The Role of GFAT-1
in *C. elegans* Protein Quality Control

Inaugural - Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln, 2019

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Tag der mündlichen Prüfung: 13.02.2019

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Acknowledgements

First, I want to thank Prof. Dr. Adam Antebi for giving me the opportunity to perform my PhD research in his laboratory. Your immense love for science and attention to detail never stopped to amaze me.

Additionally, I want to thank the members of my advisory committee, Prof. Dr. Thomas Langer, Dr. Martin Graef, Prof. Dr. Mats Paulsson and Prof. Dr. Thorsten Hoppe. I am grateful that you invested your time to provide valuable feedback for my project in recurrent meetings. Importantly, I also want to thank my thesis committee members Prof. Dr. Aleksandra Trifunovic and Prof. Dr. Ulrich Baumann for agreeing to evaluate my thesis and participate in my defense, even on short notice.

I am also very thankful to Dr. Martin Denzel, Kira Allmeroth, Dr. Moritz Horn, Sabine Ruegenberg, Isabelle Schiffer, Vignesh Karthikaisamy, and Dr. Peter Breuer for being part of the GFAT-1 community in good times and in bad. Thanks for the great collaboration and lending your sharp minds.

Thank you to all my dear former and current A-Team members and to the Denzelis. I had a great time with you sharing ideas, discussing, reassuring each other, juggling worms and enjoying coffee and cake! I wouldn´t have wanted to miss the opportunity to meet any of you beautiful nerds. Especially, I have to mention Isabelle Schiffer, Kira Allmeroth, Christian Latza, Rebecca Tharyan, and Christoph Geisen. You are great colleagues and made the lab feel like a second home. I am very happy to call you my friends now and I hope we will stay in touch for years to come.

I am really glad that I was part of the multicultural and vibrant Cologne Graduate School of Ageing. I owe a really big “Thank you” to the great team of organizers, particularly to Dr. Daniela Morick and Jenny Ostermann for their open ears and never-ending support.

Finally, I want to express my special gratitude to all my long-standing friends in Cologne and the rest of the world and my beloved, beautiful family. You managed to get my mind off “worm trouble” with shared hours of music, travelling, laughter, BBQ, dining, dancing, sun… Thank you for believing in me and always having my back. Thank you for everything.

Summary

The hexosamine pathway (HP) requires products from carbohydrate, amino acid, fatty acid and nucleotide metabolism to synthesize the aminosugar uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). This molecule is an essential precursor for diverse protein glycosylation reactions and the production of abundant biomolecules. Thus, many metabolic pathways can influence the HP and the HP, likewise, impacts multiple cellular processes. The first step of the HP determines the rate of UDP-GlcNAc synthesis and is catalyzed by glutamine fructose 6-phosphate aminotransferase (GFAT-1). Two important recent discoveries implicate GFAT-1 and the HP in the modulation of protein quality control (PQC). First, it was demonstrated that GFAT-1 is a transcriptional target of the unfolded protein response (UPR). Second, a boost in HP activity by *gain-of-function* (*gof*) mutations in *gfat-1* itself improves PQC and prolongs life in the nematode *Caenorhabditis elegans* (*C. elegans*). However, it remains unclear how GFAT-1 might regulate the complex protein homeostasis machinery and in my PhD I set out to address this important question.

In this study I confirmed a regulation of GFAT-1 protein during stress. In particular stress within the endoplasmic reticulum led to a strong induction of GFAT-1 expression specifically in the worm intestine. While *gfat-1 gof* mutations and GFAT-1 overexpression (OE) under the *gfat-1* promoter protected animals from tunicamycin toxicity, it did not, however, confer general stress resistance. Despite the broad positive effect of *gfat-1 gof* mutations on PQC mechanisms and the strong enrichment in the cellular metabolite UDP-GlcNAc we could not detect major changes in transcriptome or proteome compared to wildtype controls.

To better understand and dissect the role of GFAT-1, we generated tissue-specific GFAT-1 OE *C. elegans* lines. Intestinal GFAT-1 OE appeared detrimental as the animals of multiple transgenic lines were characterized by small body size and sick appearance. In contrast, the pan-neuronal and muscular GFAT-1 OE resulted in healthy worms. They exhibited an elevation in autophagic flux relative to controls. While we did, however, not observe a decrease in proteotoxicity in different tissues of nematodes with pan-neuronal GFAT-1 OE, we noticed a large, cell autonomous reduction in polyglutamine (polyQ) toxicity in *C. elegans* with muscle-specific GFAT-1 OE. Not only did it strongly suppress the load of polyQ aggregates but also it prevented the progressive paralysis typical for muscular protein aggregation models. Interestingly, OE of mutant enzymatic dead GFAT-1 mildly improved polyQ toxicity,

suggesting that the effect can be only partially attributed to UDP-GlcNAc production. To identify downstream effectors of GFAT-1 OE that mediate improved PQC we performed a GFAT-1 pulldown experiment to determine physical interactors. We then carried out a selective RNAi suppressor screen on these interactors as well as further candidate genes, using the strong motility phenotype as a readout. Among 15 identified candidates, we deemed ABCF-3 the most interesting. This protein is known to co-regulate the activation of GCN-2 kinase by uncharged tRNAs and to thereby modulate the integrated stress response (ISR). I showed that ABCF-3 associates with GFAT-1 and that the health benefits of muscular GFAT-1 OE in a polyQ model are completely dependent on ABCF-3. Furthermore, GFAT-1 OE led to a slight elevation of eIF2α phosphorylation and ATF-5 expression, which are critical hallmarks of an activated ISR.

We conclude that GFAT-1 exercises its positive effects on PQC by modulation of the ISR through interaction with ABCF-3 and that this novel connection of the HP and the ISR is of particular importance in the maintenance of healthy muscle tissue.

