cells after PA treatment (\( F_{(5,6)}=7.93 \) and \( F_{(5,6)}=11.08 \) in HEK cells and \( F_{(7,6)}=7.02 \) and \( F_{(7,6)}=6.15 \) in CT neurons respectively by Tukey’s post hoc). We did not detect a significant difference in RNA levels of HNRNPK targets identified by Liu et. al. in cortical neurons, or in beta or gamma actin in HEK293 cells. Wen et al specifically describe a role for HNRNPK in protection of rRNAs after stress. In order to determine if HNRNPK has an effect on rRNA levels in unstressed cells we transfected HEK cells with GFP or GFP tagged HNRNPK and assessed rRNA levels 48 hours post transfection (figure 5B). We found that overexpression of HNRNPK significantly increased the abundance of the 5.8s and 5s rRNAs, the same rRNAs which were negatively regulated by PA in both HEK cells
and cortical neurons (Two way ANOVA shows significant interaction of HNRNPK overexpression on rRNA abundance, p<0.01 and F(3,4)=10.57 and F(3,4)=4.06 respectively by Tukey’s post hoc).

This leads to the hypothesis that PA acts to inhibit HNRNPK dependent promotion of rRNA stability.

**PA Treatment does not Alter Global Levels of Translation**

Reductions in translation through knockdown of ribosomal proteins, rRNA processing proteins or changes in the abundance of ribosomal subunits have all been linked to lifespan extension in worms, and yeast\(^{139-141}\). This suggested that the neuroprotective properties of PA could potentially be mediated through its capacity to decrease rRNA abundance by inhibiting HNRNPK dependent stabilization of rRNA, potentially leading to decreased translation. We wanted to determine if changes in rRNA abundance that occur after PA treatment were affecting the abundance of ribosomes and/or levels of global translation. First we generated polysome profiles from cells treated with PA or vehicle (figure 5 D). An overlay of representative polysome profiles from PA and vehicle treated cells is shown as well as the quantification of six PA treated and six vehicle treated profiles. No significant changes were observed in the polysome profiles after treatment with PA. Overall abundance of monomeric 40s and 60s ribosomal subunits was unchanged, as was the abundance of the 80s dimer. No change in the abundance of polysomes was observed.

To confirm whether our polysome observations were of functional significance, we monitored global protein translation after PA treatment using [\(^{35}\)S]-methionine with cyclohexamide treated cells as a positive control. No changes in global protein synthesis were
observed after PA treatment (Figure 5 C). It is possible that modulation of rRNA abundance by PA results in changes that are subtle and below the sensitivity of this assay.

Figure 5.

Figure 5. A) PA treatment reduces 5s and 5.8s rRNA abundance in HEK293 cells as well as cortical neurons. B) Overexpression of HNRNPK increases 5s and 5.8s rRNA abundance in HEK293 cells. C) PA treatment does not decrease rate of total translation. D) PA treatment does not alter the cellular polysome profile.

PA Increases 5.8s rRNA Abundance in Stressed Cells
While the majority of our screening for an effect of PA on HNRNPK activity was undertaken in HEK293 cells, our previous characterization of PA’s neuroprotective action was characterized in mixed spinal cord cultures under conditions of stress associated with motor neuron disease. Now that we had characterized an alteration in rRNA abundance in HEK293 cells we wanted to explore PA’s activities on rRNA abundance in mixed spinal cord cultures under conditions of stress. To do this we infected cultures overnight with HSV- LacZ, WT SOD1, and MutSOD1. The next day cultures were treated with 250nM PA for 24 hours and then total rRNA was collected and analyzed by qPCR for the abundance of 5.8s rRNA. We found that when cultures expressed an innocuous gene such as LacZ, PA had no effect on rRNA abundance after 24 hours, similar to what we had previously seen at 24 hours in HEK293 cells (Figure 6A and data not shown). Interestingly, expression of wild type SOD1, which is mildly stressful to motor neurons, elicited a significant increase in the abundance of 5.8s rRNA; treatment with PA significantly further increased the abundance of 5.8s rRNA (figure 6A). Expression of mutant SOD1, a very stressful insult, led to even greater 5.8s rRNA expression which was again significantly enhanced by PA (figure 6A). This effect appears to be specific to rRNA and most robust in the 5.8s rRNA (figure 6B).
Discussion:

Using two parallel purification methods to identify candidate targets of PA followed by a secondary screen for direct interaction, we identified HNRNPK as a likely direct physical target of the neuroprotective compound PA. Follow up analysis of this interaction by surface plasmon resonance confirmed saturable binding of PA to HNRNPK and suggested that the interaction is
likely to be dependent on HNRNPK binding to RNA. Our original characterization of PA as being neuroprotective focused heavily on models of motor neuron disease, including ALS. The identification of FUS, TDP-43, and a hexa-nucleotide repeat expansion in the first intron of C9orf72 as causative gene mutations in ALS has put a recent focus on potential dysregulation of RNA processing as a mechanism of toxicity\textsuperscript{37,39,49}. For this reason, we were particularly intrigued by the fact that HNRPK is an RNA binding protein involved in many aspects of RNA metabolism.

Interestingly, we find that treatment of cells with PA leaves many HNRNPK dependent processes unaffected, suggesting a high level of specificity in PA’s activity. We do find a significant effect of PA on the ability of HNRNPK to influence the abundance of specific rRNAs. Although our analysis of total translation and polysome profiles did not show any significant differences in PA treated cells; it is possible that while no overt changes in translation are detectable, the cell is able to sense reduced rRNA levels and initiates a prosurvival response accordingly. Work by Li and Gu suggests that this could be the case. They find that RNAi knockdown of 5s rRNA in U2OS cells has no overt effect on translation or the generation of the large ribosomal subunit. They do observe a decrease in 5s rRNA associated with Mdmx and a subsequent decrease in Mdmx abundance. Mdmx is a negative regulator of p53 transcriptional activity and they find that knockdown of 5s rRNA leads to increased expression of p53 targets such as p21\textsuperscript{142}. These observations as well as previous studies showing that 5.8s rRNA is covalently linked to p53 suggest that the rRNAs form non-ribosomal complexes that are potentially involved in stress signaling outside of translation\textsuperscript{143}. While we find a reduction in rRNA after PA treatment in unstressed cells, possibly the more biologically relevant observation is the fact that stressed spinal cord cultures treated with PA show increased rRNA abundance.
Another possibility is that a subtle reduction in translation exists upon treatment with PA that our methods are simply not sensitive enough to detect. Knockdown of numerous ribosomal proteins (\textit{rps\textunderscore{}3,6,8,10,11,15,22,26} and \textit{rpl\textunderscore{}4,6,9,19,30}) and translation initiation factors (\textit{ifg\textunderscore{}1, iftb\textunderscore{}1, eif\textunderscore{}3.B, inf\textunderscore{}1, eif\textunderscore{}3.F, egl\textunderscore{}45}) in the worm lead to enhanced lifespan and resistance to stress\textsuperscript{139,141}. The lifespan and stress resistance phenotype of many of these knockdowns is dependent on the worm ortholog of FOXO, \textit{daf\textunderscore{}16}. PA may be initiating a similar cellular response through reduction in rRNA abundance and this may explain our previous observation that PA’s neuroprotective activities were FOXO dependent in a Drosophila model of SBMA\textsuperscript{124}.

Translation inhibition is a common response to a variety of cellular stresses. One way the cell inhibits translation is through rRNA degradation. rRNA degradation and subsequent translation inhibition could have prosurvival effects by allowing the cell to save energy under adverse conditions. Another potential mechanism of protection relies on the fact that certain apoptotic pathways require \textit{de novo} protein synthesis, inhibition of protein synthesis slows translation of apoptosis inducing proteins and therefore allows time for the cell to cope with stressful stimuli. It is possible that PA’s neuroprotective properties stem from the ability to disrupt HNRNPK stabilization of rRNAs, thus allowing the cell to inhibit translation upon stress more quickly, and or efficiently.

Conversely, apoptotic stimuli have been shown to induce extensive degradation of 5.8s rRNA in yeast\textsuperscript{144}. Reductions in rRNA are thought to potentially represent a turning point where cells commit to apoptosis as opposed to cellular repair. In neurons degradation of rRNA may act as a signal to activate p53\textsuperscript{145}. Our data from stressed neurons demonstrates that under
conditions of neuronal stress PA acts to enhance rRNA abundance, this may in turn act to prevent apoptosis. It is interesting to note that neurons seem to increase their abundance of 5.8s rRNA when stressed with wild type or mutant SOD1 suggesting that increases in 5.8s rRNA may represent an endogenous pro-survival response to stress. Assuming increased 5.8s rRNA is a consequence of stress, the fact that PA does not change 5.8s rRNA abundance in LacZ infected cells suggests that PA is specifically modifying a stress signal, likely associated with HNRNPK, and not simply stressing neurons.

Unfortunately, due to the plethora of functions of HNRNPK it was difficult for us to perform a complete analysis of every HNRNPK activity for PA dependent changes. Therefore it remains possible that PA impacts some of the HNRNPK functions that we considered to be unchanged. Based on our observation that PA alters rRNA abundance differently under conditions of stress, it is also possible that PA specifically alters a stress induced change in one of the HNRNPK activities that we considered to be unchanged. For example PA might alter the interaction between HNRNPK and other target RNAs such as those described by Liu et al under conditions of stress. Although we did not detect any changes in the abundance of these mRNAs after PA treatment, they may simply not be targeted for degradation under basal conditions.

Many of these analyses lent themselves to being done in HEK293 cells as opposed to neurons. We can not rule out the possibility that cell type specific HNRNPK activities, or complexes, exist in neurons and that we are unable to recapitulate some PA activities in HEK293 cells.

In our current analysis we identify a novel function of the small molecule PA as a modifier of rRNA abundance. Based on the role of HNRNPK in rRNA stability, we favor the view
that PA alters rRNA abundance through its interaction with HNRNPK. The extent to which this action of PA accounts for its neuroprotective activity is not entirely clear at this time.

**Methods:**

**Luciferase Assays:** HEK293 cells were transfected with pGL3-FHRE and pRL-TK plasmids for monitoring FOXO activity or pBIIx-firefly luciferase and pRL-TK for monitoring NFκB activity. 24hrs after transfection cells were trypsinized and replated into a 96-well plate in drug or vehicle treated media, cells were re-dosed at 48 hours. 72 hours after drug treatment, the ratio of firefly to renilla luciferase was determined using a Veritas Microplate Luminometer (Promega) in conjunction with the Dual-Luciferase Reporter Assay Kit (Promega).

**Psammaplysene A and Derivatives:** For synthesis of PA see: Georgiades, S. N. & Clardy, J. Total synthesis of psammaplysenes A and B, naturally occurring inhibitors of FOXO1a nuclear export. *Organic letters* **7**, 4091-4094, doi:10.1021/ol0513286 (2005). PA derivatives used for covalent crosslinking and purification were generated from reaction intermediaries. For schematic of synthesis of the PA derivative used for FG bead coupling see supplemental figure 3.

**Preparation of psammaplysene A-immobilized polymer-coated affinity magnetic beads (FG beads):** Polymer-coated affinity magnetic beads with amine groups (FG beads: Nishio, K. et al, 2008; Sakamoto, et al, 2009; TAS8848 N1130 (Tamagawa Seiki Co. Ltd., Iida, Japan)), stocked in pure water, were suspended in ethanol. Prior to immobilization step, ethanol was removed by centrifugation (15000 rpm, room temperature) of the beads. Then, ethanol, N-methylmorpholine in ethanol, PA derivative solution in ethanol, EDC•HCl (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) solution in ethanol, and 1-
hydroxybenztriazole in DMF (N,N-dimethyformamide) were added to centrifuged beads. After the beads were dispersed well into the medium, the suspension was incubated for 1 day at room temperature. This immobilization step gave psammaplysene A-immobilized FG beads with unreacted amine groups. Then, the beads were washed with ethanol once and suspended in DMF. Masking of unreacted amine groups on the beads was performed by incubation with 1% acetic anhydride in DMF for 3 hours at room temperature. DMF was removed and the PA-FG beads were resuspended in distilled water. Finally, suspension of PA-FG beads in pure water was kept at 4°C.

**PA Target Identification with 2B:** Cell lysates were prepared from HEK293 cells or *C. elegans* in Triton-X 100 buffer (50mM Tris pH 7.8, 150mM NaCl, 1% Triton-X 100, complete protease inhibitor cocktail). Lysates were incubated with 250nM of the PA derivative 2B for 1hr at 4°C with rotation. The lysate was then UV illuminated to activate the photo-affinity tag. Crosslinking was performed with 365nm UV illumination using a Spectroline 6 watt UV lamp on ice. Crosslinked lysates were then processed using the Click-iT protein reaction buffer kit (Molecular Probes) to attach either TAMRA-azide or biotin-azide (Molecular Probes) to the alkyene group on 2B. TAMRA tagged lysates were run on a 4-15% gradient gel and then visualized by in gel laser scanning with the Typhoon 9400 imaging system (GE life sciences). Biotin tagged lysates were incubated with hydrophilic streptavidin magnetic beads (New England Biolabs) at 4°C for 1 hr with rotation. Beads were washed three times with high stringency wash buffer (PBS with 1 M KCl and 500mM Urea) to decrease pulldown of indirect binding partners. Beads were submitted for mass spec analysis to identify binding partners.
**Purification of PA Targets with PA-FG Beads:** HEK293 lysates were incubated with 0x, 1x, and 5x PA-FG beads for 1 hr at 4°C. Beads were then washed 3x in PBS, moved to a new tube and washed 2 additional times in PBS. The beads were then resuspended in 1x SDS-PAGE loading buffer and boiled for 5 min. These samples were then run on a 4-15% gradient gel and visualized with coomassie stain. The bands indicated in figure 1 were excised and submitted for mass spec.

**Surface Plasmon Resonance:** PA binding to GST-HNRNPK was analyzed using a CM5 sensor chip on a BIACORE 3000. Anti GST antibody was immobilized on the sensor chip using the GST Capture Kit (GE). A capture/crosslink strategy was used to prevent dissociation of GST and GST fusions from the GST antibody over time. The anti GST antibody was saturated with GST or GST fusion protein and then briefly pulsed (60 sec) with NHS/EDC followed by a brief pulse (60 sec) of ethanolamine. The buffer used for dissolving PA and washing the surface was composed of 5% DMSO and 0.05% Tween-20 in PBS. PA was injected at a flow rate of 30μL per minute for 60 seconds followed by a 120 second wash before administration of the next drug concentration.

**In Vitro Transcription and Purification of Biotinylated Transcripts:** Plasmid DNA containing the non-coding RNA of interest downstream from a T7 promoter element was linearized by overnight restriction enzyme digest. Linearized plasmid was recovered by gel purification using the QIAquick Gel Extraction kit (Qiagen). Biotinylated transcripts were then generated using the Ampliscribe T7-Flash Biotin-RNA Transcription Kit (Epicentre) with linearized plasmid as the template. Biotinylated RNA was purified by ammonium acetate precipitation and resuspended in RNAse free water. Biotinylated RNAs were incubated with cell lysates for 1 hour and then pulled down with hydrophilic streptavidin magnetic beads (New England Biolabs). Beads were
washed 3x in TBS-T and then boiled in 1x SDS-PAGE loading buffer. Samples were run on an SDPS-PAGE gel and western blots were performed to identify associated proteins.

**Alternative Splicing Event Assay:** Total RNA was isolated from HEK293 cells using the RNeasy Plus Mini Kit (Qiagen). RNA was then analyzed for changes in alternative splicing events as described in: Venables, J. P. *et al.* Multiple and specific mRNA processing targets for the major human hnRNP proteins. *Molecular and cellular biology* **28**, 6033-6043, doi:10.1128/MCB.00726-08 (2008).

**qPCR:** Total RNA was isolated from HEK293 cells using the RNeasy Plus Mini Kit (Qiagen). RNA yield was quantified by A260 and 1ug of total RNA was used as template for reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RNA abundance was then quantified by qPCR using Power SYBR Green Master Mix (Applied Biosystems) on a Step One Plus Real Time PCR System (Applied Biosystems). All quantification was done using the ΔΔCT method to determine relative fold changes compared to GAPDH. Primers used were as follows:

5s rRNA forward 5’ GTC TAC GGC CAT ACC ACC 3’, reverse 5’ AAG CCT ACA GCA CCC G 3’

5.8s rRNA forward 5’ GGT GGA TCA CTC GGC TCG T 3’, reverse 5’ GCA AGT GCG TTC GAA GTG TC 3’

18s rRNA forward 5’ GAT ATG CTC ATG TGG TGT TG 3’, reverse 5’ AAT CTT CTT CAG TCG CTC CA 3’

28s rRNA forward 5’ TTG AAA ATC CGG GGG AGA G 3’, reverse 5’ ACA TTG TTC CAA CAT GCC AG 3’
GAPDH forward 5’ AAT GAA GGG GTC ATT GAT GG 3’, reverse 5’ AAG GTG AAG GTC GGA GTC AA 3’

Beta actin forward 5’ ATA GCA CAG CCT GGA TAG CAA CGT AC 3’, reverse CAC CTT CTA CAA TGA GCT GCG TGT G 3’

Gamma actin forward 5’ GCA AAA CCA GCT TTG CAC AT 3’, reverse CTC GCA CTC TGT TCT TCC G 3’

NCOR1 forward 5’ TTG TGA CGA GCA GTG AGA CAC G 3’, reverse 5’ TCA GCT TTG GTT TGC ACA CTC CTG 3’

RAB5A forward 5’ GGC TGC CTT TCT AAC CCA AAC TG 3’, reverse 5’ TAC ACA ACT ATG GCCGCT TGT G 3’

APP forward 5’ AAC CAG CAT TGC CAC CAC TAC C 3’, reverse 5’ AGC TTG TTC AGA GCA CAC CTC TC 3’

NF-M forward 5’ CAG TCC TCT TCG CTG CTT AAC G 3’, reverse 5’ CTG CAG CTG CTC TTT CTC GTT TG 3’

**Immunoprecipitation:** Cells were lysed in Triton-X 100 buffer (50mM Tris pH 7.8, 150mM NaCl, 1% Triton-X 100, complete protease inhibitor cocktail) and sonicated at 20% amplitude for 5 seconds with a Branson sonicator. Lysates were cleared of insoluble material by centrifugation at max speed ~13,000 g for 10 min at 4°C. Antibodies were prebound to Dynobead protein G (Invitrogen) and then incubated with cell lysates for 1hr at room temperature. Beads were washed 3x in PBS, transferred to a new tube and washed 2x in PBS. Beads were then resuspended in 1x SDS-PAGE buffer and boiled for 5 minutes. Samples were resolved on an SDS-PAGE gel and western blotted for the protein of interest.
**Polysome Profiling:** Two 100mm dishes of HEK cells were used for each sample. Cells were treated for 72 hours with PA or vehicle prior to polysome profiling. Cells were treated for 15 minutes with 100 µg/ml cyclohexamide. Cells were then washed twice with PBS + 100 µg/ml cyclohexamide and then scraped from the plate in this same solution. Cells were spun for 2 minutes at 1,000 x g to pellet cells. Supernatant was aspirated and cells were lysed in 20 mM Hepes pH 7.4, 100 mM KCl, 15 mM MgCl₂, 0.5% NP-40, 2 mM DTT, 100 µg/ml cyclohexamide, 100 U/ml RNasin plus (Promega) and 1x complete protease inhibitor cocktail by passage through a 26 gauge needle. Lysates were layered onto 15-45% continuous sucrose gradients containing 100 µg/ml cyclohexamide, 100 U/ml RNasin plus (Promega) prepared with DEPC treated water. Samples were spun in a SW-41 Ti rotor (Beckman) for 2.5 hours at 38,000 rpm with no brake. A peristaltic pump was used to through a UV monitor at 800 µl/min and A260 was plotted using Labview Signal Express.

**Incorporation of [35S]-methionine:** Cells were treated for 72 hours with PA prior to translation assay. Cells were washed two times in DMEM before replacing the media with cysteine and methionine free media for 1 hour with PA or vehicle. After 20 minutes, [35S]-methionine was added to the media at a concentration of 100 µCi/ml and translation was allowed to occur for 1hr. After labeling, media was removed and cells were washed once with PBS. Cells were lysed in TNNB (50mM Tris-HCl pH 8.0, 250 mM NaCl, 0.5% NP-40, 0.5 mM PMSF, 0.1% BSA) with 100mM iodoacetamide at a final volume of 1 ml. 3 µl of each lysate was spotted onto filter paper and dried. Filters were incubated in 10% TCA at 4⁰ C overnight. Incorporation of [35S]-methionine was quantified on a Wallac 1409 DSA liquid scintillation counter (Perkin Elmer).
Acknowledgments: We would like to thank Todd Lamitina for sharing equipment used for polysome profiling as well as for sharing his feedback on this project, Kristen Lynch for her useful discussions and HNRNPL and K plasmids, Gideon Dreyfuss for HNRNP plasmids, Maite Huarte for the Lincp21 plasmid, Michael May for the NFkB luciferase reporter and IL-1, Ramin Shiekhattar for Flag-DROSHA, Lynn Spruce and Hua Ding at the CHOP Protein Core for their assistance with LC MS/MS and surface plasmon resonance analysis, Yair Argon for help with [35S] methionine labeling, and Jinbin Zhai for his assistance with polysome profiling.

Supplemental Figures:

Suplemental Figure 1.
Figure S1. PA derivative compounds

**Suplemental Table 1**

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<th>Protein names</th>
<th>Gene names</th>
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### PA Nanobead Pulldown

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<td>DNA repair protein RAD50 (hRAD50) (EC 3.6.-.-)</td>
<td>RAD50</td>
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<tr>
<td>DNA-binding protein A (Cold shock domain-containing protein A) (Single-strand DNA-binding protein NF-GMB)</td>
<td>CSDA DBPA</td>
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<tr>
<td>DNA-dependent protein kinase catalytic subunit (DNA-PK catalytic subunit) (DNA-PKcs) (EC 2.7.11.1) (DNPK1) (p460)</td>
<td>PRKDC HYRC HYRC1</td>
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<tr>
<td>DNA-directed RNA polymerase III subunit RPC1 (RNA polymerase III subunit C1) (EC 2.7.7.6) (DNA-directed RNA polymerase III largest subunit) (DNA-directed RNA polymerase III subunit A) (RNA polymerase III 155 kDa subunit) (RPC155) (RNA polymerase III subunit C160)</td>
<td>POLR3A</td>
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<tr>
<td>Double-stranded RNA-binding protein Staufen homolog 2</td>
<td>STAU2</td>
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<td>Dynactin 1</td>
<td>DCTN1</td>
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<tr>
<td>EMILIN-2 (Elastin microfibril interface-located protein 2) (Elastin microfibril interactor 2) (Protein FOAP-10)</td>
<td>EMILIN2</td>
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<td>Eukaryotic translation initiation factor 3, subunit D (cDNA FLJ53917, highly similar to Eukaryotic translation initiation factor 3 subunit 7)</td>
<td>EIF3D</td>
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<tr>
<td>Eukaryotic translation initiation factor 3, subunit E interacting protein (Uncharacterized protein)</td>
<td>EIF3EIP EIF3L AL022311.1-001</td>
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<td>Extracellular sulfatase Sulf-2 (hSulf-2) (EC 3.1.6.-)</td>
<td>SULF2 KIAA1247 UNQ559/PRO1120</td>
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<tr>
<td>Fanconi anemia group D2 protein (Protein FACD2)</td>
<td>FANCD2 FACD</td>
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<td>Far upstream element (FUSE) binding protein 3 (HCG31253, isoform CRA_a) (cDNA FLJ58115, highly similar to Far upstream element-binding protein 3)</td>
<td>FUBP3 hCG_31253 RP11-57C19.3-002</td>
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<td>Fragile X mental retardation protein 1 (FMRP) (Protein FMR-1)</td>
<td>FMR1</td>
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<td>G patch domain-containing protein 4</td>
<td>GPATCH4 GPATC4</td>
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<td>Golgi apparatus protein 1 (CFR-1) (Cysteine-rich fibroblast growth factor receptor) (E-selectin ligand 1) (ESL-1) (Golgi sialoglycoprotein MG-160)</td>
<td>GLG1 CFR1 ESL1 MG160</td>
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<td>Guanine nucleotide-binding protein-like 3 (E2-induced gene 3 protein) (Novel nucleolar protein 47) (NNP47) (Nucleolar GTP-binding protein 3) (Nucleostemin)</td>
<td>GNL3 E2IG3 NS</td>
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<td>Heat shock 70kDa protein 1A (Heat shock 70kDa protein 1B) (cDNA FLJ75127, highly similar to Homo sapiens heat shock 70kDa protein 1A, mRNA)</td>
<td>HSPA1A HSPA1B</td>
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<tr>
<td>Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)</td>
<td>HSPA8 HSC70 HSP73 HSPA10</td>
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<tr>
<td>Protein Name</td>
<td>Gene Symbols</td>
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<td>Heterogeneous nuclear ribonucleoprotein H (hnRNP H) [Cleaved into: Heterogeneous nuclear ribonucleoprotein H, N-terminally processed]</td>
<td>HNRNPH1 HNRPH HNRPH1</td>
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<tr>
<td>Heterogeneous nuclear ribonucleoprotein L (hnRNP L)</td>
<td>HNRNPL HNRPL P/OKcl.14</td>
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<td>Heterogeneous nuclear ribonucleoprotein M (hnRNP M)</td>
<td>HNRNPM HNRPM NAGR1</td>
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<td>Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) (Glycine- and tyrosine-rich RNA-binding protein) (GRY-RBP) (NS1-associated protein 1) (Synaptotagmin-binding, cytoplasmic RNA-interacting protein)</td>
<td>SYNCRIP HNRPQ NSAP1</td>
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<td>Heterogeneous nuclear ribonucleoprotein R (hnRNP R)</td>
<td>HNRNP3 HNRPR</td>
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<td>Histone deacetylase (EC 3.5.1.98)</td>
<td>HDAC1</td>
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<tr>
<td>Insulin receptor substrate 4 (IRS-4) (160 kDa phosphotyrosine protein) (py160) (Phosphoprotein of 160 kDa) (pp160)</td>
<td>IRS4</td>
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<td>Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2 mRNA-binding protein 1) (IMP-1) (Coding region determinant-binding protein) (CRD-BP) (IGF-II mRNA-binding protein 1) (VICKZ family member 1) (Zip code-binding protein 1) (ZBP-1) (Zipcode-binding protein 1)</td>
<td>IGF2BP1 CRDBP VICKZ1 ZBP1</td>
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<td>Insulin-like growth factor 2 mRNA-binding protein 2 (IGF2 mRNA-binding protein 2) (IMP-2) (Hepatocellular carcinoma autoantigen p62) (IGF-II mRNA-binding protein 2) (VICKZ family member 2)</td>
<td>IGF2BP2 IMP2 VICKZ2</td>
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<td>Insulin-like growth factor 2 mRNA-binding protein 3 (IGF2 mRNA-binding protein 3) (IMP-3) (IGF-II mRNA-binding protein 3) (KH domain-containing protein overexpressed in cancer) (hKOC) (VICKZ family member 3)</td>
<td>IGF2BP3 IMP3 KOC1 VICKZ3</td>
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<td>Isoleucine--tRNA ligase, cytoplasmic (EC 6.1.1.5) (Isoleucyl-tRNA synthetase) (IRS) (IleRS)</td>
<td>IARS</td>
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<td>Junction plakoglobin (Catenin gamma) (Desmoplakin III) (Desmoplakin-3)</td>
<td>JUP CTNNG DP3</td>
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<td>Kelch-like protein 21</td>
<td>KLHL21 KIAA0469</td>
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<td>Keratin, type I cytoskeletal 10 (Cytokeratin-10) (CK-10) (Keratin-10) (K10)</td>
<td>KRT10 KPP</td>
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<tr>
<td>Keratin, type I cytoskeletal 16 (Cytokeratin-16) (CK-16) (Keratin-16) (K16)</td>
<td>KRT16 KRT16A</td>
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<td>Keratin, type I cytoskeletal 9 (Cytokeratin-9) (CK-9) (Keratin-9) (K9)</td>
<td>KRT9</td>
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<td>Keratin, type II cytoskeletal 1 (67 kDa cytokeratin) (Cytokeratin-1) (CK-1) (Hair alpha protein) (Keratin-1) (K1) (Type-II keratin Kb1)</td>
<td>KRT1 KRTA</td>
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<tr>
<td>Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (CK-2e) (Epithelial keratin-2e) (Keratin-2 epidermis) (Keratin-2e) (K2e) (Type-II keratin Kb2)</td>
<td>KRT2 KRT2A KRT2E</td>
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<tr>
<td>Gene Name</td>
<td>Description</td>
<td></td>
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<tr>
<td>KHDRBS1 SAM68</td>
<td>KH domain-containing, RNA-binding, signal transduction-associated protein 1 (GAP-associated tyrosine phosphoprotein p62) (Src-associated in mitosis 68 kDa protein) (Sam68) (p21 Ras GTPase-activating protein-associated p62) (p68)</td>
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<tr>
<td>KTN1</td>
<td>KTN1 protein (cDNA FLJ61494, highly similar to Kinectin)</td>
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<tr>
<td>LARP1 KIAA0731 LARP</td>
<td>La-related protein 1 (La ribonucleoprotein domain family member 1)</td>
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<td>LARP1B LARP2</td>
<td>La-related protein 1B (La ribonucleoprotein domain family member 1B) (La ribonucleoprotein domain family member 2) (La-related protein 2)</td>
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<tr>
<td>LARP7 HDCMA18P</td>
<td>La-related protein 7 (La ribonucleoprotein domain family member 7) (P-TEFb-interaction protein for 7SK stability) (PIP7S)</td>
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<td>LGALS3BP</td>
<td>Lectin, galactoside-binding, soluble, 3 binding protein (Uncharacterized protein) (cDNA FLJ53478, highly similar to Galectin-3-binding protein)</td>
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<td>MAGED1 NRAGE PRO2292 PP2250</td>
<td>Melanoma-associated antigen D1 (MAGE tumor antigen CCF) (MAGE-D1 antigen) (Neurotrophin receptor-interacting MAGE homolog)</td>
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<td>MOV10 KIAA0864 MRIP RHOIP3</td>
<td>Mov10, Moloney leukemia virus 10, homolog (Mouse) (Mov10, Moloney leukemia virus 10, homolog (Mouse), isoform CRA_a) (Uncharacterized protein)</td>
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<tr>
<td>MYH10</td>
<td>Myb-binding protein 1A</td>
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<td>MYEF2 KIAA1341</td>
<td>Myelin expression factor 2 (MEF-2) (MyEF-2) (MST156)</td>
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<td>MYH10</td>
<td>Myosin phosphatase Rho-interacting protein (M-RIP) (Rho-interacting protein 3) (RIP3) (p116Rip)</td>
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<td>MYH14 KIAA2034 FP17425</td>
<td>Myosin-14 (Myosin heavy chain 14) (Myosin heavy chain, non-muscle IIc) (Non-muscle myosin heavy chain IIc) (NMHC II-C)</td>
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<td>MYH9</td>
<td>Myosin-9 (Cellular myosin heavy chain, type A) (Myosin heavy chain 9) (Myosin heavy chain, non-muscle IIa) (Non-muscle myosin heavy chain A) (NMHC-A) (Non-muscle myosin heavy chain IIa) (NMHC-IIa)</td>
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<td>NUP160 KIAA0197 NUP120</td>
<td>Nucleolar protein 58 (Nucleolar protein 5)</td>
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<td>NOP58 NOL5 NOP5 HSPC120</td>
<td>Nucleolin, isoform CRA_c (cDNA FLJ10452 fis, clone NT2RP1000966, highly similar to NUCLEOLIN)</td>
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<td>PABPC4 hCG_2031827 RP11-426L16.2-003</td>
<td>PABPC4 protein (Poly(A) binding protein, cytoplasmic 4 (Inducible form)) (Poly(A) binding protein, cytoplasmic 4 (Inducible form), isoform CRA_c) (Uncharacterized protein)</td>
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<tr>
<td>PTCD1 hCG_2023422 tcag7.1151</td>
<td>Pentatricopeptide repeat domain 1 (cDNA FLJ76276, highly similar to Homo sapiens pentatricopeptide repeat domain 1 (PTCD1), mRNA)</td>
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<tr>
<td>Protein Name</td>
<td>Gene Name</td>
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<tr>
<td>Pentatricopeptide repeat-containing protein 3, mitochondrial (</td>
<td>PTCD3 TRG15</td>
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<tr>
<td>Transformation-related gene 15 protein) (TRG-15)</td>
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<tr>
<td>Periodic tryptophan protein 1 homolog (Keratinocyte protein IEF SSP</td>
<td>PWP1</td>
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<tr>
<td>9502)</td>
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<tr>
<td>Poly(U)-binding-splicing factor PUF60 (60 kDa poly(U)-binding-splicing</td>
<td>PUF60 60kDa poly(U)</td>
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<tr>
<td>factor (FUSE-binding protein-interacting repressor) (FBP-interacting</td>
<td>factor)</td>
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<tr>
<td>repressor) (Ro-binding protein 1) (RoBP1) (Siah-binding protein 1)</td>
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<td>(Siah-BP1)</td>
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<tr>
<td>Polyadenylate-binding protein 1 (PABP-1) (Poly(A)-binding protein 1)</td>
<td>PABPC1 PAB1 PABP1 PABPC2</td>
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<td>Prelamin-A/C [Cleaved into: Lamn- A/C (70 kDa lamin) (Renal carcinoma</td>
<td>LMNA LMN1</td>
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<td>antigen NY-REN-32)]</td>
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<td>Probable ATP-dependent RNA helicase DDX41 (EC 3.6.4.13) (DEAD box protein</td>
<td>DDX41 ABS</td>
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<td>41) (DEAD box protein abstrakt homolog)</td>
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<td>Probable ATP-dependent RNA helicase DDX5 (EC 3.6.4.13) (DEAD box protein</td>
<td>DDX5 G17P1 HELR HLR1</td>
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<td>5) (RNA helicase p68)</td>
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<td>Probable ATP-dependent RNA helicase DHX36 (EC 3.6.4.13) (DEAH box protein</td>
<td>DHX36 DDX36 KIAA1488</td>
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<tr>
<td>36) (MLE-like protein 1) (RNA helicase associated with AU-rich element</td>
<td>MLE11 RHAU</td>
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<td>ARE)</td>
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<td>Probable ATP-dependent RNA helicase YTHDC2 (EC 3.6.4.13)</td>
<td>YTHDC2</td>
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<tr>
<td>Protein angel homolog 1</td>
<td>ANGEL1 KIAA0759</td>
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<td>Protein angel homolog 2</td>
<td>ANGEL2 KIAA0759L</td>
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<td>Protein C16orf88 (Testis-specific gene 118 protein)</td>
<td>C16orf88 TSG118</td>
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<td>Protein FAM98A</td>
<td>FAM98A</td>
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<tr>
<td>Protein LYRIC (3D3/LYRIC) (Astrocyte elevated gene-1 protein) (AEG-1)</td>
<td>MTDH AEG1 LYRIC</td>
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<td>(Lysine-rich CEACAM1 co-isolated protein) (Metadherin) (Metastasis</td>
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<tr>
<td>adhesion protein)</td>
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<tr>
<td>Protein phosphatase 2 (Formerly 2A), regulatory subunit A (PR 65), alpha</td>
<td>PPP2R1A hCG_19686</td>
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<tr>
<td>isoform (cDNA FLJ78455, highly similar to Homo sapiens protein</td>
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<td>phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform</td>
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<td>(PPP2R1A), mRNA) (cDNA, FLJ96799, Homo sapiens protein phosphatase 2</td>
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<td>(formerly 2A), regulatory subunit A (PR 65), alpha isoform (PPP2R1A),</td>
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<td>mRNA)</td>
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<td>Protein transport protein Sec16A (SEC16 homolog A)</td>
<td>SEC16A KIAA0310 SEC16</td>
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<td>Putative ATP-dependent RNA helicase DHX57 (EC 3.6.4.13) (DEAH box protein</td>
<td>DHX57</td>
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<td>57)</td>
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<td>Putative protein FAM90A7</td>
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<td>Putative RNA helicase Ski2w</td>
<td>SKI2W</td>
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<td>Putative uncharacterized protein DKFZp686A13234 (Fragment)</td>
<td>DKFZp686A13234</td>
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<td>RBM39 protein (Uncharacterized protein) (cDNA FLJ59214, highly similar</td>
<td>RBM39</td>
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<td>to RNA-binding region-containing protein 2)</td>
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<tr>
<td>Protein Name</td>
<td>Gene IDs</td>
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<td>Regulator of nonsense transcripts 1 (EC 3.6.4.1) (ATP-dependent helicase RENT1) (Nonsense mRNA reducing factor 1) (NORF1) (Up-frameshift suppressor 1 homolog) (hUpf1)</td>
<td>UPF1 KIAA0221 RENT1</td>
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<td>Ribonuclease 3 (EC 3.1.26.3) (Protein Drosha) (Ribonuclease III) (RNase III) (p241)</td>
<td>DROSHA RN3 RNASE3L RNASEN</td>
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<td>RNA-binding protein 14 (Paraspeckle protein 2) (PSP2) (RNA-binding motif protein 14) (RRM-containing coactivator activator/modulator) (Synaptotagmin-interacting protein) (SYT-interacting protein)</td>
<td>RBM14 SIP</td>
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<td>RSL1D1 protein (Fragment)</td>
<td>RSL1D1</td>
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<td>SAFB protein (Scaffold attachment factor B)</td>
<td>SAFB</td>
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<td>Scaffold attachment factor B2 (SAF-B2)</td>
<td>SAFB2 KIAA0138</td>
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<td>SNW domain-containing protein 1 (Nuclear protein SkiP) (Nuclear receptor coactivator NCoA-62) (Ski-interacting protein)</td>
<td>SNW1 SKIIP SKIP</td>
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<td>Splicing factor 3A subunit 2 (SF3a66) (Spliceosome-associated protein 62) (SAP 62)</td>
<td>SF3A2 SAP62</td>
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<tr>
<td>Splicing factor 3B subunit 1 (Pre-mRNA-splicing factor SF3b 155 kDa subunit) (SF3b155) (Spliceosome-associated protein 155) (SAP 155)</td>
<td>SF3B1 SAP155</td>
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<td>SRP72 protein (Fragment)</td>
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<td>Stress-70 protein, mitochondrial (75 kDa glucose-regulated protein) (GRP-75) (Heat shock 70 kDa protein 9) (Mortalin) (MOT) (Peptide-binding protein 74) (PBP74)</td>
<td>HSPA9 GRP75 HSPA9B</td>
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<td>Structural maintenance of chromosomes protein 1A (SMC protein 1A) (SMC-1-alpha) (SMC-1A) (Sb1.8)</td>
<td>SMC1A DXS423E KIAA0178 SB1.8 SMC1 SMC1L1</td>
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<td>Synembryn-A (Protein Ric-8A)</td>
<td>RIC8A</td>
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<td>TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa (TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa, isoform CRA_b) (cDNA, FLJ94920, Homo sapiens TAF15 RNA polymerase II, TATA box binding protein(TBP)-associated factor, 68kDa (TAF15), transcript variant 1, mRNA)</td>
<td>TAF15 hCG_1992163</td>
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<td>TBC1 domain family, member 9B (With GRAM domain)</td>
<td>TBC1D9B</td>
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<td>T-complex protein 1 subunit alpha (TCP-1-alpha) (CCT-alpha)</td>
<td>TCP1 CCT1 CCTA</td>
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<tr>
<td>T-complex protein 1 subunit epsilon (TCP-1-epsilon) (CCT-epsilon)</td>
<td>CCT5 CTE KIAA0098</td>
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<tr>
<td>T-complex protein 1 subunit gamma (TCP-1-gamma) (CCT-gamma) (hTRiC5)</td>
<td>CCT3 CCTG TRIC5</td>
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<td>Tetrastricopeptide repeat protein 37 (TPR repeat protein 37) (Thespin)</td>
<td>TTC37 KIAA0372</td>
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<td>THO complex subunit 2 (Tho2) (hTREX120)</td>
<td>THOC2 CXorf3</td>
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<td>Titin (EC 2.7.11.1) (Connectin) (Rhabdomyosarcoma antigen MU-RMS-40.14)</td>
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<td>Transcription factor 25 (TCF-25) (Nuclear localized protein 1)</td>
<td>TCF25 KIAA1049 NULP1</td>
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<tr>
<td>Gene Name</td>
<td>Gene ID</td>
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<td>Trifunctional enzyme subunit alpha, mitochondrial (78 kDa gastrin-binding protein) (TP-alpha) [Includes: Long-chain enoyl-CoA hydratase (EC 4.2.1.17); Long chain 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.211)]</td>
<td>FKSG26</td>
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<td>Tubulin beta chain (Tubulin beta-5 chain)</td>
<td>TUBB TUBB5 OK/SW-cl.56</td>
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<td>Tubulin beta-4B chain (Tubulin beta-2 chain) (Tubulin beta-2C chain)</td>
<td>TUBB4B TUBB2C</td>
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<td>Twinkle protein, mitochondrial (EC 3.6.4.12) (Progressive external ophthalmoplegia 1 protein) (T7 gp4-like protein with intramitochondrial nucleoid localization) (T7-like mitochondrial DNA helicase)</td>
<td>PEO1 C10orf2</td>
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<td>U2 small nuclear RNA auxiliary factor 2 isoform b</td>
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<td>UACA KIAA1561</td>
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Suplemental Table 2