Zusammenfassung

Der Hexosamin Biosyntheseweg (HP) setzt metabolische Intermediate aus Kohlenhydrat-, Aminosäure-, Fettsäure- und Nukleotidmetabolismus um und sythetisiert den Aminozucker Uridindiphosphat-N-acetylglucosamin (UDP-GlcNAc). Dieses Molekül ist ein essentieller Baustein für diverse Protein-glykosylierungsreaktionen und für die Produktion weit verbreiteter Biomoleküle. Viele Stoffwechselwege können somit den HP beeinflussen und der HP nimmt gleichermaßen Einfluss auf zahlreiche zelluläre Prozesse. Der erste enzymatische Schritt des HP bestimmt die Geschwindigkeit der Produktion von UDP-GlcNAc und wird durch Glutamin-Fructose-6-phosphataminotransferase (GFAT-1) katalysiert.

Zwei wichtige aktuelle Entdeckungen zeigen zudem, dass GFAT-1 und der HP in die Modulation der Proteinqualitätskontrolle (PQC) eingebunden sind. Zum einen wurde gezeigt, dass GFAT-1 ein transkriptionelles Zielgen in der Antwort auf ungefaltete Proteine (UPR) im endoplasmatischen Retikulum (ER) ist. Zum anderen erhöhen funktionsverstärkende (*gof*) Mutationen in *gfat-1* die HP Aktivität und verbessern dadurch die PQC und verlängern die Lebensspanne des Fadenwurms *Caenorhabditis elegans* (*C. elegans*). Es blieb jedoch weiterhin unklar, wie GFAT-1 die komplexen Systeme zur Erhaltung der Proteinhomöostase beeinflusst. Ziel meiner Doktorarbeit war es diese wichtige Frage zu beantworten.

Mit den Ergebnissen dieser Studie konnte ich bestätigen, dass die Expression von GFAT-1 durch Stress reguliert ist. Insbesondere ER-Stress führte zu einer starken Induktion von GFAT-1 im Darm der Würmer. Obwohl *gfat-1 gof* Mutationen und GFAT-1-Überexpression (OE) unter Kontrolle des *gfat-1* Promotors vor Tunicamycin Toxizität schützten, verlieh dies keine allgemeine Stressresistenz. Trotz der umfassenden positiven Wirkung von *gfat-1 gof* Mutationen auf PQC-Mechanismen und der starken Anreicherung des zellulären Stoffwechselprodukts UDP-GlcNAc konnten wir im Vergleich zu Wildtyp-Kontrollen keine größeren Veränderungen des Transkriptoms oder Proteoms nachweisen.

Um die verschiedenen Funktionen von GFAT-1 besser zu verstehen stellte ich gewebespezifische GFAT-1 OE *C. elegans* Linien her und untersuchte diese. Die OE von GFAT-1 im Darm schien hierbei nachteilig zu sein, da auch die Herstellung mehrerer transgener Linien nur zu Tieren mit geringer Körpergröße und krankem Aussehen führte. Im Gegensatz dazu waren transgene Würmer mit neuronaler oder muskulärer GFAT-1 OE augenscheinlich gesund und nicht von wildtypischen Würmern zu unterscheiden. Gegenüber den Kontrollen zeigten sie eine leichte

Erhöhung des Autophagie Prozesses. Im Falle der neuronalen GFAT-1 OE führte dies nicht zu einer Abnahme der Proteotoxizität in verschiedenen untersuchten Geweben. Im Gegensatz dazu konnte ich eine starke zellautonome Reduktion der Polyglutamin (polyQ)-Toxizität in Nematoden mit muskel-spezifischer GFAT-1 OE feststellen. Nicht nur war die Zahl der polyQ Aggregate stark reduziert, sondern es wurde auch die für Protein-aggregationsmodelle im Muskel typische, progressive Lähmung verhindert. Interessanterweise verbesserte die OE von mutiertem GFAT-1 ohne enzymatische Aktivität die PolyQ Toxizität noch in geringem aber signifikantem Maße, was darauf hindeutet, dass der Effekt der GFAT-1 OE nur teilweise auf die UDP-GlcNAc Produktion zurückzuführen ist.

Im Folgenden führte ich Ko-Immunpräzipitation durch um mögliche Bindungspartner von GFAT-1 zu identifizieren. Anschließend testete ich die Abhängigkeit der beobachteten PQC Verbesserung von diesen GFAT-1 Interaktoren und von weiteren Kandidaten. Hierfür führte ich einen selektiven RNAi Screen mit Motilitätsverbesserung als kritischem Phänotyp durch. Aus den 15 identifizierten Kandidaten wählten wir ABCF-3 zur weiteren Analyse aus. Es ist bekannt, dass dieses Protein die Aktivierung der GCN-2 Kinase durch ungeladene tRNAs reguliert und dadurch die integrierte Stressantwort (ISR) moduliert. Ich konnte zeigen, dass ABCF-3 ein mit GFAT-1 assoziiert und dass die gesundheitlichen Vorteile der muskulären GFAT-1 OE im PolyQ-Modell vollständig von ABCF-3 abhängen. Darüber hinaus führte GFAT-1 OE zu einer leichten Erhöhung der eIF2α Phosphorylierung und der ATF-5 Expression, beides wichtige Merkmale einer aktivierten ISR.

Zusammenfassend schließen wir, dass GFAT-1 seine positive Wirkung auf die PQC durch direkte Interaktion mit ABCF-3 und resultierender Modulation der ISR ausübt und dass diese bisher nicht beschriebene Verbindung von HP und ISR für die Erhaltung von gesundem Muskelgewebe von besonderer Bedeutung ist.