### Screened PA Target Candidates

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<td>Histone deacetylase 6 (HDAC6)</td>
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<td>YTH domain containing 2 (YTHDC2)</td>
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Suplemental Figure 2
Figure S2. Heat map of alternative splicing events.
Author Contributions: Conceived and designed the experiments: MB, SS, RK. Performed the experiments: MB, NL, SS, BC. Analyzed the data: MB, BC. Contributed reagents/materials/analysis tools: JC, HH, BC. Wrote the paper: MB, RK.

Chapter 5

Future Directions:

Effects of IIS Signaling on SOD1 Toxicity in C. elegans

Are the Beneficial Effects of Reduced IIS on SOD1 Toxicity Cell Autonomous?

Our work with the Psnb-1::G85R::YFP; sid-1(pk3321)[Punc119::sid-1] strain suggested a specific requirement of reduced IIS in the nervous system in order to rescue G85R induced toxicity. While this approach allowed us to preferentially direct RNAi to the nervous system it did not provide us with cell specific activation of DAF-16. This prevented us from definitively determining whether increased DAF-16 activity was required cell autonomously to rescue mutant SOD1 toxicity. If so this finding would be in contrast to effects of reduced IIS on longevity. Reduced IIS in specific tissues has been found to coordinate organismal aging and provide much of the same benefits on longevity as reducing IIS in the entire organism17. This observation was made by mosaic analysis. Similar studies have not been performed to analyze the effects of reduced IIS on stress resistance or more specifically, beneficial effects on proteotoxicity in a tissue/lineage specific fashion. One potential approach to answering this question would be to rescue daf-16 function in the Psnb-1::G85R::YFP;daf-2(e1370);daf-
16(mgDf50) background using tissue specific promoters. Using the snb-1 promoter to rescue daf-16 function in this background, one could determine if restoration of DAF-16 activity exclusively in neurons that express G85R is sufficient to rescue locomotor function in these worms. If expression of daf-16 under the snb-1 promoter offered similar protection against G85R toxicity as daf-2(e1370) does in a wild type daf-16 background, this would suggest that the beneficial effects of reduced IIS on proteotoxicity are being mediated in a cell autonomous manner. Similarly, this approach would allow you to selectively restore daf-16 in any tissue of the Psnb-1::G85R::YFP;daf-2(e1370);daf-16(mgDf50) worm in order to determine if this was beneficial to locomotion. If Psnb-1::daf-16 rescued locomotor function in the Psnb-1::G85R::YFP;daf-2(e1370);daf-16(mgDf50) worm but expression of daf-16 in other tissues did not this would suggest a cell autonomous effect of decreased IIS reducing SOD1 toxicity. An alternative interpretation could be that snb-1 expressing neurons act to coordinate an organismal response to SOD1 toxicity. In order to control for this possibility, one could develop a SOD1 toxicity assay in a non-snb1 expressing tissue and ensure that rescue of daf-16 function under control of the snb-1 promoter does not affect SOD1 toxicity in this assay. This would ensure that the effects of increased DAF-16 activity on G85R toxicity are truly cell autonomous and not coordinated by snb-1 expressing cells. A cell autonomous effect of decreased IIS on SOD1 toxicity could be further validated through cell specific knockdown of daf-16 on the G85R;daf-2(e1370) background. A daf-16 RNAi driven by Psnb-1 would be predicted to eliminate the rescue typically seen in these worms if the benefits of daf-2(e1370) are mediated cell autonomously.

Developing an accurate model of the cell autonomous versus nonautonomous effects of reduced IIS on stress resistance is of clinical importance due to the potential for reduced IIS to
ameliorate toxicity associated with a broad range of neurodegenerative diseases. Reduced IIS might have adverse effects in certain tissues. If this were true therapies could be developed that target reductions in IIS to specific tissues or potentially even regions within the brain. Conversely if tissues or brain regions were found that were able to coordinate organismal stress response, as certain cell types regulate aging in the worm, reduced IIS in these cell types could be exploited to ameliorate disease globally.

**Molecular Mechanisms of PA Neuroprotection**

Although we were able to identify the target of PA as HNRNPK and determine that PA alters rRNA abundance presumably through HNRNPK, a number of questions remain.

**Are Reductions in rRNA Abundance Neuroprotective?**

Reductions in rRNA abundance after stress are well documented, and are typically thought to be associated with apoptosis.\(^{144,146}\). Consistent with these observations our data demonstrates an ability of PA to increase rRNA abundance under stressful conditions. By preventing decreases in rRNA abundance PA may be acting to prevent apoptosis. The fact that stressed neurons not treated with PA increase their abundance of rRNA suggests that increasing rRNA may be an endogenous mechanism for coping with cellular stress. Increased rRNA abundance may facilitate increased translation that allows for translation of pro-survival factors. Alternatively increased rRNA could function in a translation independent fashion. Non-coding RNAs are capable of having signaling pathways and the 5s rRNA has been shown to act as a brake on p53 activation by stabilizing mdmx.\(^{142}\). Possible pro-survival activities of rRNAs could be
investigated through overexpression of rRNA. A Herpes Simplex Virus (HSV) could be engineered to express 5s, 5.8s, 18s or 28s rRNA and the neuroprotective capacity of overexpression of rRNA could be monitored in motor neuron cultures exposed to various stressors.

A potential role for decreased rRNA in survival has not been investigated. Knockdown of components necessary for translation, such as ribosomal proteins and translation initiation factors, is not only tolerated in *C. elegans*, such manipulations can significantly extend lifespan\textsuperscript{139,141}. Due to the proven record of knockdown of transcriptional machinery in worms, *C. elegans* might be an optimal system to begin investigating the effects of knockdown of 5s, 5.8s, 18s, and 28s rRNAs. It would be interesting to determine if knockdown of any of the rRNAs is tolerated and, if so, if this knockdown increases longevity or stress resistance. If knockdown of rRNA is tolerated it would also be interesting to determine the effect of rRNA knockdown on *daf-16::*GFP reporter worms. These worms show robust nuclear accumulation of DAF-16::GFP upon exposure to stimuli which activate DAF-16. If RNAi to rRNAs increases stress resistance and DAF-16 activation in worms this would suggest that reduction of rRNA stability might represents the neuroprotective mechanism of PA. Some manipulations in the worm that extend lifespan through inhibition of translation are not *daf-16* dependent\textsuperscript{139}, therefore it would be informative to determine whether *daf-16* is necessary to mediate any lifespan extending or stress resistance effects of rRNA knockdown. A positive result in the worm would suggest follow up experiments to determine if rRNA knockdown can be protective in mammalian cells as well. A HSV could be generated to knockdown rRNAs in neurons and its protective effects could be screened in the neuroprotection assays for which PA has already been determined to be effective.
Does Altered rRNA Abundance Represent the Only Functionally Significant Change in HNRNPK After PA Treatment?

Although we were unable to find an effect of PA on many other HNRNPK functions, such as alternative splicing, activation of NFκB, or abundance of HNRNPK target mRNAs, our assays were somewhat limited in scope and therefore do not represent absolute evidence that PA is not altering these functions. Our investigation into HNRNPK’s role as a transcription factor was limited to effects on NFκB. HNRNPK effects the transcription of a number of other genes which we did not investigate. Similarly HNRNPK is known to effect alternative splicing of a number of the genes in the alternative splicing assay we performed, but these do not represent all of the HNRNPK mediated alternative splicing events. In order to further investigate potential effects of PA on HNRNPK mediated splicing and gene expression, total RNA sequencing could be performed on PA treated cells and compared to vehicle. This approach would allow for simultaneous quantification of changes in transcript abundance as well as alternative splicing. The ability to quantify non-coding RNA is another important advantage of this approach. Since we already know that PA alters rRNA abundance, other non-coding RNAs might be important targets of HNRNPK which are altered by PA as well. Alternatively this could be accomplished with an exon array, although the ability to quantify non-coding RNAs would be lost.

Another important experiment would be to identify all of the RNAs whose binding to HNRNPK is modified by PA treatment. We know that PA decreases rRNA stability. Work by Jiaqi Shi and colleagues suggests that HNRNPK binds to and is an important stabilizer of rRNAs and displacement of HNRNPK from rRNA leads to its degradation. Based on this model, PA’s ability to decrease rRNA abundance could potentially be mediated by a displacement of HNRNPK from
rRNA resulting in decreased rRNA stability. There could be numerous other RNAs which are displaced, or preferentially bound, by HNRNPK after PA treatment. One might be able to identify some of these transcripts simply by changes in transcript abundance, but they may be difficult to identify if they are not being actively targeted for degradation. If they are not actively being degraded their abundance may be similar whether they are HNRNPK bound or not. Stress or other stimuli may target these RNAs for degradation, at which point the effect of PA would become biologically significant, but the PA effect may be masked under basal conditions. One way to identify all the RNAs whose binding to HNRNPK are altered by PA would be through Immunoprecipitation of HNRNPK followed by sequencing of HNRNPK bound transcripts under vehicle and PA treated conditions. This would give a quantitative analysis of all the transcripts which are dissociated or preferentially bound to HNRNPK after PA treatment.

Another limitation of our analysis of PA activity was the use of HEK293 cells for most screening assays. The neuroprotective properties of PA were described in spinal cord cultures which raises the possibility of cell type specific effects of PA. It may be important to perform the experiments described above in neurons and, it may even be necessary to repeat some of the previously performed assays in neurons as well.

**Does PA Alter Other HNRNPK Activities in a Stress Dependent Fashion?**

A number of studies have shown that HNRNPK plays an important role in p53 biology, both as a transcriptional co-activator of p53 targets and as a transcriptional repressor of genes downregulated by LINCp21 upon p53 activation\(^{54,55}\). This is of particular interest in light of work implicating p53 in neurodegeneration and preclinical data suggesting that p53 inhibitors may be effective therapeutics for neurodegenerative disease\(^ {147}\). Based on our observations regarding
rRNA and the fact that PA is neuroprotective, it is possible that PA plays a role in modifying other stress specific functions of HNRNPK. For this reason it may prove important to do many of the experiments we already performed, as well as some of the ones described above, under basal as well as stressed conditions. Unfortunately adding stress to the analysis of HNRNPK function after PA treatment makes data interpretation significantly more difficult; as you now have to tease apart what changes in HNRNPK function are cause by stress, and which are caused by PA under conditions of stress.

**Can Functional Data be Inferred by Determining the Structure of PA Bound HNRNPK?**

Another potential direction to take this project would be to try to determine where PA binds HNRNPK. Several approaches could be considered to answering this question. 1) The BIACORE analysis that was performed on GST-HNRNPK could be repeated using overlapping truncated versions of the protein in order to define a minimal binding domain. 2) A truncated overlapping HNRNPK constructs could also be transfected into cells and analyzed using the 2B binding assay. 3) One could also attempt to crystalize PA bound to HNRNPK. These approaches could be used to determine where PA is binding HNRNPK. Many of the domains of HNRNPK are well characterized and knowing which domains PA is interacting with might give insight into its activity. If PA is acting to occlude a KH domain this might suggest which HNRNPK might be displaced by PA binding. If PA binds in or near the KI domain, this might suggest that PA is disrupting an HNRNPK protein-protein interaction. Similarly, binding on or near a site of known post-translational modification might suggest a role for that residue in stabilizing rRNA, or interaction with an important protein partner.

**Could PA Have Therapeutic Potential Against Cancer?**
While we set out trying to identify the neuroprotective mechanism of PA, we may have identified a cancer fighting activity of PA. PA was originally described as potentially having cancer fighting properties based on its ability to relocalize FOXO1 to the nucleus in PTEN deficient cells. Our work potentially suggests another cancer fighting property of PA. Work by Jiaqi Shi, and colleagues, has described a significant reduction in eIF3f in various human cancers, including pancreatic cancer and melanoma. eIF3f appears to play an important role in decreasing translation by increasing rRNA degradation. The molecular mechanism by which this is achieved is by dissociating HNRNPK from rRNA, thus decreasing their stability and facilitating their degradation. As we have found that PA decreases rRNA abundance, it is conceivable that administration of PA to eIF3f deficient cells might act to correct dysregulated translational control in these cells. This could be of therapeutic benefit. Shi and colleagues describe numerous cell growth and proliferation assays used to characterize cells that are transformed by a loss of eIF3f. Monitoring changes in these assays in PA treated cells could be informative about whether PA treatment could be of therapeutic potential.

**Screening for Compounds with Similar Properties as PA.**

The intent of this study was to identify the mechanism of action of PA so that compounds with more “drug-likeness” than PA could eventually be identified. Assuming it can be verified that altering the abundance of rRNA is PA’s protective mechanism, then a screen for rRNA modulating compounds could be undertaken. This could be done by high throughput qPCR. A potentially confounding aspect of this approach is that PA appears to change rRNA abundance in different ways under stressed and unstressed conditions. It may be necessary to determine which represents the neuroprotective activity before undertaking this screen.
A more technically challenging but potentially more informative approach could be to utilize a fluorescent RNA aptamer to monitor the interaction between HNRNPK and rRNA. Since HNRNPK binding to rRNA has been suggested to increase rRNA stability, PA might be changing HNRNPKs binding to rRNA. Paige et al recently described an RNA aptamer, called spinach, which mimics GFP upon addition of the cell permeable fluorophore DFHBI. In their work they attach spinach to 5s rRNA and are able to monitor its cellular localization and abundance in live cells in real time\textsuperscript{151}. While they do not perform any FRET experiments, it is possible that FRET between spinach and a fluorescently tagged protein (such as HNRNPK) could be used to monitor protein RNA interactions. A screen could be designed where fluorescence from a fluorophore on HNRNPK excites 5s rRNA spinach and spinach fluorescence is used to monitor their interaction in the presence of various drugs. This could be set up as a high throughput imaging screen looking for compounds that modify FRET between 5s rRNA and HNRNPK.

**Summary:** Our work has identified HNRNPK as the target of the neuroprotective compound PA. We have described a role for PA in modifying HNRNPKs ability to regulate rRNA abundance. We show that neurons endogenously upregulate the abundance of the 5.8s rRNA when stressed and PA acts to enhance this effect. rRNA degradation has been associated with apoptosis and the ability of PA to upregulate rRNA may represent PA’s mechanism of neuroprotection. Alternatively PA appears to decrease rRNA abundance when chronically administered under non-stressed conditions. This may also represent a potential neuroprotective activity as disruption of translation machinery has been shown to increase lifespan in various model organisms.
Appendix

Appendix 1


Regulation of Foxo-Dependent Transcription by Post-translational Modifications

Marco Boccitto and Robert G. Kalb

Department of Pediatrics, Division of Neurology, Abramson Research Center, Children’s Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Abstract

The Forkhead Box O (Foxo) proteins represent an evolutionarily conserved family of transcription factors that play an important role in regulating processes including metabolism, longevity, and cell death/survival. How is it that a single transcription factor can initiate such divergent cellular responses? We will review the evidence that specific patterns of post-translational modifications play a key role in directing Foxo into various transcriptional readouts. This regulation appears to take on a two tiered regulatory model; with a group of well defined post-translational modifications regulating nuclear localization and transcriptional activity while a second set of modifications regulate the transcriptional specificity of Foxo.

Introduction

The Forkhead Box O (Foxo) transcription factors are members of the Forkhead superfamily of winged helix transcription factors. The Foxo family regulates cellular processes
including metabolism, stress response, DNA damage repair and cell death. The Foxo family is highly conserved evolutionarily, with Foxo orthologs exhibiting similar physiological functions in model organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and in vertebrates. There are four mammalian Foxos, FOXO1, FOXO3, FOXO4 and FOXO6. FOXO2 is identical to FOXO3 and FoxO5 is the fish ortholog of FOXO3. While all Foxo members are capable of binding the same core DNA sequence, 5’-TTGTTTAC-3’, the expression of individual Foxos is tissue specific. In addition, some Foxos undergo specific regulation or lack the regulatory interactions of other isoforms. In most cases groups study the regulation of a single Foxo isoform. Frequently regulatory pathways that are discovered in a single Foxo isoform are conserved between isoforms, therefore, for the sake of simplicity, the general term Foxo will be used in this article instead of listing each individual isoform.

While much is known about signaling pathways that influence its nuclear localization, the necessary antecedent for transcriptional activity of Foxo, less is known about signaling pathways that control Foxo transcription of specific genes in specific contexts. The ability of Foxo to regulate so many cellular processes would suggest that such signaling pathways exist in order to integrate cellular context and direct Foxo to specific targets. For example nuclear localization of Foxo through Paclitaxel treatment causes cell death in breast cancer cells\textsuperscript{152}, while nuclear localization of Foxo through Psammaplysene A treatment is neuroprotective in multiple models of motoneuron disease\textsuperscript{124}. Even within the same cell type Foxo can have opposing actions (mediating either apoptosis or survival of neurons\textsuperscript{100,124} depending on the cellular context), raising the question of how its opposing activities are regulated. One mechanism of regulating Foxo transcriptional output is through its pattern of posttranslational modifications (i.e. phosphorylation, acetylation and ubiquitination). We will review the evidence that these
patterns of posttranslational modification are responsible for regulating both transcriptional specificity and transcriptional activation. In order to fully understand Foxo signaling it is important to consider both pathways – those that generally activate or inhibit Foxo (by regulating its subcellular localization), and those that direct Foxo to transcribe specific genes.

**Foxo Inhibitory Kinases**

The following kinases are known, general inhibitors of Foxo signaling. Their signaling activities are responsible for promoting translocation of Foxo out of the nucleus and regulating its degradation. Because of their ability to turn off Foxo transcription, and therefore repress, for example, Foxo’s pro-apoptotic activity, these kinases are often considered in terms of Foxo’s role in cancer biology. While it is clear that these signaling pathways play a role in controlling Foxo’s subcellular localization, the extent to which these posttranslational modifications (at any of these sites) play a role in the specificity of Foxo transactivation is unclear.

**PI3’K Regulates Foxo Activation through Its Downstream Kinases AKT and SGK**

The Phosphoinositide 3 Kinase (PI3’K) pathway is a major regulator of Foxo activation. Activation of PI3’K leads to phosphorylation and activation of downstream targets including Protein Kinase B (also known as AKT) and Serum and Glucocorticoid inducible Kinase(SGK). AKT and SGK are both capable of recognizing the same motifs for substrate phosphorylation, RXRXXS/T, and are responsible for phosphorylation of Foxo at 3 key residues: Thr32, Ser253 and Ser315 (of Foxo3a). When Foxo is phosphorylated at these residues it associates with 14-3-3 proteins and is exported from the nucleus, where it is transcriptionally inactive\(^7,153,154\).
While AKT and SGK are capable of phosphorylating the same residues, there does seem to be some preference for specific sites between these two kinases. In particular, the C-terminal most phosphorylation site, Ser315, seems to be preferentially phosphorylated by SGK, while Ser253 is preferentially phosphorylated by AKT\textsuperscript{155}. The N-terminal Thr32 is efficiently phosphorylated by both kinases. Experiments using dominant negative forms of these two kinases have demonstrated that disruption of either AKT or SGK kinase activity leads to nuclear Foxo, suggesting a non-redundant role for these kinases in efficiently phosphorylating Foxo at these three sites and targeting it to the cytoplasm.

Each of these three phosphorylation sites appears to mediate its own important function in the nuclear export of Foxo. The most N-terminal phosphorylation site, Thr32, is involved in Foxo association with 14-3-3, which is essential for Foxo nuclear export. The central phosphorylation site at Ser253 is located in the Foxo Nuclear Localization Sequence (NLS) and acts to disrupt NLS activity by introducing a negative charge to the basic NLS region. This prevents re-entry of Foxo into the nucleus. The C-terminal most phosphorylation site, Ser315, plays a key role in the rate of nuclear export. Phosphorylation of Ser315 un masks the Foxo Nuclear Export Sequence (NES) thus increasing its rate of nuclear export. The importance of this residue is demonstrated by Foxo6, which lacks this phosphorylation site, and unlike the other three Foxos, is predominantly nuclear\textsuperscript{156}. Nuclear export of Foxo is an active process that depends on the association between CRM1 and Foxo. Treatment of cells with the CRM1 specific inhibitor leptomycin B (which covalently modifies the CRM1 NES binding domain) results in a robust nuclear localization of Foxo even when Foxo is phosphorylated at Thr32, Ser253, and Ser315\textsuperscript{153}.
Acetylation of Foxo influences AKT phosphorylation at Ser-253. Foxo mutants that are acetylation-mimetic at Foxo lysine residues 242, 245, and 262 become highly phosphorylated under basal growth conditions. Similarly, phosphorylation of wild type Foxo (Ser253) increases with deacetylase inhibitor treatment, while phosphorylation at Ser253 of non-acetylatable mutants is unaffected by deacetylase inhibitors. Acetylation of Foxo by the histone acetyl transferases CBP and p300 inhibit Foxo DNA binding capacity in vitro. Phosphorylation of Foxo at Thr32 disrupts the interaction between Foxo and CBP/p300. These data suggest a model in which acetylation inhibits Foxo transcription in a sequential manner. Once Foxo is acetylated on lysine 242, 245, and 262, its DNA binding capacity decreases. Acetylated Foxo, which does not interact with DNA, is a superior substrate for phosphorylation by AKT at nuclear export sites. Phospho Thr32/Ser253 Foxo loses the interaction between Foxo and CBP/p300 and thus negates the favorable chromatin remodeling that this interaction promotes.

The ability of PI3′K to activate AKT and SGK is negatively regulated by the lipid phosphatase, phosphatase and tensin homolog (PTEN) which degrades PIP3 thus inhibiting PI3′K. Inactivation of PTEN is common in glioblastomas and prostate cancer. Similarly, decreased PTEN activity has been characterized in breast cancer. The ability of this pathway to robustly control Foxo nuclear localization helps explain the oncogenic properties of PTEN. Normally, PTEN hydorlyzes PIP3, the product of PI3′K, and therefore decreases the activity of AKT and SGK. PTEN deficiency results in a constitutively cytoplasmic Foxo. The lack of nuclear Foxo in these cells could contribute to oncogenic transformation through a loss of the cell cycle arrest and proapoptotic capabilities of Foxo.
Phosphorylation of Ser315 by SGK Primes Foxo for CK1 Phosphorylation and Increases the Rate of Nuclear Export

Casein Kinase 1 (CK1) plays a role in cell differentiation and circadian rhythm control and its dysregulation can contribute to neurodegeneration and cancer development. CK1 can phosphorylate Ser318 and Ser321 but only after phosphorylation of Ser315 by SGK. Phosphorylation of Ser315 by SGK creates a consensus sequence for CK1 phosphorylation, S/T(P)XXS/T, at Ser318 which in turn creates a CK1 consensus sequence at Ser321. Ser to Ala mutations and phosphospecific antibodies have been used to demonstrate that phosphorylation at these residues is, in fact, hierarchical, with phosphorylation of Ser315 by SGK being necessary for the subsequent phosphorylation of Ser318 and Ser321 by CK1. As mutations at any of these sites have no effect on nuclear import rates; they probably function by increasing nuclear export. It is thought that in conjunction with Ser325 this grouping of highly phosphorylated residues creates an acidic patch that increases Foxo’s association with its nuclear export complex.

Dual Specificity Tyrosine Phosphorylated and Regulated Kinase 1A (DYRK1A) Increases Nuclear Export of Foxo

DYRK1A is a serine/threonine kinase found in both the nuclear and cytoplasmic compartments. While some DYRK1A targets are known, a detailed understanding of its cellular functions is lacking. Decreased levels of the Drosophila ortholog, MiniBrain Kinase, results in decreased brain size suggesting a role in the regulation of neurogenesis. Based on its chromosomal location, and the role of MiniBrain, DYRK1A has been implicated in the pathogenesis of Down syndrome. DYRK1A has been shown to phosphorylate Foxo at Ser325.
Phosphorylation at this residue decreases the amount of Foxo in the nuclear fraction and accordingly decreases Foxo transcriptional activity. DYRK1A activity is SGK-and-CK1-independent and a Ser to Ala mutation at Ser325 increases the amount of nuclear Foxo\textsuperscript{165}. Although DYRK1A activity is PI3’K independent, phosphorylation of Foxo at Ser325 by DYRK1A seems to have a synergistic effect with SGK and CK1 perhaps by enhancing the acidic patch effect that promotes Foxo to interact with its nuclear export complex.

**IKK Regulates Foxo Inactivation and Degradation in an AKT Independent Fashion**

The IкB Kinase (IKK) is best known for its regulation of the Nuclear Factor-κB family of transcription factors\textsuperscript{166}. Activation of the IKK has also been shown to inactivate Foxo. Constitutive activation of IKK has been linked to breast carcinoma, suggesting a model where constitutive activation of IKK increases pro-survival factor expression and inhibits the pro-apoptotic functions of Foxo. Hu et.al. demonstrated that the ability of IKK to induce cytoplasmic localization of Foxo is AKT independent\textsuperscript{167}. This AKT independence was characterized using mutant forms of Foxo that are non-phosphorylatable at the three AKT/SGK sites described above, as well as cell lines lacking AKT activity. Activation of IKK has been shown to induce phosphorylation of Foxo at ser644. Phosphorylation at this residue results in both nuclear exclusion and degradation of Foxo. Phosphorylation at ser644 has been shown to induce ubiquitination of Foxo and a subsequent decrease in Foxo levels that can be inhibited using the proteasomal inhibitor MG132.

**ERK Inhibits and Degrades Foxo**

The RAS-ERK pathway is a signaling cascade that regulates cellular proliferation and survival\textsuperscript{168}. Many tumors are RAS overexpressors or express a mutant, constitutively active,
form of RAS. When this is the case, ERK activity is upregulated and there is an accompanying decrease in Foxo transcriptional activity in two ways. First, ERK has been shown to phosphorylate Foxo (at Ser294, Ser344, and Ser425 on Foxo3a) and this phosphorylation results in increasing amounts of cytoplasmic Foxo. Second, Foxo phosphorylated at these three residues is less stable, and is degraded in a proteasome dependent fashion. This decreased stability may be based on its increased interaction with the E3-ligase, MDM2.  

**Foxo Activating Kinases**

**Stressors Induce Nuclear Accumulation of Foxo**

A number of different kinases (MST1, JNK, and CDK1) are capable of promoting translocation of Foxo into the nucleus under conditions of cellular stress. Once localized to the nucleus, Foxo seems to execute either a pro-survival or pro-death transcriptional pattern dependent on the cellular context and severity of the insult. While phosphorylation at any of the sites described below seems to be sufficient to induce nuclear localization, little is known about the consequences of activating these pathways together. Do these multiple stress dependent kinases represent redundant pathways of Foxo activation, thus ensuring that Foxo responds to stressors, or might they act to integrate data to help determine the severity of the stressor and thus signal the appropriate transcriptional pattern to execute?

**Oxidative Stress Activates JNK which Activates Foxo**

c-Jun N-terminal Kinase (JNK) is a kinase which is activated by cell stress, including inflammatory signals, ultraviolet light and Reactive Oxygen Species (ROS), and upon activation can increase Foxo transcription. ROS activate the small GTPase Ral, and Ral, in turn activates
JNK. JNK then phosphorylates Foxo at Thr447 and Thr451 leading to an increase in its transcriptional activity\textsuperscript{170}. The JNK dependent effect on Foxo transcription appears to be antagonized by the AKT/SGK signaling pathway. ROS are known to induce nuclear localization of Foxo even in the presence of serum (when AKT is active and would otherwise lead to cytoplasmic localization of Foxo). Nevertheless, Foxo proteins which are phosphomimetic at the JNK sites show similar association with 14-3-3 upon insulin treatment as wild type Foxo, and undergo cytoplasmic localization. This suggests that the nuclear localization of Foxo after ROS treatment is not regulated by JNK. While Foxo is regulated normally by PI3’K after JNK phosphorylation, JNK phosphorylation of Foxo makes Foxo transcriptionally more active.

**CDK1 Activates Foxo as a Key Check in the Cell Cycle**

Cyclin Dependent Kinase 1 (CDK1) phosphorylates Foxo in the forkhead domain at Ser249. CDK1 is a kinase associated with cell cycle progression, predominantly the G\textsubscript{2}/M transition. As opposed to the PI3’K/AKT pathway, phosphorylation at Ser249 by CDK1 disrupts the interaction between Foxo and 14-3-3, thus leading to its nuclear localization. Phosphorylation at this site varies with the cell cycle, with Foxo being highly phosphorylated at Ser249 during the G\textsubscript{2}/M transition (when CDK1 is most active) and relatively unphosphorylated during the G\textsubscript{0}/G\textsubscript{1} transition. The ability of CDK1 to increase Foxo transcription likely correlates with Foxo’s ability to induce expression of DNA damage repair genes such as GADD45 and genes that eliminate ROS. These genes are turned on at the G\textsubscript{2}/M transition and ensure the cell is in an optimal state before the cell cycle continues\textsuperscript{171}.

Another interesting aspect of this pathway is that aberrant activation of the cell cycle has been associated with cell death in post-mitotic neurons. For example depriving cerebellar
granule neurons of action potential firing (through blockage of calcium flux) causes cell death. This activity deprivation is also associated with an increase in CDK1 activity, leading to Foxo activation. Foxo-dependent cell death is mediated by the expression of pro-apoptotic factors such as Bim. There is some disagreement about this as some have suggested that phosphorylation at Ser249 results in inhibition of Foxo potentially leading to tumorogenesis.

**MST1 Phosphorylates Foxo Leading to its Activation**

_Mammalian Sterile 20-like kinase 1 (MST1) is known to control cell proliferation, survival and morphology_. The fly ortholog of MST1, Hippo, is a known tumor suppressor and it is suspected that MST1 has a tumor suppressor capacity as well. MST1 is capable of phosphorylating Foxo at Ser207. Once phosphorylated at this site the interaction between Foxo and 14-3-3 is disrupted and Foxo localizes to the nucleus. Phosphorylation at this site can be initiated by ROS or activity/trophic factor deprivation in neurons. Neuronal death induced by trophic factor/activity withdrawal or ROS exhibit a dependence on MST1 and Foxo. While not characterized, it is likely that this signaling pathway may also be adaptive allowing for survival depending on the severity of the insult. Insults resulting in cell death are generally studied due to the fact that they result in an easily quantifiable phenotype (i.e. death/survival). For this reason it is difficult to say definitively that this pathway is adaptive when less severe insults are administered, but the Foxo transcriptome would suggest that this is the case.

**Kinases That Dictate a Transcriptional Profile**

Foxo is the transcriptional regulator of a group of proteins whose cellular functions, when considered as a whole, are not directed towards a single purpose. For example Foxo is the
regulator of GADD45a and manganese superoxide dismutase, which are involved in DNA damage repair and ROS scavenging respectively. Yet Foxo also regulates expression of FAS-ligand and BIM which are involved in apoptosis\textsuperscript{177}. These four proteins would likely never be activated in unison as there is no clear purpose in clearing cellular ROS and repairing DNA in a cell which has initiated an apoptotic pathway. This suggests that Foxo is capable of integrating cellular signals in order to select the transcriptional profile suited to affect a specific outcome. This is analogous to the cellular function of the transcription factor p53, which acts to initiate cell survival and DNA damage repair in the case of mild insults but initiates cell death when insults are severe\textsuperscript{178}.