Abbreviations

Acetyl-CoA	Acetyl-Coenzyme A
ACN	acetonitrile
AD	Alzheimer´s disease
AMPK	adenosine monophosphate (AMP)-activated protein kinase
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATFS-1	activating transcription factor associated with stress-1
ATP	adenosine triphosphate
C. elegans	*Caenorhabditis elegans*
cAMP	cyclic adenosine monophosphate
CFP	cyan fluorescent protein
CITE	cap-independent translation enhancer
CMA	chaperone mediated autophagy
CP	20S core particle
DAF/*daf*	abnormal dauer formation
dsRNA	double-stranded RNA
ECM	extracellular matrix
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ERSE	ER stress response element
FDR	false discovery rate
FOXO	forkhead box class O
Fruc-6-P	Fructose 6-phosphate
FT	flow-trough
GAG	glycosaminoglycan
GALE	UDP-galactose-4-epimerase
GCN2	general control non-depressible 2
GEF	guanine nucleotide exchange factor
GFAT-1	Glutamine-fructose 6-phosphate aminotransferase 1, also abbreviated as GFPT1 (mice, humans) or GFA1 (yeast)
GFAT-2	Glutamine-fructose 6-phosphate aminotransferase 2
Glc-6-P	Glucose 6-phosphate
GlcN-6-P	Glucosamine 6-phosphate
GlcNAc-1-P	N-acetylglucosamine 1-phosphate
GlcNAc-6-P	N-acetylglucosamine 6-phosphate
GlmS	glucosamine-6-phosphate synthase
Gln	Glutamine
gna-2	glucosamine phosphate N-Acetyl transferase
gof	gain-of-function
HD	Huntington´s disease
HLH-30	helix loop helix 30
HP	hexosamine pathway
HRI	heme-regulated inhibitor
HSF1	heat shock transcription factor 1
HSR	heat shock response
IP	immunoprecipitation

IRE1	inositol-requiring protein-1
Met-tRNA^{Met}	methionine-loaded methionyl-transfer RNA
mRNA	messenger RNA
MS	mass spectrometry
mTORC1	mechanistic target of rapamycin complex 1
NGM	nematode growth medium
OE	overexpression
OGA	O-GlcNAcase
OGT	O-GlcNAc transferase
OGT	O-GlcNAc transferase
ORF	open reading frame
OST	oligosaccharyltransferase
PAM	protein aggregate myopathy
P-eIF2α	phosphorylated eukaryotic initiation factor 2α
P-PMK-1	phosphorylated PMK-1
PD	Parkinsons´s disease
PERK	PKR-like ER kinase
PIC	pre-initiation complex
PKA	protein kinase A
PKR	protein kinase R
PMK-1	P38 Map Kinase family member 1
polyQ	polyglutamine
polyUb	polyubiquitin
ppGalNAcT	polypeptide N-acetyl-α-galactosaminyl-transferase
PTM	posttranslational modification
qRT-PCR	quantitative reverse transcription PCR (polymerase chain reaction)
RIDD	regulated IRE1-dependent decay
RNAi	RNA interference
RP	19S regulatory particle
S. cervisiae	*Saccharomyces cervisiae*
SDS	sodium dodecyl sulfate
Ser51	serine 51
SILAC	Stable isotope labeling with amino acids in (cell culture)
TFEB	transcription factor EB
TOR	target of rapamycin
tRNA	transfer RNA
Ub	ubiquitin
UDP	uridine diphosphate
UDP-GalNAc	UDP-N-Acetyl galactosamine
UDP-GalNAc	UDP-N-acetylgalactosamine
UDP-GlcNAc	UDP-N-Acetyl glucosamine
UDP-HexNAc	UDP-GlcNAc and UDP-GalNAc
uORF	upstream open reading frame
UPR	unfolded protein response
UPR	unfolded protein response
UPRE	UPR element
UPR^{ER}	unfolded protein response of the ER
UPR^{mt}	the mitochondrial unfolded protein response
UPS	ubiquitin-proteasome system
UTP	uridine triphosphate
UTR	untranslated region
UTR	untranslated region

xbp-1s	spliced *xbp-1* (active form)
XBP1	X-box binding protein 1
XBP1s	spliced XBP1

1. INTRODUCTION

1.1 The aging process

1.1.1 Regulation of aging

Aging can be defined as the universal time-dependent decline in physiological functions essential for organismal integrity, fertility, and survival. This entropic process is not regulated, however, longevity and the rate of aging are. Life- and healthspan are under evolutionary selection, depend on the ecological context and can be genetically manipulated (Kirkwood and Austad, 2000, Bonsall, 2006). In the following passage I will describe several pieces of evidence that emphasize the genetic basis of longevity and of healthspan plasticity.

First, the lifespan of organisms can differ strongly even between related species that might share similar environments and physical properties. Probably the most famous example of an exceptionally long-lived species is the naked mole rat (*Heterocephalus glaber*). It can reach beyond 30 years, thereby growing almost ten times as old as its relative the house mouse (*Mus musculus*) (Gorbunova et al., 2014). Strikingly, the mortality hazard of the naked mole rat does not increase as a function of chronological age. While the mortality risk of other mammals grows exponentially starting with sexual maturity, the risk of dying remains constant and very low for the naked mole even when they exceed the age of sexual maturity by 25-fold (Ruby et al., 2018). Although the molecular basis for this extreme longevity cannot be completely explained yet, it was found that the naked mole rat is extremely resistant to cancer, shows elevated protein translation fidelity at the ribosome, and largely maintains physiological youthfulness into high age (Buffenstein, 2008, Delaney et al., 2013, Azpurua et al., 2013). Another particularly long-lived species is the ocean quahog (*Arctica islandica*), a clam species endemic at the Icelandic shelf, with a lifespan of up to 400 years (Wanamaker et al., 2008). The maximal reported lifespan of the taxonomically related northern quahog (*Mercenaria mercenaria*) is just above 100 years. Again, the underlying cause for the outstanding longevity could not

yet be conclusively clarified, but it was hypothesized that an increased resistance to oxidative stress might be a contributor (Abele et al. 2008, Ungvari et al., 2011).

Second, life- and healthspan are clearly determined by genetic factors and can be altered by single gene mutations. On the one hand, specific mutations can lead to progeroid syndromes, dramatically accelerating the occurrence of age-associated clinical symptoms and diseases and leading to premature death. The Werner syndrome is one such genetic disorders. Loss of function of the WRN RecQ like helicase involved in DNA replication, repair and recombination, telomere maintenance, and apoptosis is the underlying cause for this dramatic syndrome (Yu et al., 1996, Huang et al., 1998, Spillare et al., 1999, Edwards et al., 2015). It is striking how mutations in this single gene potently accelerate whole organism aging while leaving early development unaffected. Classically, the first symptom is the missing characteristic growth spurt during teenage years resulting in a short stature. Manifestations of premature aging such as graying and receding hair, skin atrophy and cataracts appear as early as the third decade of life. Because patients with Werner syndrome also develop typical age-associated disorders like cancer, arteriosclerosis and cardiovascular diseases prematurely, the median age at death is between only 47 and 54 (Werner, 1904, Epstein et al. 1966, Muftuoglu et al., 2008). In any case, conclusions gained by studying models of premature aging should be treated with caution, as they can never fully recapitulate the phenotype of normally aged individuals. It is debated whether these models merely lead to sick animals with an aging-like phenotype or whether they can truly stand in for naturally aged subjects (Miller, 2004, Vanhooren and Libert, 2013). On the other hand, single gene mutations can stave off the aging process and cause an extraordinarily long health- and lifespan. One well-established example is reduced function of the insulin/IGF receptor tyrosine kinase. Originally, it was discovered that missense mutations in abnormal dauer formation *2* (*daf-2*), the insulin receptor ortholog in *Caenorhabditis elegans* (*C. elegans*), can extend the lifespan of nematodes about twofold and that this longevity is dependent on downstream signaling via the forkhead transcription factor *daf-16* (FOXO) (Kenyon et al., 1993, Larsen et al., 1995, Kimura et al., 1997, Lin et al., 1997, Ogg et al., 1997). Consecutive studies in flies and mice, as well as association studies between phenotype and genome in humans could prove a strong conservation of this nutrient sensing pathway and its implication in lifespan regulation (Clancy et al., 2001, Tatar et al., 2001, Blüher et al., 2003, Holzenberger et al., 2003, Bonafè et al., 2003, Hwangbo et al., 2004, van Heemst et al., 2005, Willcox et al., 2008, Soerensen et al., 2010, Deelen et al., 2013). It is important to note that longevity is often directly linked to general stress resistance and extended health

span. Indeed, this holds true for genetic variants which modulate insulin signaling (Hitt et al., 1999, Murakami, 2006, Ambrogini et al., 2010, Demontis and Perrimon, 2010, Martins et al., 2016). Consequently, genes that maximize life expectancy are of outstanding interest as they very probably also control the general fitness and susceptibility to disease of an organism. It is of paramount importance to better understand the aging process as it is accompanied by decreased fitness and by a strongly increased risk for severe diseases posing a public health problem and a major socioeconomic challenge to our aging society.

1.1.2 Demographic changes in our society

Our world currently hosts roughly 7.6 billion people. While the world population increased only very little during human history until the year 1800 when it reached around 1 billion, it has seen a vast growth since then as the number of people multiplied by seven in just above 200 years (World Population Prospects: The 2017 Revision, United Nations) As global birth rates are steadily decreasing this is especially remarkable and traces back to a substantial increase in life expectancy. This is caused by a strong reduction of mortality of young children under the age of five, which posed a problem in the least developed countries. Within the last fifteen years it could be decreased from 7% to 4.8% worldwide. Additionally, the number of people reaching high and very high age is constantly growing, which is reflected in a sharp increase in the population´s median age (Figure 1A). Globally, the proportion of people aged 60 and above is ever rising and in 2017 amounts to 25% within Europe (World Population Prospects: The 2017 Revision, United Nations). This trend can also be represented by the more and more rapidly growing number of centenarians throughout recent history. In 1990 the number of centenarians worldwide was estimated to be 96,000 and has reached 451,000 in 2015 (World Population Prospects: The 2015 Revision, United Nations, Robine and Cubaynes, 2017). And while this exceptionally long-lived cohort of people show increased resistance towards certain common causes of death like cancer, aging in general is accompanied by deteriorating health (Smith, 1997, Bonafè et al., 2002, Niccoli and Partridge, 2012, Partridge et al., 2018).