It is clear that post-translational modifications play an important role in specifying the transcriptional outputs of Foxo. It has been technically challenging thus far to profile the Foxo transcriptome in the case of every post translational modification due to the sheer number of potential modifications and pathways that act upon Foxo. While it is likely that some of the aforementioned inhibitory and activating kinases are specifying more than an on/off type signal, AMPK is the only kinase whose effect on Foxo’s transcriptional profile has been well characterized.

**AMPK acts to Integrate Data on Energy Levels into Foxo Transcriptional Activity**

The AMP-activated Protein Kinase (AMPK) is a key regulator of energy homeostasis in cells. It is regulated by the ratio of AMP to ATP in a cell, with higher AMP/ATP ratios resulting in AMPK activation. AMPK can phosphorylate Foxo at six sites. While phosphorylation at these sites appears not to have any effect on the localization or overall activity of Foxo, these sites do influence the transcriptional profile of Foxo. Microarray studies of cells expressing mutant Foxo
that is non-phosphorylatable at the six AMPK sites has revealed a transcript profile in which genes involved in ROS resistance are up regulated\textsuperscript{179}. Simultaneously, others are turned down and many remain unchanged. This is particularly interesting because it represents a second layer of control dictated by post translational modification. AMPK does not regulate Foxo in a binary manner (i.e. transcriptionally active versus inactive) instead it is dictating a specific transcriptional output. The mechanism by which phosphorylation at these sites alters Foxo’s transcriptional pattern been well explored. Potential mechanisms for AMPK’s control of Foxo dependent transcription include: recruitment of additional proteins to a transcriptional complex or direction of Foxo to specific promoter regions.

**Figure 1.** Numerous residues on Foxo are regulated via phosphorylation. These sites influence Foxo’s subcellular localization, transcriptional potency and specify gene targets for activation.

*This SGK site is not present in Foxo6. ** This JNK site is only present in Foxo4.

**Acetylation of Foxo**
The effect of acetylation on Foxo activity represents a complex balance between histone deacetylases (hDACs) such as Silent Information Regulator 2 (SIRT1) and (SIRT2) and the Histone Acetyl Transfersases (HATs) CREB Binding Protein (CBP) and p300. The histone acetyltransferase activity of the coactivators CBP/p300 could enhance Foxo transcription indirectly through their ability to acetylate histones and favorably remodel chromatin. In contrast, acetylation of Foxo has been shown to inhibit its DNA binding capacity\textsuperscript{157}. This negative feedback mechanism is most likely offset by the activity of SIRT1 and 2 which are capable of deacetylating Foxo. Monoubiquitination of Foxo lysines which can prevent acetylation, should both keep Foxo non-acetylated and extend its association with DNA promoter regions.

**SIRT1 and 2**

SIRT1 and SIRT2 are members of the sirtuin family of deacetylases and are the mammalian homologs of the yeast deacetylase Sir2. In yeast, Sir2 plays an important role in organismal longevity through histone deacetylation and gene silencing. Deacetylation of other contributory proteins by Sir2, such as acetyl-coenzyme A, may also play a role in longevity through regulation of intermediary metabolism\textsuperscript{180}. In mammals, SIRT1 and SIRT2 interact with Foxo in a ROS-dependent manner. ROS promotes nuclear localization of Foxo and nuclear localization of Foxo is necessary for the Foxo SIRT1 interaction since SIRT1 is constitutively nuclear. Yet, nuclear localization alone is not sufficient to initiate the Foxo-SIRT1 interaction; cells must be exposed to increased levels of ROS to induce this protein-protein interaction. SIRT2 on the other hand shuttles between the nucleus and the cytoplasm, so it is conceivable that SIRT2 could deacetylase Foxo in the nucleus or the cytoplasm.
Based on the work of several groups, it appears that deacetylation of Foxo by SIRT1 imparts transcriptional specificity, shifting Foxo’s transcriptional profile away from apoptosis and towards expression of survival promoting genes\textsuperscript{181-184}. This is consistent with findings that inhibition of SIRT1 or disruption of its interaction with Foxo promotes the expression of apoptotic genes controlled by Foxo in prostate cancer derived cell lines\textsuperscript{185,186}. Experiments in mouse embryonic fibroblasts that are null for SIRT1 suggest that deacetylation of Foxo by SIRT1 selectively increases the expression of ROS resistance genes while having little effect on pro-apoptotic gene expression\textsuperscript{181}. SIRT1 deacetylation of Foxo also seems to increase the ability of Foxo to induce cell cycle arrest. These two findings taken together suggest the role for deacetylation of Foxo, by SIRT1, is to pause the cell cycle in order to allow cells to repair DNA damage and remove ROS prior to cell division. SIRT2 has also been reported to upregulate transcription of Foxo targets that allow cells to cope with ROS, such as p27\textsuperscript{kip} and manganese superoxide dismutase\textsuperscript{187}. In addition over expression of SIRT2 is capable of inducing transcription of the pro-apoptotic factor Bim\textsuperscript{188}. This scenario is quite reminiscent of p53\textsuperscript{178}.

**CBP/p300**

The HATs CBP and p300 have two distinct activities on Foxo transcription. Acetylation of Foxo by CBP/p300 results in a decrease in Foxo transcriptional activity. CBP is able to acetylate Foxo at Lys242, 245 and 262\textsuperscript{157}. These residues are all located in the DNA binding domain of Foxo, and in vitro experiments using mutants which mimic the acetylated state at these residues (by replacing the basic lysine residues with Alanines or Glutamines) have severely diminished DNA binding capacity \textit{in vitro}\textsuperscript{157}. This is a mechanism through which CBP/p300 can act to inhibit Foxo-dependent transcription.
The second activity of CBP/p300 is to acetylate histones in a manner that allows Foxo increased access to promoter regions\(^\text{189}\). One example of this activity is during treatment of cells with ROS. Treatment with ROS has been shown to increase the association of CBP/p300 with Foxo. This interaction has been shown to be mediated by a redox sensitive disulfide bridge that covalently links Foxo to CBP/p300 through Cys477 on Foxo\(^\text{190}\). This could potentially lead to acetylation of Foxo and decreased levels of Foxo transcription, yet ROS are known to increase the expression levels of many Foxo regulated genes. The most probable explanation for this result is that CBP/p300 acetylation of histones favorably remodels chromatin while SIRT1/2 prevents acetylation of Foxo and allows it to bind DNA. Thus while CBP/p300 can act as repressors of Foxo through their protein acetyltransferase activity, they also act as coactivators, increasing Foxo transcription through their histone acetyltransferase activity. A structural representation of how CBP/p300 binds Foxo and acts as a coactivator to increase Foxo transcription has recently been solved\(^\text{189}\).

**Ubiquitination of Foxo**

Ubiquitination plays a key role in the regulation of Foxo proteins. Foxo, like many other proteins, is targeted for proteasomal degradation through polyubiquitination. In addition to this canonical role for ubiquitination in protein degradation, monoubiquitination also plays a role in Foxo response to ROS. This is achieved by controlling transcriptional activity and localization of Foxo. This suggests a complex role for ubiquitination in Foxo signaling. Monoubiquitination of Foxo leads to activation of its transcriptional activity. This is balanced by polyubiquitination which leads to degradation – essentially depression of Foxo transcription through degradation. Therefore, from the monoubiquitinated state, polyubiquitination can be used for long term
inactivation of Foxo transcription or deubiquitination can occur, allowing for quick reactivation of Foxo if necessary.

**Monoubiquitination by MDM2**

Exposure of cells to ROS results in monoubiquitination of Foxo at multiple sites. This monoubiquitination is lost upon treatment of cells with MDM2 siRNA suggesting this E3 ligase directly ubiquitinates Foxo. Expression of Foxo-ubiquitin fusion constructs suggest that monoubiquitination of Foxo results in an increase in nuclear localization of Foxo as well enhancement of Foxo transcriptional activity. While MDM2 likely acts as a monoubiquitinating E3 ligase for Foxo, this event probably primes Foxo for branching ubiquitination by SKP2. This is suggested by the observation that very high levels of MDM2 expression (or phosphorylation of Foxo by ERK), which increases MDM2-Foxo interaction, results in decreased levels of Foxo protein\(^{191}\).

Mutant Foxo constructs and treatment with deacetylase inhibitors suggests that the lysine residues that are ubiquitinated are the same residues that are acetylated by CBP/p300\(^{192}\). Under some circumstances a competition exists between the ubiquitin ligases and acetylases for these residues. This is potentially important as it suggests a posttranslationally modified state of Foxo where acetylases could interact with Foxo as cofactors (allowing optimal chromatin structure) without acetylating Foxo (which would inhibit its transcriptional activity). It is important to note that this state is independent of SIRT deacetylation.

**Deubiquitination by USP7**
Deubiquitination of Foxo is mediated by the deubiquitinating enzyme USP7. Foxo has been shown to interact with USP7 both through yeast two hybrid and immunoprecipitation experiments. This interaction is enhanced by increased levels of ROS with kinetics which are slower than the monoubiquitination of Foxo\textsuperscript{192}. These observations suggest that Foxo exists in a dynamic equilibrium: Foxo monoubiquitination by MDM2 is induced by ROS, and this monoubiquitination is offset by USP7-dependent deubiquitination. Deubiquitinated Foxo is less transcriptionally active, thus USP7 acts as a negative regulator on Foxo transcription. While USP7 inhibits Foxo through deubiquitination it has no effect on protein levels of Foxo reinforcing the view that monoubiquitination of Foxo acts independent of protein degradation.

**Polyubiquitination by SKP2**

SKP2 physically interacts with Foxo in a phosphorylation-dependent manner. Phosphorylation of Foxo at Ser253 (preferentially phosphorylated by AKT) is required for Foxo SKP2 interaction and its subsequent ubiquitination\textsuperscript{193}. SKP2 polyubiquitinates Foxo after binding and the ability of SKP2 to decrease Foxo protein levels is proteasome-dependent (it is abolished by MG132 treatment\textsuperscript{194}). Elevated SKP2 levels are found in a wide variety of human cancers, and overexpression of this protein in mice leads to tumor formation. The ability to degrade Foxo (and thus eliminate its pro-apoptotic activity) may help explain the oncogenic properties of SKP2. This is also consistent with the oncogenic properties of PTEN deficiency. The loss of PTEN activity enhances AKT signaling and phosphorylation of Foxo at Ser253. This not only promotes nuclear exclusion of Foxo but polyubiquitination by SKP2 which leads to degradation of Foxo. This link between SKP2 and AKT phosphorylation also helps to explain the capacity of PTEN deficiency to induce tumorogenesis.
Figure 2. The acetylases CBP and p300 play an important role in chromatin remodeling, allowing Foxo to effectively transcribe its targets. Interestingly CBP/p300 also have an inhibitory effect on Foxo DNA binding through acetylation of the Foxo DNA binding domain. This negative regulatory effect is counteracted by the histone deacetylases SIRT1 and SIRT2. Foxo acetylation can also potentially be prevented through monoubiquitination of the lysines in Foxo’s DNA.
bidning domain. Monoubiquitination also primes Foxo for potential polyubiquitination and eventual degradation.

**Foxo Cofactors and Foxo as a Cofactor**

Foxo transcriptional activity can be affected by cofactors. These cofactors may have a general effect on Foxo transcriptional activity or they may confer Foxo transcriptional specificity. In addition, Foxo acts as a cofactor for a number of other transcription factors, affecting their transcriptional activity independently of Foxo’s own DNA binding activity. This increases the scope of Foxo activity beyond the already impressively large number of Foxo transcriptional processes.

**β-Catenin Increases Foxo Transcriptional Activity**

β-Catenin directly binds to Foxo and increases its transcriptional activity. ROS increase the interaction between β-catenin and Foxo\(^{195}\). This interaction may play a role in determining whether cells progress through the cell cycle or arrest. In the absence of ROS, β-catenin interacts with members of the T cell factor (TCF) family of transcription factors, which act to promote progression of the cell cycle. In contrast, increased Foxo transcription results in higher levels of cell cycle inhibitors such as p27\(^{kip}\). In this way the ROS dependent interaction between Foxo and β-catenin may act like a switch, allowing cells to pause and clear harmful ROS when Foxo and β-catenin interact. When oxidative stress subsides, β-catenin preferentially interacts with TCF, allowing cells to proceed through the cell cycle.
Foxo acts as a Corepressor of HIF1

As mentioned before, PTEN deficiency results in a lack of nuclear Foxo and is associated with tumorogenesis. PTEN-deficient tumors are known to be quite aggressive and highly vascular. This knowledge led to a study of the effects of PTEN on Hypoxia Inducible Factor 1 (HIF1) a key transcription factor controlling the expression of many angiogenic genes. PTEN alters HIF1 transcriptional activation via Foxo’s cellular localization\textsuperscript{196}. Even in PTEN deficient cells, if Foxo is localized to the nucleus, then HIF1 transactivation is inhibited. This is accomplished by Foxo complexing with HIF1 and interfering with HIF1’s interaction with its coactivator p300\textsuperscript{196}. This discovery adds to the complexity of Foxo’s anticancer properties. In addition to affecting cell cycle progression, clearing ROS, and proapoptotic capacity, Foxo also seems to act as a repressor of the HIF1 transcription factor and neovascularization.

Foxo is a Corepressor of the Androgen Receptor

Much like its effect on HIF-1, Foxo is capable of repressing the transcriptional activity of the Androgen Receptor (AR). This inhibition is not dependent on the transcriptional capacity of Foxo but simply on its nuclear localization. Transcriptionally inactive Foxo (mutant in its DNA binding domain) inhibits AR as robustly as the wild type protein\textsuperscript{197}. Much like HIF-1, Foxo and the AR form a complex on AR promoter regions. HDAC3 is also a part of this complex and seems to play an important role in Foxo’s ability to inhibit AR transcription. Foxo inhibition of the AR prevents both adrenergic and non-adrenergic activation of the AR\textsuperscript{197}.

Foxo Acts as a Coactivator of Ets-1

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There are 15 consensus phosphorylation sites on Foxo which are predicted to be regulated by members of the Mitogen Activated Protein Kinase (MAPK) family. Nine of the serine residues are capable of being phosphorylated \textit{in vivo} by ERK and of these, five can also be phosphorylated by p38. Mutational analysis has revealed that phosphorylation at these sites can influence Foxo interaction with another transcription factor, Ets-1. Foxo interaction with Ets-1 leads to an increase in Ets-1 transcriptional activity as assessed by Ets-1 activity on the Flk-1 promoter, which promotes angiogenesis. Foxo has no activity at the Flk-1 promoter region but Ser to Ala mutations at the putative ERK/p38 sites that disrupt the Foxo-Ets-1 interaction greatly diminish Ets-1 interaction with Flk-1\textsuperscript{198}. This suggests that Foxo can act as a cofactor for other transcription factors indicating an even broader role of Foxo in transcriptional regulation. As discussed previously, phosphorylation of Foxo by ERK decreases Foxo stability potentially acting as a negative regulator of this signaling pathway.

**Foxo Signaling as a Potential Therapeutic Target for Disease**

As of yet, the only clinical use of drugs that target the Foxo signaling pathway is in cancer. Inactivation of Foxo through aberrant PTEN, AKT, IKK or ERK signaling have all been shown to promote tumorigenesis. A number of chemotherapeutic drugs have been shown to mediate their activity at least in part through restoring Foxo activity to cells, inducing Foxo pro-apoptotic target genes such as Bim. For example, Paclitaxel is known to increase Foxo activity through repression of AKT and induction of JNK signaling which cooperatively lead to nuclear localization and activation of Foxo\textsuperscript{152}. While no drugs that function through the Foxo signaling pathway are currently available for treating diseases other than cancer, some research has suggested that under certain circumstances promoting Foxo signaling may be used in a pro-
survival manner as well. The marine sponge compound Psammaplysene A has been shown to be neuroprotective both *in vitro* and *in vivo* against neurotoxic insults\textsuperscript{124}. This reinforces the observation that induction of Foxo can have varying activity based on the cellular context of Foxo activation. This raises the intriguing possibility of developing drugs in the future which act on Foxo to modulate its transcriptional profile. Current drug strategies take advantage of the fact that Foxo, through the integration of various posttranslational modifications, is able to activate pro-survival or pro-death genes depending on the state of cell. If we fully understood the signaling pathways that mediate Foxo gene expression choice, we could develop more potent drugs that not only activate Foxo, but ensure that it is transcribing only those genes which will give the greatest benefit in a specific disease state.

**Appendix 2**


**FOXO3a is broadly neuroprotective in vitro and in vivo against insults implicated in motor neuron diseases.**

Jelena Mojsilovic-Petrovic\textsuperscript{1}, Natalia Nedelsky\textsuperscript{2}, Marco Boccitto\textsuperscript{1}, Itzhak Mano\textsuperscript{3}, Savvas N. Georgiades\textsuperscript{4}, Weiguo Zhou\textsuperscript{1}, Yuhong Liu\textsuperscript{5}, Rachael L. Neve\textsuperscript{6}, J. Paul Taylor\textsuperscript{2}, Monica Driscoll\textsuperscript{3}, Jon Clardy\textsuperscript{4}, Diane Merry\textsuperscript{5} and Robert G. Kalb\textsuperscript{1,2*}

\textsuperscript{1}Department of Pediatrics, Division of Neurology, Abramson Research Center, Children’s Hospital of Philadelphia, 3416 Civic Center Boulevard, Philadelphia, PA 19104; \textsuperscript{2}Department of Neurology, University of Pennsylvania School of Medicine, 3400 Spruce Street, Philadelphia, PA 19104; \textsuperscript{3}Department of Molecular Biology and Biochemistry, Nelson Biological Labs., Rm A232,
Abstract:

Aging is a risk factor for the development of adult-onset neuro-degenerative diseases. While some of the molecular pathways regulating longevity and stress resistance in lower organisms are defined (i.e., those activating the transcriptional regulators DAF-16 and HSF-1 in *C. elegans*), their relevance to mammals and disease susceptibility are unknown. We studied the signaling controlled by the mammalian homolog of DAF-16, FOXO3a, in model systems of motor neuron disease. Neuron death elicited *in vitro* by excitotoxic insult or the expression of mutant SOD1, mutant p150<sub>glued</sub> or polyQ-expanded androgen receptor was abrogated by expression of nuclear-targeted FOXO3a. We identify a compound (Psammaplysene A, PA) that increases nuclear localization of FOXO3a *in vitro* and *in vivo* and show that PA also protects against these insults *in vitro*. Administration of PA to invertebrate model systems of neurodegeneration similarly blocked neuron death in a DAF-16/FOXO3a-dependent manner. These results indicate that activation of the DAF-16/FOXO3a pathway, genetically or pharmacologically, confers protection against the known causes of motor neuron diseases.