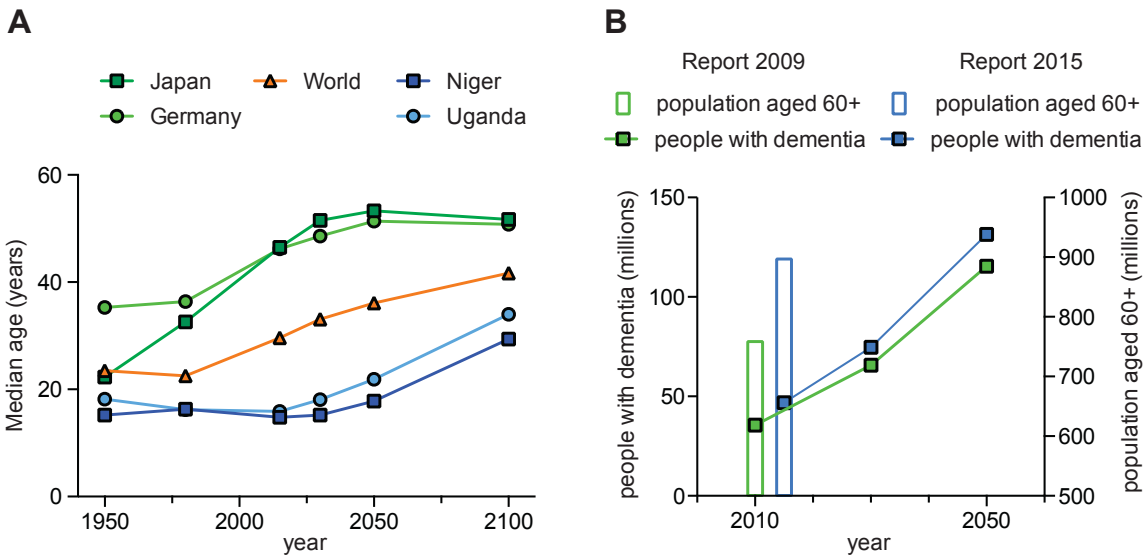


Figure 1. Strong increase in cases of dementia in our aging society. A. Development of median age from 1950 to 2015 and expected future trends. Japan and Germany (green) are shown as the top two countries, Niger and Uganda (blue) as the last two countries regarding median lifespan in 2015. Worldwide average is depicted in orange. Datasource. United Nations, Department of Economic and Social Affairs, Population Division (2015). World Population Prospects: The 2015 Revision. **B**. Increasing numbers of people living with dementia. Current estimates of worldwide population aged 60 years and above and anticipated rise in population affected by dementia as published by the 2009 (green) and 2015 (blue) World Alzheimer Report.

1.1.3 Age-associated diseases

Aging is accompanied by a decline in essential molecular machines, which for example maintain proteostasis, genome stability and stem cell function (López-Otín et al., 2013). Consequently, aging is the main risk factor for multiple, very distinct diseases. And while some of these disorders such as cardiovascular diseases and cancer also occur in the very young, albeit with much lower prevalence, some neurodegenerative diseases like Alzheimer´s (AD), Parkinson´s (PD) and Huntington´s disease (HD) are tightly linked to advanced age (Niccoli and Partridge, 2012, Masters et al., 2015). Worldwide, the number of people over the age of 60 years diagnosed with AD, the most common form of dementia, amounted to 46.8 million in 2016 (Prince et al., 2016). The progressive loss of cognitive ability most often completely disrupts the lives of the affected individuals, their family and friends. Beyond that, the huge and growing number of patients with AD and other forms of dementia (Figure 1B) poses a major socio-economic challenge. The U.S. alone spent an estimated $259 billion on the health care, long-term care and hospice of Alzheimer´s patients in 2017. The cost is expected to rise to $1.1 trillion by the year

2050 if no effective treatment will be developed (www.alz.org). This specific example helps to demonstrate the necessity and importance of aging research. Only if the fundamental consequences of increasing age on cellular and molecular integrity are understood, can we hope to decrease the burden of age-associated disease and promote healthy aging.

Although progressive diseases of the nervous system, such as AD, PD, and HD manifest with distinctive pathological symptoms, they share a key characteristic: the gradual aggregation and accumulation of misfolded proteins in the brain. Gene mutations coding for aggregation-prone protein variants are at the basis of the disorder in case of HD and the rare familial forms of AD and PD (Goate et al., 1991, The Huntington's Disease Collaborative Research Group, 1993, Levy-Lahad et al., 1995, Rogaev et al., 1995, Polymeropoulos et al., 1996, Selkoe, 2001, Funayama et al., 2002, Singleton et al., 2003, Zimprich et al., 2004). The multifactorial causes for the much more common sporadic forms of AD and PD are less well defined: genetic variants of multiple genes as well as several environmental conditions leading to mitochondrial dysfunction and production of oxidative stress were identified to be risk factors for the manifestation of disease (Chai and Lim, 2013, Zetterberg and Mattsson, 2014). While these risk factors may concur and combine, young individuals are still protected from AD and PD by cellular proteostasis mechanisms that fold, maintain and degrade proteins. With age, these processes become faulty and deregulated and, when facing extraordinary challenges, are no longer sufficient to sustain a healthy proteome (Blake et al., 1991, Terman, 1995, Carrard et al., 2002, Ferrington et al., 2005, Ben-Zvi et al., 2009, Brehme et al., 2014, Labbadia and Morimoto, 2015). It is of paramount importance to understand this age-specific decline to be able to counteract these severe diseases.

1.1.4 *C. elegans* as a genetic model for aging research

In the 1970s, the soil nematode *C. elegans* was first described as a tool for genetic research by Sydney Brenner (Brenner, 1974). Since then, it evolved into a popular model organism in basic biological research due to its many positive features: Its small size (about 1 mm) and cost-effective culture allows maintenance and analysis of large numbers of individuals. The transparency of the nematodes makes it ideally suited to study expression patterns of proteins, cell divisions or the embryonic cell lineage *in vivo* (Sulston et al., 1983). *C. elegans* can reproduce by

self-fertilization as well as sexually, after the production of males in adverse environmental conditions. Therefore, clonal expansion and genetic variation by crossing males and hermaphrodites are both feasible. Furthermore, the completely sequenced and curated genome, big databases and extensive worm libraries enable fast analysis of single gene deletions, mutations and overexpression. Additionally, targeted gene knock-down is very straightforward as nematodes can be fed bacteria, expressing double-stranded RNA of a specific gene, leading to RNA interference (RNAi) (Fire et al., 1998, Timmons and Fire, 1998, Timmons et al., 2001). This technique as well as random chemical mutagenesis combined with whole genome sequencing make it is possible to interrogate the whole genome for modifiers of certain traits like lifespan or drug resistance.