Introduction:

Although motor neuron diseases due to single gene mutations are unusual (~10% of cases), the affected genes have been successfully used to model these diseases in experimental contexts.
systems. Rare, genetic forms of motor neuron disease that arise from mutations in superoxide dismutase (SOD) or p150glued have a disease phenotype that strongly resembles sporadic ALS with lower motor neuron predominance. Another predominantly lower motor neuron disease, called spinobulbar muscular atrophy (SBMA or Kennedy’s Disease) is due to a polyglutamine expansion in the androgen receptor. Despite identification of the “disease protein”, the underlying pathogenic mechanism(s) remain incompletely understood.

In most cases of sporadic ALS, motor neuron death is triggered by the interaction of a genetic pre-disposition and environmental factors. Genome-wide association studies have failed to reveal consistent susceptibility loci. Correlative evidence suggests that aging is a risk factor for the development of ALS as well as other adult-onset neurodegenerative disorders. Studies from a variety of experimental systems have provided insight into the genetic factors controlling aging, in particular, the insulin/insulin-like growth factor signaling pathway. In Caenorhabditis elegans, hypomorphic alleles of the daf-2 gene (mammalian homolog, insulin/insulin-like growth factor receptor) and the downstream signaling molecule age-1 (mammalian homolog, phosphotidylinositol-3’-kinase, PI3’K) promote longevity and lifespan extension. These effects require the activity of the DAF-16 transcription factor (mammalian homolog, FOXO3a).

DAF-16/FOXO3a shuttles between the cytoplasm (where it is inactive) and the nucleus in a process that is controlled by its phosphorylation state. Phosphorylation of DAF-16/FOXO3a by the PI3’K substrate kinases Akt and SGK leads to the 14-3-3 protein-dependent export of nuclear DAF-16/FOXO3a and re-entry into the nucleus requires dephosphorylation and release of 14-3-3. Within the nucleus, DAF-16/FOXO3a leads to the expression of a number
of genes that have context-dependent effects on cellular physiology. Expression of a constitutively nuclear FOXO3a can promote the death of purified motor neurons and cerebellar granule cells and this has been linked to the expression of FasL. In contrast, active FOXO3a protects a variety of quiescent cells against death evoked by oxidative stress or glucose deprivation and this has been linked to the expression of manganese superoxide dismutase (MnSOD) and catalase. Thymocyte survival and differentiation is FOXO3a-dependent. Differences in beneficial versus harmful effects of FOXO3a probably relate to cell-circumstance-specific level of activation and post-translational modifications.

Some of the stresses that contribute to motor neuron death in ALS include excitotoxicity, reactive oxygen species, accumulation of insoluble aggregates of neurofilaments, and defects in axonal transport. Since DAF-16/FOXO3a-dependent gene transcription, in some contexts, combats cellular stresses, we inquired whether manipulating FOXO3a signaling protected neurons from insults relevant to motor neuron diseases. We show FOXO3a activation is neuroprotective across phyla and identify a toxicity-sparing pharmacological approach for enhancement of DAF-16/FOXO3a signaling activation.

**Methods:**

Source of Reagents: Trophic factors (ciliary neuronotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), neurotrophin 4 (NT 4), cardiotrophin 1 (CT 1) and glial-derived neurotrophic factor (GDNF)) were obtained from Alomone Labs (Jerusalem, Israel). Psammaplysene A was synthesized as described. All other reagents were obtained from Sigma (St. Louis, MO) and were of the highest grade available.
Tissue Culture: Embryonic Sprague-dawley rat spinal cord neurons were grown on confluent monolayers of cortical astrocytes, as previously described. The substratum was acid washed glass coverslips when imaging was performed and Primaria tissue culture plasticware (Falcon, Becton-Dickinson) when biochemistry was performed. Culture media consisted of astrocyte-conditioned media supplemented with 10 ng/ml CNTF, BDNF, NT 4, CT 1 and GDNF and 50% of media was replaced with fresh media every 3 days.

Male SBMA mice transgenic for the prion protein promoter-driven androgen receptor cDNA containing an expanded (112) CAG repeat tract were mated with non-transgenic C57Bl/6 female mice. Dissociated, mixed spinal cord cultures obtained from 13.5-day embryos were grown for 3 weeks in conditioned medium (conditioned on normal mouse astrocytes) containing charcoal-stripped serum to remove hormones. At 3 weeks, motor neurons were well differentiated and distinguishable from other neurons, including sensory neurons, by size and morphology. At this time, cultures were treated with indicated conditions for 7 days; cells were then fixed with 4% paraformaldehyde and immunostained using antibodies to neurofilament heavy chain (NF-H) (SMI32; Sternberger Monoclonal Inc.). Motor neurons were visualized using a Leica DMR fluorescence microscope, photographed, and analyzed using IP Labs software. Statistical analyses of results were carried out using Student's t-test (viral infections) or ANOVA (compound treatments; SigmaStat).

Recombinant HSV: cDNAs were cloned into the PrpUC amplicon plasmid to generate recombinant HSV as previously described. The titer of virus used in these studies was routinely 3-5 x 10^7 plaque-forming units/ml. The sources of constructs were: Michael
Greenberg, Harvard University (HA-tagged wild type and triple mutant human FOXO3a), David Borchelt, University of Florida (WT and G85R mutant SOD), Erika Holzbaur, University of Pennsylvania (WT and mutant p150\textsuperscript{glued}) and Anne Brunet (3xFHRE-luciferase).

Excitotoxicity Assay: After 14 days \textit{in vitro} (DIV), culture media was removed (and saved) and cells were exposed to 100 µM kainic acid (KA) for 1 hour. Subsequently they were washed three times in Locke’s buffer not containing KA, the original media was replaced and incubated for another 24 hours at 37 °C. in 5% CO\textsubscript{2} before fixation in 4% paraformaldehyde. Motor neurons were identified in mixed culture by immunostaining for nonphosphorylated neurofilaments and counting only labeled cells with cell body diameter of 25 µm or greater. We have previously validated this method as a means of specifically recognizing motor neurons (Figure 1 in \textsuperscript{123}). In experiments involving recombinant HSV, 1 µL of viral stock was added to 1mL of culture media 24 hours or more prior to the next manipulation. Tubes containing viruses were color coded so that the operator was blinded to the specific virus used.

Quantification of motor neurons: The # of immunostained cells were counted in 3 randomly selected fields/coverslip and the mean value obtained. In each experiment 3+ independent coverslips were used per condition and the results presented were obtained for 4+ independent cultures and experiments.
Immunocytochemistry: Tissue culture cells were fixed in freshly prepared 4% paraformaldehyde in 0.1 M pH 7.4 phosphate buffer for 30 minutes prior to extensive washing in phosphate buffered saline. Overnight incubation with primary antibody was performed at room temperature and after washing, coverslips were incubated with Alexafluor conjugated secondary antibody (2 – 4 hrs.). When double labeling experiments were performed, species-specific secondary antibodies with distinct emission spectra were employed. Coverslips were washed prior to mounting in PermaFluor (Thermo Electron Corporation) and viewing on an Olympus FV300 Fluoview laser confocal microscope.

Western Blots and quantification: Cultures were lysed in NP-40 lysis buffer (1% NP-40, 40 mM Tris pH = 7.4, 0.15 M NaCl, 10% glycerol, 0.1% SDS, 0.1% deoxycholate + protease inhibitors and phosphatase inhibitors), sonicated, particulate matter removed by centrifugation and subjected to PAGE-SDS prior to transfer to nitrocellose. Equal amounts of protein (determined using BCA reagents from Pierce) were loaded in each lane. After blocking in 5% milk in phosphate buffer saline, membranes were incubated in primary antibody overnight, washed, incubated with secondary antibody, washed and visualized to GE Healthcare (Buckinghamshire, U.K.) chemiluminescent substrate according to the manufacturers directions. Densitometric analysis of films was obtained using TINA (Isotopenmeßgeräte, GmbH) from 4+ independent experiments, the results averaged and mean values ± S.E. formed the basis of the statistical comparisons. Quantitative data on band intensity was expressed as the fold change in comparison with values of HSV-LacZ infected cultures. The displayed western blot data are representative of the results obtained from at least 4 independent cultures. In some
experiments the LI-COR Odysseus system was used for visualizing and quantifying western blot bands. Secondary antibodies were from LI-COR Biosciences (IR 800 or 680 goat anti-mouse IgG or anti-rabbit IgG). The source of primary antibodies was: (Calbiochem, Oncogene Research Products, rabbit anti-MnSOD (Stressgen Bioreagents, Victoria, British Columbia, Canada), rabbit anti-FKHRL1/FOXO3a and anti-phospho FKHRL1/FOXO3a (T32) (Upstate, Lake Placid, NY), anti-phosphoFOXO3a (S253) (Abcam) and rabbit anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA). Species-specific HRP-conjugated secondary antibodies were from Amersham (GE Healthcare, Buckinghamshire, U.K.) and Alexa 488-conjugated anti rabbit secondary antibody from Molecular Probes, Invitrogen (Eugene, OR).

Subcellular fractionation

Nuclear protein lysate was prepared using Sigma N-Xtract Tm kit. Briefly, cells collected from one 60 mm dish were suspended in 150 ul hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, and 10 mM KCl). After incubating on ice for 10 minutes, 10% IGEPA CA-630 was added to final concentration of 0.6%. Vortexed vigorously for 10 seconds and centrifuged immediately at 10,000-11,000g for 30 seconds. The supernatant is cytoplasmic fraction and the pellet is nuclear fraction.

Luciferase reporter assay

Human embryonic kidney 293 cells were grown to approximately 80% confluence and then transfected either 3x Fork Head Response Element Luciferase (FHRE), the parent vector lacking
the FHREs (pGL3), or pGL3 vector containing 3x Retinoic Acid Response Element (RARE-luciferase from Addgene). In all cases, the Renilla luciferase reporter construct, driven by the thymidine kinase promoter (pRL-TK), was co-transfected as an internal control for transfection efficiency and cell death. After 24 hrs the cells were trypsinized and replated into media containing the appropriate concentration of PA or vehicle. After 48 hrs the cells were trypsinized again and replated into a 96 well plate in fresh media containing PA or vehicle (12 replicates per condition). Twenty four hours later the plates were analyzed using the Dual-Glo Luciferase Assay System (Promega) on the Xenogen In Vitro Imaging System. The ratio of Luciferase signal to Renilla signal for each well was calculated and the various treatment groups were compared to vehicle.

Bioinformatics

In order to identify genes that were differentially expressed in ALS we accessed the NCBI GEO database and searched the available data sets for ALS-related microarray data. Five data sets were identified and these were analyzed using the two-tailed t-test analysis with a 0.050 significance level, as provided on the GEO site, to compare the control group to the ALS group in each of the data sets. The data from these differentially expressed genes was then downloaded into a spreadsheet and searched for genes that were previously determined to be controlled by FOXO. Genes that were present in both lists were added to the Supplement Table and their direction of regulation was indicated.

Study of mice. B6SJL-Tg(SOD1-G93A)1Gur/J mice were obtained from Jackson labs and were bred to the F1 generation of C57Bl/6 x SJL mice. The offspring of this cross was used for all of
the biochemical studies (n=4 in each experimental group). Lumbar spinal cord was obtained at P87, frozen on dry ice until use. The spinal cord segments were homogenized with a dounce in NP-40 buffer as above (10:1 volume to weight). All other manipulation of the lysates was as described tissue culture cells.

Study of *Drosophila*

*Drosophila* stocks were crossed on standard cornmeal agar media at 29°C. Food was supplemented with DHT (Steraloids) and Psammaplysene A once it had cooled to <50 °C, to final concentrations of 1 mM and 0.5 mM, respectively. Eye phenotypes of female flies of each condition were examined and blindly scored according to the criteria described with one scoring modification to increase sensitivity to differences in affected eye areas. Flies haploinsufficient for dFOXO were generated by mating GMR-GAL4, AR52Q flies to the following fly stock lines: foxo^{B6G1018} (Bloomington stock 12530); foxo^{21}, foxo^{25} and foxo^{c01841} (Exelisis, Harvard).

Study of *C. elegans*

The following *C. elegans* strains were obtained from the *C. elegans* Genetic Center (biosci.umn.edu/CGC) or constructed by us: Δglt-3: ZB1096 glt-3(bz34) IV; nuls5: KP742[glr-1::gfp; glr-1::G_{ex}(Q227L) V; lin-15(+)]; Δglt-3; nuls5: ZB1102 glt-3(bz34) IV; nuls5 V; age-1; Δglt-3; nuls5 Strain ZB1102 glt-3(bz34) IV; nuls5 V was crossed with strain BE13 sqt-1(sc13) II. sqt-1 was then kicked out and replaced by age-1 using strain TJ1052 age-1(hx546) II. P_{myo-3::daf-16::gfp}
Strain ZB2283 was produced by Jian Xue and Carolina Ibanez-Ventoso by making transgenic animals carrying an extra-chromosomal array containing $P_{myo-3}$: $daf-16$:gfp.

For PA treatment, we soaked the diluted drug into standard NGM culture plates. We transferred freshly growing nematode cultures to these plates and allowed them to grow for 2 days before assessing the effect. To determine the ratio of nuclear vs. cytoplasmic DAF-16 levels we used a strain transgenic for $P_{myo-3}$: $daf-16$:gfp, where the DAF-16::GFP reporter is expressed in body wall muscle cells. We compared the intensity of GFP labeling in the nucleus and adjacent cytoplasm using NIH-Image. We monitored the effect of PA or age-1 on nematode excitotoxicity by measuring the extent of neurodegeneration in $\Delta glt$-3; nuls5 animals ± PA or ± age-1 mutation. We observed free-moving animals with an inverted scope under Nomarski DIC optics with no anesthetics. Swollen cells in the nerve-ring region were counted as head necrotic figures indicative of neurodegeneration.

Statistics. Pairwise comparisons employed two-tailed Students’s t-test and when three or more groups were compared we used Analysis of Variance (ANOVA) and post hoc analysis with significance set at p< 0.05.

Results:

In fibroblasts, expression of FOXO3a with alanine substitutions at three phosphorylation sites (Threonine 32, “T32”; Serine 253, “S253” and Serine 315, “S315”) leads to nuclear retention of the transcriptionally active protein. Cultures of rat spinal cord neurons were infected with recombinant HSV engineered to express the triple mutant (TM) or wild type (WT) FOXO3a. Immuno-staining for the transgene demonstrated that the WT-FOXO3a is restricted to
the neuronal cytoplasm and the TM-FOXO3a is largely nuclear (Figure 1A). The transgene products were detectable in all neurons for >5 days without any apparent toxicity. To determine if these transgenes influenced the susceptibility of motor neurons to excitotoxic insult, 14 days in vitro (DIV) mixed spinal cord cultures were infected with HSV- WT-FOXO3a, HSV- TM-FOXO3a, HSV-LacZ or no virus, the following day exposed to an excitotoxic challenge (100 µM kainic acid (KA) or vehicle for 1h) and the number of surviving motor neurons determined 24 hours later.\textsuperscript{199,200} While approximately 45% of motor neurons were killed by KA in the HSV-LacZ and HSV-WT-FOXO3a infected cultures, no KA-induced motor neuron death occurred in the HSV-TM-FOXO3a infected cultures (Figure 1B). ANOVA using transgene expression as the between-group factor and survival as the within-group factor demonstrated a significant difference between groups ($F_{(5,12)} = 15.68; p < 0.001$, ANOVA). The post-hoc comparisons between groups using Scheffé's $F$ test with significance set at $p < 0.05$ showed that significant motor neuron death only occurred in the cultures expressing the WT-FOXO3a or LacZ. Thus expression of the TM-FOXO3a protects cultured motor neurons from excitotoxic insult.

Next we explored the capacity of TM-FOXO3a to protect motor neurons in vitro from a variety of proteotoxic insults relevant to motor neuron diseases. In spinobulbar muscular atrophy a polyglutamine expansion in the androgen receptor (AR) leads to testosterone-dependent motor neuron death.\textsuperscript{121} We made cultures from spinal cord of mutant AR-expressing mice and found that a 7 day treatment with dihydrotestosterone (DHT) led to the loss of approximately 25% of motor neurons compared with the vehicle-treated cultures ($p < 0.05$) (Figure 1C). We observed an equivalent rate of DHT-dependent motor neuron death in cultures treated with HSV-WT-FOXO3a ($p < 0.05$). Infection of cultures with HSV-TM-FOXO3a,
however, completely blocked DHT-dependent death of motor neurons, with all motor neurons surviving in the presence of DHT (Figure 1C).

Proteotoxic motor neuron death can also be precipitated by expression of mutant forms of human superoxide dismutase or p150\textsuperscript{glued}\textsuperscript{201}. We asked if treating cultures expressing these mutant proteins with TM-FOXO3a affected motor neuron death. We began these studies by determining concentrations of viruses that led to co-expression of both transgenes in neurons but did not lead to toxicity owing to the viral burden. We established that infection of spinal cord neurons with \(~8 \times 10^4\) pfu of HSV-mutant SOD/ml culture media reliably induced 50% motor neuron loss 7 days post infection. Addition of \(~8 \times 10^4\) more pfu of HSV-LacZ to these wells did not exacerbate motor neuron death. Immunocytological localization studies revealed that 97 ± 2% of motor neurons expressing mutant SOD also expressed \(\beta\)-galactosidase (Figure 1D). In all subsequent studies we confirmed a >95% co-expression of the toxicity-inducing mutant protein (SOD or p150\textsuperscript{glued}) and LacZ or TM-FOXO3a. We also established that co-expression of LacZ with either WT SOD or WT p150\textsuperscript{glued} had no adverse effect on motor neuron survival. We previously have shown that this concentration of HSV-mutant SOD infects all motor neurons at this plating density\textsuperscript{120,201}.