C. elegans is an especially popular model organism in aging research for several reasons: First, worms exhibit phenotypic aging. The visible signs of tissue deterioration associated with aging for example include disintegration of germline, pharynx and muscle, also leading to a functional decline in fertility, pharyngeal pumping and motility (Herndon et al., 2002, Garigan et al., 2002, Glenn et al., 2004, Chow et al., 2006). Second, many diseases can be modeled in *C. elegans*. Transgenically expressed Aβ or polyglutamine repeats for example are used to model AD or HD, respectively. In both cases, protein aggregates are deposited and, if expressed in muscle, a progressive paralysis can be observed (Link, 1995, Link, 2001, Wang et al., 2006, Kaletta and Hengartner, 2006). Third, survival curves can be determined easily and quickly, because the nematode has a short lifecycle - from egg over for larval stages to young adult and gravid, reproducing adult - and mean lifespan of roughly 20 days under standard conditions (Tissenbaum and Guarente, 2002).

Variations in genetic makeup or in environmental conditions can, however, strongly influence life expectancy of the nematodes. For instance, modulation of insulin/IGF-1 signaling by specific genetic polymorphisms for example in downstream effector kinase *ageing alteration 1* (*age-1*) (phosphoinositide 3-kinase, PI3K) or phosphatase *daf-18* (phosphatase and tensin homolog, PTEN), was discovered to greatly prolong *C. elegans* lifespan (Friedman and Johnson, 1988, Kenyon et al., 1993, Larsen et al., 1995, Morris et al., 1996, Mihaylova et al., 1999). Consecutive studies also associated this pathway with longevity in *Drosophila*, mice and even humans (Clancy et al., 2001, Tatar et al., 2001, Hsieh et al., 2002, Holzenberger et al., 2003, Bonafè et al., 2003, van Heemst et al., 2005, Kappeler et al., 2008, Harries et al., 2012, Ortega-Molina et al., 2012, Proshkina et al., 2015). The progression of

research proved *C. elegans* a valuable tool in discovering novel regulators of health- and lifespan.

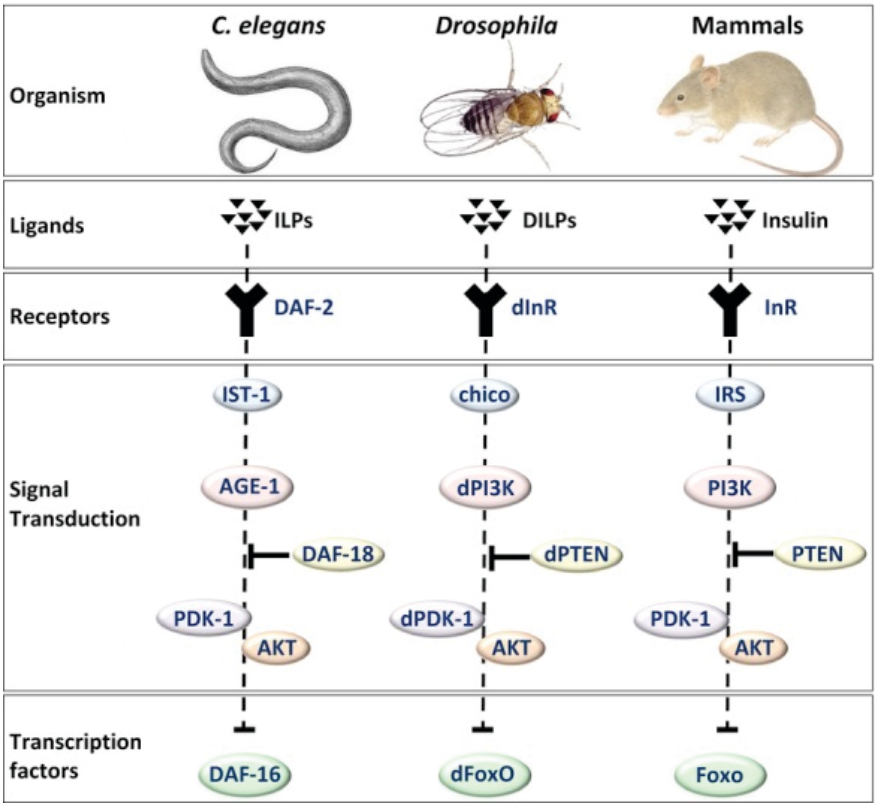


Figure 2. Conserved players of the PI3K signal transduction pathway downstream of the insulin receptor. Many components, like the insulin receptor (DAF-2/ dInR/ InR), its downstream effector kinase (AGE-1/dPI3K/PI3K) and its respective phosphatase (DAF-18/ dPTEN/ PTEN), are strongly connected to longevity. While single genes code for most components in *C. elegans* and *D. melanogaster*, mammals often express several isoforms. AKT, v-akt murine thymoma viral oncogene homologue 1; dFoxO, *Drosophila* forkhead box, sub-group O transcription factor; DILPs, *Drosophila* insulin‐like peptides; dInR, *Drosophila* insulin receptor; dPDK-1, *Drosophila* phosphatidylinositide‐dependent protein kinase 1; dPI3K, *Drosophila* phosphoinositide 3-kinase; dPTEN, *Drosophila* phosphatase and tensin homolog; Foxo, forkhead family of transcription factor; ILPs, insulin‐like peptides; InR, insulin receptor; IRS, insulin receptor substrate; IST-1, insulin receptor substrate ‐like adaptor; PDK-1, phosphatidylinositide‐dependent protein kinase 1 (adapted from Martins *et al*. 2016)

1.2 Synthesis, maintenance and degradation of proteins

1.2.1 Regulation of protein synthesis

Proteostasis describes the concept of multiple integrated biological processes that ensure integrity of the intra- and extracellular proteome by controlling and executing protein synthesis, folding, trafficking and degradation. Proteostasis is essential for cells to survive. Maintaining a healthy proteome is, however, very costly. The synthesis of proteins alone constitutes one of the most energy intensive processes in a cell, accounting for roughly 20 percent of total energy demand (Reeds et al., 1985, Buttgereit and Brand, 1995). For this reason the translation of mRNA into protein is regulated at the level of initiation, elongation, termination as well as ribosome recycling. The tightest control happens hereby at the level of translation initiation, which is rate-limiting in protein synthesis (Sonenberg and Hinnebusch, 2009).