We compared next the outcome of cultures infected with HSV-mutant SOD + HSV-LacZ or HSV-TM-FOXO3a as well as cultures infected with HSV-mutant p150\textsuperscript{glued} + HSV-LacZ or HSV-TM-FOXO3a (Figure 1E). The ANOVA indicates that statistically significant differences between groups existed (\(F_{(5,12)} = 70.14, p < 0.0001\)). Infection of cultures with HSV-mutant SOD + HSV-LacZ led to significantly lower motor neuron numbers when compared with cultures infected with HSV-mutant SOD + HSV-TM-FOXO3a (35.0 ± 4.0 versus 69.0 ± 2.0, \(p < 0.05\) in post hoc
Similarly motor neuron numbers from cultures infected with HSV- mutant p150<sub>glued</sub> + HSV-LacZ were significantly lower than motor neuron numbers from cultures infected with mutant p150<sub>glued</sub> + HSV-TM-FOXO3a (36.0 ± 4.0 versus 68.0 ± 3.0, p < 0.05 in post hoc analysis) (Figure 1E). Thus the toxicity of three different mutant proteins that cause motor neuron disease can be blocked by expression of a version of FOXO3a that constitutively resides in the nucleus.

Figure 1

Figure 1. Triple-mutant FOXO3a is retained in the nucleus and protects against excitotoxic and proteotoxic insults. Panel A. Mixed spinal cord neurons were transfected using Lipofectamine 2000 (Invitrogen) with HA-tagged wild type FOXO3a (WT-FOXO3a) or
triple mutant FOXO3a (TM-FOXO3a) and 24 hours later fixed and immunostained with anti-HA antibody and Alexa-488 conjugated secondary antibody. In the left frame, WT-FOXO3a is present in neurites and the soma cytoplasm while the nucleus appears devoid of immunoreactivity. In the right frame, TM-FOXO3a is seen largely concentrated in the nucleus while soma and neurite staining is weak. Calibration bar = 24 microns. Panel B., Mixed spinal cord cultures were maintained for 14 DIV before infection with HSV-LacZ, HSV-WT-FOXO3a, or HSV-TM-FOXO3a. 24 hrs later, the cultures underwent a 1 hr excitotoxic insult and after 24 hrs, the cultures were fixed and stained for SMI-32. The number of SMI32(+) motor neurons surviving each treatment is noted on the ordinate axis. A statistically significant reduction in motor neuron number is seen in all groups except the cultured treated with HSV-TM-FOXO3a. Panel C., 14 DIV mixed spinal cord cultures from mice engineered to express polyQ expanded AR were infected with HSV-LacZ, HSV, WT-FOXO3a, or HSV-TM-FOXO3a and then treated with DHT or vehicle. After 7 days, fixed cultures were stained and the number of motor neurons surviving was determined. A statistically significant, DHT-dependent death was found in the uninfected group and the HSV-WT-FOXO (but not HSV- TM-FOXO3a) group. Panel D., Cultures were infected with HSV-mutant SOD (green) and HSV-LacZ (red) and subsequently stained with specific antibodies. A high degree of co-localization is evident (yellow, merge image). Calibration bar = 40 microns. Panel E., The number of motor neurons surviving each treatment was determined after infecting 14 DIV cultures with combinations of HSV-LacZ, TM-FOXO3a, mutant SOD, mutant p150glued, WT-SOD or WT p150glued. Mutant SOD and mutant p150glued motor neuron death is blocked when TM-FOXO3a is expressed.

Inhibition of PI3’K should lead to accumulation of FOXO3a dephosphorylated at T32, S253 and S315 and enhanced partitioning of FOXO3a into the nucleus. Given this, we wondered if PI3’K inhibition would confer neuroprotection. We began these experiments by determining that application of the PI3’K inhibitor LY294002 (final concentration, 20 µM) every other day for six days, did not adversely affect motor neuron survival under our culture conditions (data not shown). Next we examined the biochemical effects of LY294002. For this set of experiments, the culture media was supplemented with CNTF, CT-1, NT4 and GDNF but lacked BDNF. Neurons were pre-treated with LY294002 or vehicle for 30 minutes prior to acute stimulation with BDNF (50 ng/ml) or vehicle and then lysates were prepared 30 minutes later (Figure 2A). In the absence of LY294002, BDNF evoked an increase in the phosphorylation of AKT, MAPK and T32 and S253 of FOXO3a. No changes in the abundance of the non-phosphorylated species were noted. In cultures pre-treated with LY294002, application of BDNF evoked an increase in
MAPK phosphorylation but there were no changes in the phosphorylation of AKT and T32 and S253 of FOXO3a. No changes in the abundance of the non-phosphorylated species were noted. These results suggest that LY294002 selectively inhibit the PI3′K signaling pathway and phosphorylation of FOXO3a. Next we investigated the effects of chronic LY294002 application to cultures supplemented with all five of the above trophic factors. Twenty-four hour incubation with LY-294002, in comparison with vehicle treated cultures, led to a decrease in the abundance of phosphoAKT and phosphorylation at the S253 site on FOXO3a (Figure 2B). No other changes were noted in the phosphorylated proteins studied and no changes were detected in the abundance of the non-phosphorylated species. Next we looked at the effects of 6 day incubation with LY294002 (fresh drug applied every other day – Figure 2 C). We found that incubation with LY-294002, in comparison with vehicle treated cultures, led to a decrease in the abundance of phosphoAKT, phosphoMAPK and phosphorylation of T32 and S253 of FOXO3a. In sum, these experiments indicate that inhibition of PI3′K has the desired effect of decreasing the abundance of phosphorylated FOXO3a.

To determine if LY294002 was neuroprotective, cultures were infected with either WT or mutant SOD expressing viruses and LY294002 or vehicle added to the culture media every other day for 6 days (Figure 2D). In the presence of WT SOD, equivalent levels of motor neuron survival was found in the presence or absence of LY294002. In addition, in the presence of mutant SOD, equivalent levels of motor neuron death were found in the presence or absence of LY294002. These results indicate that LY294002 is not toxic on its own and does not protect motor neurons from mutant SOD toxicity. Since LY294002 reduces FOXO3a phosphorylation but is not neuroprotective, it may be that phosphorylation of other PI3′K targets in mammalian cells offset any potential beneficial actions of dephosphorylated FOXO3a.
Figure 2. Inhibition of PI3’K reduces FOXO3a phosphorylation but does not protect against mutant SOD toxicity. Mixed spinal cord cultures were grown in the absence of BDNF and pretreated with LY294002 or vehicle prior to acute stimulation with BDNF. Thirty minutes after application of BDNF, lysates were prepared for western blot analysis. Panel A shows that LY294002 blocks the BDNF stimulated increase in P-AKT, P-MAPK and phosphorylation of FOXO3a on T32 and S253. Panel B shows the effect of a 24 hours treatment with LY294002 of cultures chronically grown in 5 trophic factors including BDNF. By western blot analysis, LY294002 suppresses P-AKT and phosphorylation of FOXO3a on S253. Panel C shows the effect of a 6 day treatment with LY294002 of cultures chronically grown in 5 trophic factors including BDNF. By western blot analysis, LY294002 suppresses P-AKT, P-MAPK and phosphorylation of FOXO3a on T32 and S253. Panel D shows the number of motor neurons surviving 6 days of infection with HSV-WT-SOD or HSV-mutant SOD grown in the presence or absence of chronic LY294002. Mutant SOD led to approximately 50% motor neuron loss and LY294002 did not abrogate the process.

A chemical-genetic screen recently reported the identification of a series of compounds that can inhibit FOXO1a nuclear export. Compounds fell into two classes: 1) inhibitors of general nuclear export machinery and 2) inhibitors specific to the FOXO1a pathway. We inquired whether compounds in the second class would also block the nuclear export of FOXO3a since they would be predicted (based on the results above) to display neuro-protective activity. We focused on Psammaplysene A (PA), which was isolated from a marine sponge, because it was the most potent of the class 2 inhibitors (Figure 3A). In addition PA was reported to have no effect on AKT phosphorylation suggesting it acted either downstream of AKT or in a synergistic pathway.
We began by looking at the effect of a synthetic sample of PA (for synthesis see \textsuperscript{110}) on the distribution of FOXO3a endogenously expressed by neurons. We biochemically isolated nuclei from spinal cord cultures treated with PA or vehicle (Figure 3B). Based on the distribution of the nuclear envelope protein lamin, it is clear that our subcellular fractionation procedure greatly enriched nuclei. There was an \approx 2.5 fold increase in the nuclear FOXO3a/nuclear lamin ratio in the PA treated cultures in comparison to vehicle-treated cultures. Total FOXO3a and lamin levels were unaffected by drug treatment. These observations indicate that PA promotes the sequestration of FOXO3a into nuclei.

We next tested the ability of PA to promote FOXO-dependent transcription. HEK 293 cells were transfected with a plasmid containing a minimal promoter containing 3x Fork Head Response Elements (3x FHRE) driving luciferase expression and then treated with various concentrations of PA or vehicle for 72 hours. As a control for a non-specific action of PA on transcription, a parallel set of cells were transfected with the parent plasmid (pGL3) that lacked the 3x FHRE. In all experiments, an internal control plasmid expressing Renilla luciferase was cotransfected and all results are expressed as the ratio Luciferase/Renilla fluorescence. PA treatment led to a dose-dependent increase in luciferase signal in 3x FHRE, but not pGL3, -expressing cells (Figure 3C). As an added control for specificity we looked at the ability of PA to enhance transcription from a 3x retinoic acid response element (RARE) luciferase construct and found that PA had no effect on this promoter. These results indicate that PA can promote FOXO transcriptional activity.

To determine if PA had neuroprotective activity, spinal cord cultures were treated with the drug (10 nM) for two days and then subjected to an excitotoxic challenge (Figure 3D). The
percent of KA-induced cell death was ~50\% in vehicle-treated cultures (65 ± 2 versus 35 ± 3 vehicle versus KA, \( p < 0.01 \), Student’s \( t \)-test) and ~3\% in PA treated cultures (67 ± 3 versus 65 ± 2 vehicle versus KA, \( p < 0.6 \), Student’s \( t \)-test) indicating that PA protected motor neurons from excitotoxic challenge. Next we looked at mutant AR proteotoxicity (Figure 3D). Significant differences between groups (\( F_{(2,6)} = 18.84 \), by ANOVA) were found in the three-way comparison of 1) No DHT, 2) DHT + vehicle, and 3) DHT + PA). The post hoc analysis demonstrated a DHT-dependent ~25\% loss of motor neurons in vehicle-treated cultures (\( p < 0.01 \)) and neuroprotection in the PA treated cultures (\( P > 0.05 \) in the comparison of no DHT versus DHT + PA).

We followed up these observations by asking if PA blocked the proteotoxicity of SOD or \( p150^{\text{glued}} \). Spinal cord cultures were infected with HSV engineered to express the WT or mutant forms of SOD or the WT or mutant forms of \( p150^{\text{glued}} \) and received PA (or vehicle) every other day for 4 days. The drug treatment had no effect on transgene expression (not shown). After 4 days, the cultures were fixed and motor neuron number was determined. ANOVA revealed statistically significant differences between groups in LacZ versus WT SOD versus mutant SOD (± PA) comparisons (\( F_{(5,12)} = 18.41, \ p < 0.001 \)) as well as LacZ versus WT \( p150^{\text{glued}} \) versus mutant \( 150^{\text{glued}} \) (± PA) comparisons (\( F_{(5,12)} = 19.26, \ p < 0.001 \)) (Figure 3E). The post hoc analysis revealed that statistically significant protection against the toxicity of mutant SOD or \( p150^{\text{glued}} \) was conferred by PA treatment on motor neuron survival. PA had no adverse effect on survival of motor neurons expressing LacZ or wild type versions of SOD or \( p150^{\text{glued}} \). Thus PA is non-toxic on its own, but can protect against four different insults \textit{in vitro} that are directly relevant to motor neuron diseases.
Figure 3. Psammalysisene A (PA) drives FOXO3a into the neuronal nucleus, promotes FOXO-dependent transcription and protects against the proteotoxicity of mutant SOD and mutant p150glued. Panel A. The chemical structure of PA. Panel B. Subcellular fractionation of DIV14 spinal cord culture lysates treated with PA or vehicle. Nuclear fraction (enriched for the nuclear marker, lamin) contains more FOXO3a in PA- versus vehicle-treated cultures. The abundance of total FOXO3a, lamin or actin is the same under both conditions. Quantification of band intensities show that PA treatment leads to a statistically significant increase in nuclear FOXO3a/nuclear lamin in comparison with vehicle treated cultures. Panel C. PA promotes transcription from a 3x FHRE-luciferase reporter in a dose-dependent manner. PA affected neither transcription from the parent vector (pGL3 that lacks the FHREs) nor from a 3x RARE luciferase reporter. There was a statistically significant difference between vehicle treatment of 3xFHRE
versus PA concentrations 10 nM (*, p < 0.05), 50 nM (**, p < 0.01) and 100nM (***, p < 0.001). Panel D. Pretreatment of mixed spinal cord cultures with PA (+PA) protected motor neurons from excitotoxic insult (left bar graph) and DHT-dependent death of polyQ expanded AR toxicity (right bar graph). Panel E. One set of 14 DIV mixed spinal cord cultures was infected with HSV-LacZ, HSV-WT-SOD or HSV-mutant SOD and a second set of cultures was infected with HSV-LacZ, HSV-WT-p150glued or HSV-mutant p150glued. Survival of SMI-32 stained motor neurons was determined 4 days later. Infected cultures were either treated with Vehicle ("- PA") or PA ("+ PA"). Mutant SOD led to a statistically significant reduction in motor neuron number (by ANOVA, see text for details) and this was prevented in PA-treated cultures. Similarly mutant p150glued led to a statistically significant reduction in motor neuron number and this was prevented in PA-treated cultures. * p < 0.05.

Although the direct molecular target of PA is unknown, we examined the effect of PA on some candidate biochemical and cell biological processes that have previously been implicated in neuron death. Neurotrophins (such as brain-derived neurotrophic factor, BDNF) can promote neuronal survival by activating its receptor, TrkB, both during development and after insult (202-204, but see 201,205) and so we wondered if PA had demonstrable effects on this signaling pathway. Spinal cord cultures grown for 14 DIV were infected with HSV-mutant SOD and then PA or vehicle was added to the cultures. Under these conditions, in the absence of BDNF, the level of active, phosphoTrk receptor is very low (Figure 4A). Similarly, downstream signaling involving AKT and MAPK are only modestly active. In response to BDNF addition to the media, there is a rapid and robust activation of the Trk receptor (as monitored by assaying for the phosphorylated form of the receptor) as well as phosphoAKT and phosphoMAPK. The temporal pattern of receptor activation and downstream signaling in our cultures conforms to previous observations 120,200(Figure 4A). Identical results were obtained in cultures uninfected with viruses (not shown). Thus we find no evidence that pre-treatment of cultures with PA has any effect on the magnitude or duration of BDNF-TrkB signaling.

Insoluble aggregates of mutant SOD are detectable within cells from transgenic mice engineered to express mutant SOD 206,207. We wondered if WT or mutant SOD aggregated in
neurons in vitro and if treating cultures with PA influenced the cellular distribution of human transgene SOD. Immunocytological location of human SOD in cultures infected with HSV-WT-SOD revealed that the protein is homogeneously distributed throughout the cytoplasm and extends centrifugally for >100 microns into axons and dendrites (Figure 4B). In contrast, in cultures infected with HSV- mutant SOD immunoreactivity is concentrated into puncta (the cytological signature of insoluble aggregated proteins) in the soma cytoplasm and neurites. Double labeling studies reveal that mutant SOD puncta are present in motor neurons (identified by SMI32 immunoreactivity) as well as non-motor neurons in our cultures (Figure 4B).

Treatment of cultures with PA had no effect on the subcellular distribution of human SOD in cultures infected with either of the recombinant HSVs. Although the pathophysiological significance of aggregated protein is controversial, these results indicate that the neuroprotective action of PA is dissociable from the accumulation of aggregated mutant SOD. A similar observation has been made in C. elegans wherein DAF-16 protects against Aβ1-42 toxicity but does not influence the accumulation of protein aggregates.

Since the neuroprotection conferred by PA does not seem to be linked to alterations in trophic factor signaling or the generation of macroscopic mutant SOD aggregates, perhaps PA action is linked to its capacity to promote nuclear localization of FOXO3a. This would be consistent with the observations that PA causes nuclear partitioning of FOXO3a in neurons and constitutive localization of FOXO3a in the nucleus is broadly neuroprotective. We began with biochemical studies of spinal cord neurons in vitro expressing WT SOD or mutant SOD. In the absence of PA, the abundance of phosphorylated FOXO3a at T32 (or its ratio to the non-phosphorylated species) is the same in mutant- versus WT-SOD, expressing cultures (Figure 4C). The same was true when we assayed phosphorylation of S253 (not shown). On the other hand,
one transcriptional target of FOXO3a is MnSOD and the abundance of this protein is markedly depressed in mutant SOD, in comparison with WT-SOD-expressing cultures. Expression of neither mutant- nor WT-SOD led to measurable changes in total FOXO3a or actin. In the presence of PA treatment, the abundance of phosphorylated FOXO3a (or its ratio to the non-phosphorylated species) is the same in mutant- versus WT-SOD, expressing cultures (Figure 4C). Interestingly, PA treatment rescued the mutant SOD effect on the level of MnSOD such that MnSOD abundance was equal in the mutant- versus WT-SOD expressing cultures. PA had no effect on the abundance of total FOXO3a or actin. These findings suggest that mutant SOD proteotoxic stress in vitro is not associated with a change in the state of phosphorylation of FOXO3a at sites that regulate its subcellular distribution. On the other hand, mutant SOD expression results in a reduction in the abundance of a FOXO3a transcriptional target (MnSOD) raising the possibility that proteotoxic stress might influence FOXO3a signaling pathway. How PA treatment reverses the mutant SOD-dependent effect on MnSOD expression, and if it is linked to FOXO3a, will require further study.