In eukaryotes, the canonical pathway of translation initiation is the cap-dependent scanning mechanism which comprises multiple steps (Kozak, 1989, Hinnebusch, 2014). First, the 40S ribosomal subunit forms the pre-initiation complex (PIC) together with the ternary complex consisting of GTP bound eukaryotic initiation factor (eIF) 2 and methionine-loaded methionyl-transfer RNA (Met-tRNA^{Met}). The PIC attaches proximally to the capped 5´untranslated region (UTR) of the mRNA with the assistance of several other eIFs and scans the 5´UTR until it identifies the first start codon (Figure 4A). Here, eIF2 bound GTP is hydrolyzed with the help of even further eIFs. Thus, the interaction between PIC, mRNA and tRNA stabilizes and eIFs dissociate, resulting in an arrest of the scanning process. Subsequently, the 60S ribosomal subunit is recruited and the 80S initiation complex is assembled, whereupon the transition into the elongation phase of protein synthesis begins (Kozak, 2002, Jackson et al., 2010, Aitken and Lorsch, 2012, Lacerda et al., 2017).

Besides the canonical pathway, there are cap-independent mechanisms of translation initiation. A well-studied example is the internal ribosome entry site (IRES)-mediated translation initiation (Pelletier and Sonenberg, 1988, Macejak and Sarnow, 1991). The 40S ribosome can be recruited to the proximity of a start codon on the mRNA regardless of the 5´cap structure. This mechanism can even function without the assistance of eIFs and is relatively prevalent (Lozano and Martínez-Salas, 2015, Lacerda et al., 2017). It is estimated that about 10% of cellular mRNAs

are translated using IRES-mediated translation initiation, which is particularly important under conditions of stress when protein synthesis via the canonical pathway is stalled (Spriggs et al., 2008, Thakor and Holcik, 2012, Weingarten-Gabbay et al., 2016, Shi et al., 2016, Philippe et al., 2016).

Another way to ensure mRNA translation under circumstances that shut down the canonical pathway is via cap-independent translation enhancers (CITEs). These elements, first identified in the 3´UTR of plant viruses (Wu and White, 1999), interact with factors of the translation initiation machinery and direct them to the AUG start codon. In mammals as well, CITEs located in the 5`UTR of certain mRNAs can attract multiple components of the translation apparatus to facilitate translation initiation (Shatsky et al., 2010, Andreev et al., 2012, Lacerda et al., 2017).

Generally, functioning mRNA translation and its fine-tuned regulation are absolutely essential for survival under standard conditions, adaption to stress and even controlled cell death under continuous stress. Intriguingly and opposingly, mutations that disturb normal translation and decrease the overall rate of protein synthesis can promote longevity in baker´s yeast, fruit flies and nematodes (Hansen et al., 2007, Pan et al., 2007, Syntichaki et al., 2007, Bjedov et al., 2010, Mehta et al., 2010). Additional studies suggest a conservation of this mechanism to extend lifespan up to mammals (Hsieh and Papaconstantinou, 2004, Sharp and Bartke, 2005, McElwee et al., 2007, Karunadharma et al., 2015). Currently, there are different hypotheses that try to explain this paradox: The differential expression of a protein or a select group of proteins that directly influence aging under conditions of perturbed translation is one possibility. Another one is that a decreased load of newly synthesized proteins would allow for improved protein quality control of existing proteins. Furthermore, it was suggested that the energy balance might be improved and shifted from reproduction and anabolic processes towards somatic maintenance, when the generation of biomass is limited (Ravikumar et al., 2004, Hansen et al., 2007, Pan et al., 2007, Steffen et al., 2008, Mehta et al., 2010, Dhondt et al., 2016). It is also conceivable, that a reduction in translation rate poses stress to cells and is not beneficial itself. Instead, it activates stress responses, which then impart resistance to unfavorable conditions and lifespan extension (Wang et al., 2010b, Li et al., 2011, Robida-Stubbs et al., 2012). However, there is still insufficient evidence to definitively understand this phenomenon.

1.2.2 Challenges to the healthy proteome and protein misfolding diseases

The great majority of proteins can only fulfill their function if they assume their correct, thermodynamically stable, three-dimensional conformation. Alterations in the amino acid sequence can lead to non-functional proteins or to misfolded, aggregation prone and toxic protein species. It was reported that only one random missense mutation causes a functional loss of a protein in roughly 35% of the cases (Guo et al., 2004). To achieve and maintain proper protein folding and to degrade misfolded proteins, a lot of energy is allocated to the elaborate proteostasis network (PN). But protein homeostasis is constantly challenged by extrinsic factors, like radiation, toxins or heavy metals, and especially intrinsic factors:

First, reactive oxygen species (ROS) are a natural byproduct of a cell´s energy metabolism, detoxification mechanisms and enzymatic activity of certain proteins, but the modifications they evoke on biomolecules can be extensive. In proteins, ROS can modify certain amino acids like arginine, proline or cysteine specifically or have a more general effect on protein structure, for example by leading to carbonyl formation (Hawkins and Davies, 2001, Dröge, 2002). All of those modifications, some of which are irreversible, compromise protein function. They do so by leading to conformational changes or to alterations in biophysical properties of affected proteins, by interfering with binding of interaction partners, or by resulting in aggregation (Höhn et al., 2014).