Given these in vitro observations, we wondered if a similar phenomenon occurs in mutant SOD mice. We examined lysates from spinal cords of mice expressing the G93A mutant form of human SOD or wild type controls for the expression of phosphoFOXO3a and target genes. The mice were 87 days old, a time when they are asymptomatic in terms of weakness, but do manifest other subtle abnormalities. A consistent increase in the phosphorylation of T32 of FOXO3a (and the ratio of phosphoFOXO3a to the non-phosphorylated species) was seen in the G93A mice in comparison with the wild type animals (n=4 in each experimental group) (figure 4D). This was associated with a reduction in MnSOD in the G93A mice in comparison to the wild type animals. No differences were noted in the abundance of actin in
the mutant versus wild type animals. Thus in both \textit{in vitro} and \textit{in vivo} experiments, mutant SOD expression is associated with a reduction in MnSOD expression. The effect of mutant SOD expression on FOXO3a phosphorylation differed in the \textit{in vitro} versus \textit{in vivo} paradigms.

Figure 4

A. Vehicle PA

\begin{verbatim}
phosphoTrk
Trk
phosphoAKT
AKT
phosphoMAPK
MAPK
\end{verbatim}

0’ 5’ 30’ 120’ 0’ 5’ 30’ 120’ BDNF addition

B. WT-SOD G87R-SOD

\begin{verbatim}
Vehicle PA
\end{verbatim}

Panel A. Mixed spinal cord cultures infected with HSV-WT-SOD or HSV-G87R-SOD were treated with PA or vehicle overnight prior to stimulation with 50 ng/ml BDNF. Lysates of unstimulated cells or stimulated cells at the given time points were subjected to Western blot analysis. The kinetics and degree of TrkB pathway stimulation is the same in PA- versus vehicle-treated cells. Panel B. Mixed spinal cord cultures infected with HSV-WT-SOD or HSV-G85R-SOD were treated with PA or vehicle for 3 days prior to fixation and immunostaining for human SOD. WT-SOD is homogenously distributed throughout the cytoplasm in the neuronal soma and processes in the absence of PA (“- PA”) and in the presence of PA (“+ PA”). In contrast, G87R SOD assumes a
punctate pattern of expression indicative of aggregates of the protein, both in the absence of PA ("-PA") and in the presence of PA (+PA). Calibration bar = 20 microns. Below, using cultures infected with HSV-G85R-SOD, motor neurons are stained with SMI-32 (left panel) and anti-human SOD (middle panel). Aggregated mutant SOD is localized within motor neurons (yellow in right panel). Calibration bar = 15 microns. Panel C. In the absence of PA ("-PA") treatment, spinal cord cultures expressing mutant SOD or WT SOD have the same abundance of phosphoFOXO3a (T32) and the non-phosphorylated species. In addition, mutant SOD expressing cultures have decreased abundance of MnSOD. In the presence of PA ("+PA"), there is no difference in the abundance of phospho- or non-phosphorylated FOXO3a in cultures expressing WT or mutant SOD. In addition, the level of expression of MnSOD is no longer suppressed by mutant SOD expression in PA treated cultures. Panel D. Western blots of lumbar spinal cord lysates from 2 wild type (labeled 1 and 2) and G93A mutant SOD mice (labeled 1 and 2). Representative western blots show an increase in the abundance of T32 phosphoFOXO3a in the lysates from the mutant mice as well as a decrease in the abundance of MnSOD. Quantification of these results are shown in the histograms below.

To further explore this issue we employed bioinformatics tools to query existing microarray profiles for a potential connection between motor neuron disease and FOXO3a-dependent transcription. We identified five microarray datasets located in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) server that are relevant to motor neuron disease. These datasets come from studies of postmortem tissue from ALS patients and mutant SOD mouse or rat tissues at various stages of disease. To our knowledge the only description of the FOXO3a transcriptome comes from a study of PTEN null 786-O renal carcinoma cells. This study identified 198 transcripts whose level of expression is changed ≥2-fold when a DNA binding-competent, constitutively nuclear (triple mutant) FOXO3a is expressed in these cells. When we asked how many genes in the motor neuron disease data set were also components of the FOXO3a transcriptome, we identified 22 transcripts in both datasets (Supplemental Table). Even given the disparate experimental platforms employed in these studies, the presence of any overlapping dataset is intriguing. A better-controlled prospective study is required to obtain a more full understanding of the potential importance of FOXO3a in motor neuron disease pathogenesis.
In light of the neuroprotective effect of PA *in vitro*, we examined the effects of the drug in two *in vivo* model systems of neuronal degeneration. Expression of polyglutamine-expanded AR in the *Drosophila* eye leads to DHT-dependent degeneration\(^{111,112}\). We found that flies reared on food supplemented with 0.5 mM PA had a reduced degenerative phenotype when compared with vehicle treated flies (Figure 5a, b). These *in vivo* results complement the observations made in spinal cord cultures from mice expressing polyglutamine-expanded AR wherein we found expression of TM-FOXO or treatment with PA blunts DHT-dependent mutant AR toxicity.

We next asked if the neuroprotective action of PA depended on FOXO. To this end, we examined the efficacy of PA in flies that both expressed the polyglutamine expanded AR and were haploinsufficient for *Drosophila* Foxo (dFOXO) (Figure 5c, d). In a dFOXO-deficient background (foxo\(^{BG01018}\) allele)\(^{113}\), PA lost its ability to protect against DHT-dependent degeneration. To corroborate these observations, we studied two additional dFOXO loss-of-function alleles (foxo\(^{21}\) and foxo\(^{25}\)) and one predicted loss-of-function allele (foxo\(^{C01841}\))\(^{114,115}\). As above, on all of these dFOXO-deficient backgrounds, PA lost its ability to protect against DHT-dependent degeneration. Using a quantitative rating score, we found that PA led to a statistically significant mitigation of polyglutamine-expanded AR degeneration but this was lost in the dFOXO haploinsufficient flies (Figure 5 bar graph). Variation in the baseline (no PA provided) level of neurodegeneration phenotype among the studied fly lines is likely due to differences in the background genotype. These observations indicate that PA confers protection against mutant AR proteotoxicity in a FOXO-dependent manner.
Figure 5: PA suppresses degeneration in a *Drosophila* model of SBMA in a dFOXO-dependent manner. Right panel of each diptych shows higher magnification of the posterior margin of the eye, where degeneration is concentrated. Panel a, flies expressing polyglutamine-expanded human androgen receptor (GMR, AR52Q) show degeneration when reared on food containing 1 mM dihydrotestosterone (+DHT). Panel b, this degeneration is suppressed when food is supplemented with 0.5 mM PA. Panels c and d, PA fails to rescue AR52Q-mediated degeneration in a dFoxo-deficient background. Bar graph below - Quantification of the
degenerative eye phenotype (scored according to Pandey et al. 111) shows that the neuroprotective effect of PA is lost when flies are haploinsufficient for dFOXO. Number of eyes scored: GMR, AR52 – DMSO n=34, PA n = 41; GMR, AR52; foxoBG01018 DMSO n=37, PA n = 47; GMR, AR52; foxo11 DMSO n=20, PA n=16; GMR, AR52; foxo25 DMSO n=8, PA n=14; GMR, AR52; foxoC01841 DMSO n=28, PA n=20. Mean values are indicated with standard error. P value was determined using Student’s t-test. * p < 0.05

We developed a C. elegans model system of neurodegeneration by combining a null mutation in the glutamate transporter GLT-3 (∆glt-3) with a transgenic strain (nuls5 - 116) in which the glr-1 promoter drives expression of an activated form of Gxs (abbreviated Gxs*) and GFP in glutamatergic neurons 117. The ∆glt-3; nuls5 double mutants exhibit necrotic neuron death at all stages of postembryonic development, with the strongest effect seen in developmental stage L3. PA had a dose-dependent neuroprotective effect at the L3 stage, with complete rescue from death using 10 nM PA (Figure 6A). This concentration of PA had no adverse effect on WT nematodes. We asked if the effect of PA is mediated by changing the timing of neurodegeneration or by reducing it throughout development (Figure 6B). To examine this, we studied the effect of PA on the ∆glt-3; nuls5 double mutants as a function of larval stage and we found neuronal death was reduced in all developmental stages, with the strongest effect observed in the developmental stages most prone to excitotoxicity. Neuron death was reduced in a statistically significant manner in larval stages L2 (3.4 ± 0.2 versus 2.5 ± 0.2 dying neurons/animals, n = 44 versus n = 49, vehicle versus PA, p = 0.006) and L3 (4.2 ± 0.2 versus 2.2 ± 0.1 dying neurons/animals, n = 63 versus n = 65, vehicle versus PA, p < 0.001) (Figure 6B).

In C. elegans, stress resistance and longevity is promoted by a reduction in the activity of insulin growth factor receptor (IGFR) signaling pathway (i.e., hypomorphic alleles of IGFR, daf-2, and PI3’K, age-1) and this requires daf-16, the nematode homolog of FOXO3a. This led us to wonder if reducing activity in the IGFR signaling pathway would alleviate nematode
excitotoxicity. To that end, we crossed the Δglt-3; nuls5 double mutants with an age-1 mutant that carries the hx546 allele. This allele has a specific anti-aging effect but does not affect development. We generated triple mutant nematodes (age-1; Δglt-3; nuls5) and found a robust neuroprotective effect of age-1 at larval stages L1, L2 and L3 (all p values < 0.001) (Figure 6C).

Finally, while we showed that PA leads to accumulation of FOXO3a in mammalian neuronal nuclei, we wished to determine if the same was true in C. elegans. To this end, we studied nematodes in which a DAF-16::GFP fusion protein was expressed in body wall muscles. Addition of PA, but not vehicle, to the growth substrate led to nuclear localization of the fusion protein and quantification of the nucleus/cytoplasm ratio of DAF-16::GFP revealed a statistically significant effect of PA (1.46 ± 0.05 versus 3.44 ± 0.55, n = 36 versus n=27, vehicle versus PA, p < 0.0001) (Figure 6D). This result indicates that PA has an evolutionarily conserved capacity to promote nuclear localization of the DAF16/FOXO3a transcription factor and this is associated with resistance to necrotic neuron death. Another method for promoting nuclear localization of DAF-16 (i.e., mutation of age-1) has a neuroprotective effect and further re-enforces the notion that manipulation of the IGFR signaling pathway could have promise as a neuroprotective strategy.
Figure 6. PA treatment or reducing the activity of IGFR signaling pathway alleviate nematode excitotoxicity. Panel A. PA has a dose-dependent neuroprotective effect on nematode excitotoxicity: ∆glt-3; nus5 double mutant nematodes were cultured in presence of increasing concentrations of PA in the medium. The number of swollen, degenerating head neurons was scored under Nomarski DIC optics in live animals at the L3 developmental stage. PA shows a statistically significant, dose-dependent neuroprotective effect.

Panel B. The neuroprotective effect of PA is mediated by reducing the extent of neurodegeneration throughout development. Panel C. age-1(hx546), a mutation that reduces IGFR signaling pathway without altering developmental decisions, protects C. elegans from excitotoxicity. Degenerating head neurons were scored in live animals as above. Panel D. PA causes preferential nuclear localization of DAF-16, the nematode homolog of FOXO3a. Animals express a transgene (Pmyo-3::daf-16::gfp) that directs the expression of DAF-16::GFP fusion protein in body-wall muscle cells. In control animals (i) DAF-16::GFP is abundantly present in the cytoplasm. Animals grown in the presence of 10nM PA (ii) show preferential (though not uniform) accumulation of DAF-16::GFP in the nucleus.
Discussion:

Biochemical pathways that regulate longevity in yeast, *C. elegans*, *Drosophila melanogaster* and mice also play a fundamental role in resistance to stresses such as UV radiation, oxidative conditions, heat shock and misfolded and aggregation-prone proteins \(^{118}\). Two transcription factors, Heat-shock factor 1 and DAF-16/FOXO3a, are essential mediators of this longevity/stress resistance program in nematodes \(^4,^{119}\). Here we show that genetic and pharmacological maneuvers that enhance nuclear localization of FOXO3a protect mammalian motor neurons *in vitro* from 4 insults directly relevant to motor neuron diseases and abrogate neurodegeneration in two *in vivo* invertebrate model systems. The broad neuroprotective action of PA suggests it acts on a phylogenetically conserved, core stress resistance pathway.

*Nuclear FOXO3a and PA can evoke a neuroprotective program*

We have employed several complementary experimental platforms (each with its own unique advantage) to investigate the biological actions of PA. In sum the experimental result suggest that the neuroprotective actions of PA are mediated, at least in part, by FOXO3a/DAF-16. First, in both rat neuron cultures and *C. elegans*, application of PA promotes nuclear accumulation of FOXO3a or DAF-16. Second, PA evokes a dose-dependent increase in FOXO-dependent transcription. Third, constitutive nuclear localization of FOXO3a mimics the neuroprotective action of PA. Fourth, PA-mediated protection against mutant AR-evoked degeneration is dFOXO-dependent. Even without knowledge of the direct molecular target of PA, this evidence favors the view that PA acts, at least in part, in a FOXO3a transcription-dependent manner.
Upon nuclear localization, members of the FOXO family of transcription factors can control the expression of messages that impact glucose metabolism, tumor suppression (through effects on cell cycle progression and apoptotic responses), stress resistance and longevity\textsuperscript{99,176}. The specific effects of FOXOs are both cell-type and context-dependent and specific posttranslational modifications (i.e. phosphorylation, ubiquitination, acetylation) play a key role in controlling the transcriptional read-out\textsuperscript{97,167,181,212}. For example, work from the Brunet lab has demonstrated that AMPK phosphorylates FOXO3a at 6 sites and the state of phosphorylation at these sites does not affect nuclear localization\textsuperscript{179}. Instead these sites are vital for expression of genes involved in oxidative stress management and energy metabolism. Our finding that induction of MnSOD by PA in cultures expressing mutant SOD parallels the observation of Greer et al. that AMPK induction of sod-3 expression in nematodes is FOXO/DAF-16-dependent\textsuperscript{213}. Our preliminary studies suggest that expression of mutant SOD ± PA does not influence the state of phosphorylation of FOXO3a at AMPK sites S413 or S588 (unpublished observations). Further work is required to determine if FOXO3a undergoes posttranslational modifications in cells expressing mutant SOD, if PA influences such a change, and whether this has functional consequences.

It is noteworthy that expression of mutant SOD in neurons \textit{in vivo}, prior to neuron loss, is associated with an increase in FOXO3a phosphorylation at T32 (one of the three key sites controlling nuclear/cytoplasmic partitioning) and a reduction in the abundance of MnSOD. These observations may indicate that as a consequence of expressing mutant SOD, neurons upregulate the activity of FOXO3a kinases (and/or decrease the activity of FOXO3a phosphatases) and this results in a reduction in the expression of FOXO3a-regulated transcripts that combat proteotoxicity. In fact, there is a 2.5 fold increase in PI3’K activity and protein in
spinal cords of ALS patients (the activator of the FOXO3a kinases AKT and SGK\textsuperscript{214}) as well as a 3.6 fold increase in phosphoAKT itself in mutant SOD mice\textsuperscript{215}. These observations may indicate that a putatively beneficial response to stress, such as increased expression and activity of an anti-apoptotic pathway, may have an unintended adverse additional action on FOXO3a transcription.

While we found that constitutive nuclear localization of FOXO3a is neuroprotective, other groups obtained the opposite result. Heterologous expression of TM-FOXO3a led to death of purified motor neurons and cerebellar granule neurons \cite{97} as well as PTEN-deficient tumor cells\textsuperscript{216}. One factor that may contribute to these disparate observations is experimental platform. A second contributory factor may be the level of transgene expression. It has been suggested that the level of nuclear FOXO3a dictates the cellular read-out\textsuperscript{102} and the promoter in the HSV-based system we employ generally evokes moderate level of transgene expression\textsuperscript{122}.

\textit{Invertebrate models support PA acts via a conserved mechanism}

Given the potent neuroprotective activity of PA \textit{in vitro} we sought to examine its effects \textit{in vivo}. The two model systems we chose have features that are reminiscent of human motor neuron diseases. Neurons in the \textit{Δglt-3: nusIS} nematode die an excitotoxic cell death and there is abundant evidence that implicating excitotoxic mechanisms in ALS\textsuperscript{106}. Spinobulbar muscular atrophy is caused by expression of the proteotoxic polyQ expanded AR and transgenic expression of this mutant in the fly eye similarly causes degeneration\textsuperscript{111}. The neuroprotective activity of PA in these model systems complements the \textit{in vitro} studies with mammalian motor neurons. A simple explanation for these observations is that PA operates on a biochemical
pathway common in all these experimental systems that can overcome a diversity of noxious insults.

Extending these observations to the mutant SOD mouse model of ALS at the present time is hindered by the lack of evidence that PA can cross the blood-brain barrier. In preliminary studies we have not detected PA in brain homogenates after intraperitoneal administration. Administration using an intrathecal catheter/osmotic minipump may be a viable option\textsuperscript{217}, although the long-term stability of PA at body temperatures is not known. It may be possible to synthesize derivatives of PA that retain the essential biological activity and cross the blood-brain barrier\textsuperscript{218}.

\textit{Healthful aging and the FOXO3a signaling pathway}

The onset of many neurodegenerative diseases in adulthood raises the possibility that the causes of aging are specific contributors to disease pathogenesis. To the extent that this is true, applying our understanding of the molecular biology of longevity may lead to new types of therapy for neurodegenerative diseases. The present work on FOXO3a is an example of how manipulation of a phylogenetically conserved, longevity-promoting signaling pathway can effectively block neurodegeneration. This is particularly interesting in light of a recent population-based study of human longevity showing a strong association of FOXO3a and healthy aging\textsuperscript{27}. Agents that trigger neurodegeneration may act in part by subverting the normally healthful actions of an aging pathway.

\textbf{Acknowledgements:}
We thank Dr. David Borchelt for the gift of the anti-human SOD rabbit serum and Ernst Hafen and Julia Lüdke (Institute of Molecular Systems Biology, Zurich) for the Foxo^{21} and Foxo^{25} fly lines. This work was supported by the U.S. Public Health Service (NS34435, MD, CA24487, JC; NS32214, DEM; NS053825, JPT and NS52325, RGK) and the ALS and Muscular Dystrophy Associations.

**Author Contributions:** Conceived and designed the experiments: JM-P, NN, MB, IM, SG, YL, JPT, MD, DM, RK. Performed the experiments: JM-P, NN, MB, IM, SG, WZ. Analyzed the data: NN, MB, IM, SG, WZ, JPT, RK. Contributed reagents/materials/analysis tools: JC, RN. Wrote the paper: MB, RK.


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