Second, the process of protein synthesis is less than perfect. It was estimated that missense errors in translation happen every 1.000 to 10.000 codons (Neidhardt, 1987, Drummond and Wilke, 2008). Although this number and therefore the fidelity of mRNA translation seems quite high, it means that about 15% of newly synthesized proteins contain an incorrect amino acid (Drummond and Wilke, 2009).

Finally, DNA mutations in protein coding genes can result in alterations of the amino acid sequence, potentially leading to truncated, non-functional, misfolded, or aggregation prone and toxic proteins.

With increasing age, the exposure time to all the extrinsic and intrinsic risk factors rises while, at the same time, the efficiency of the protective mechanisms declines (Bulteau et al., 2000, Morley and Morimoto, 2004, Cuervo, 2008). This is the starting point for diverse age-associated degenerative disorders such as AD, PD or polyglutamine (polyQ) diseases. AD and PD are sporadic diseases in the majority of cases. Contrarily, polyQ diseases as HD, spinal and bulbar muscular atrophy or spinocerebellar ataxia always have a genetic component, which is the repeat expansion of the glutamine coding CAG trinucleotide in distinct genes (La Spada et

al., 1991, Di Prospero and Fischbeck, 2005). This expansion, once it reaches a specific threshold, causes aggregation of the affected protein and constitutes the molecular basis of disease. The length of the CAG stretch directly correlates with the protein's propensity to aggregate and thus, with its toxicity, which is reflected in age of disease onset, rate of progression and severity of symptoms (Duyao et al., 1993, Brinkman et al., 1997, Reddy et al., 1998, Scherzinger et al., 1999). Loss of function of the affected protein is likely to promote disease manifestation, but gain of toxic function is very probably causative for disease development (Duyao et al., 1995, Faber et al., 1999, Dragatsis et al., 2000, Reiner et al., 2001). In fact, it is not only the mutated protein that aggregates, but other unaffected proteins can be sequestered within the aggregates (Perutz et al., 1994). It was shown that natural interaction partners of the wildtype protein as well as non-pathogenic proteins that contain a short glutamine stretch and associate via glutamine-glutamine interactions can be enclosed in the polyQ aggregates (Preisinger et al., 1999, Chai et al., 2001, Chen et al., 2001, Perez et al., 1998, Kazantsev et al., 1999). Oligomers of the disease protein can additionally expose sticky, hydrophobic surfaces that trigger aberrant interactions with any protein in close proximity (Olzscha et al., 2011, Breydo and Uversky, 2015). The resulting imbalance and depletion of important cellular components might contribute to disease phenotypes (Steffan et al., 2000, Nucifora et al., 2001, Kim et al., 2016). Beyond this, players of the PN recognize and bind to the misfolded proteins. In the attempt to re-fold or degrade them, chaperones and the 26S proteasome are trapped within aggregates (Cummings et al., 1998, Chai et al., 1999). This further contributes to protein folding stress as key components of the protein quality control machinery are missing in action (Bence et al., 2001, Holmberg et al., 2004).

It is important to understand that a single misfolding protein can, consequently, impact the folding of other vulnerable proteins by consuming an abnormally high proportion of the limited capacity of the PN, which fails to adapt to chronic protein misfolding stress (Gidalevitz et al., 2006, Morimoto, 2008, Park et al., 2013). It is still unclear why proteostasis mechanisms are effectively induced by acute stress to restore homeostasis but are so ill-adapted to respond to persistent proteotoxic stress. If the PN could be modified to recognize and counteract chronic stress, posed for example by the accumulation of misfolding and aggregating proteins, it could be a great asset in preventing numerous age-related diseases.

1.2.3 Network of protein quality control mechanisms

An intricate and interlinked network of protein quality control (PQC) pathways manages protein folding, disaggregation and degradation (Figure 3). All branches of the PN are constantly active to maintain protein homeostasis but still have the capacity to improve their efficiency under challenging conditions like heat stress, hypoxia, or burst in protein synthesis (Lindquist and Craig, 1988, Gass et al., 2002, Dohi et al., 2012).

The correct three-dimensional structure of a protein is installed in its amino-acid sequence and many proteins can spontaneously fold into their correct shape *in vitro* (Dobson et al., 1998). Still, in the crowded environment of a cell, where proteins tend to aggregate, most proteins require assistance to achieve their native state (Ellis and Hartl, 1999)

Chaperones bind to nascent polypeptide chain during synthesis at the ribosome and ensure its folding into a thermodynamically stable, yet flexible protein (Preissler and Deuerling, 2012). It can also become necessary to refold or unfold proteins, after their stress-induced denaturation. These processes are always dependent on adenosine triphosphate (ATP) hydrolysis and the executing chaperones, large and complex multicomponent machines, often require cofactors for specific protein binding (Hartl et al., 2011). One well-studied example of ER resident chaperones involved in *de novo* protein folding is the highly expressed and stress-inducible heat-shock-protein 70 (HSP70) binding immunoglobulin protein (BiP/ HSP-4) (Gething, 1999). Like many other chaperone families, the HSP70 chaperones have additional functions in protein disaggregation, translocation and degradation (Chiang et al., 1989, Bercovich et al. 1997, Glover and Lindquist, 1998, Kabani et al., 2003, Hartl et al. 2011, Mattoo et al., 2013, Rout et al., 2014, Clerico et al., 2015, Craig, 2018) (Figure 3).

In response to heat stress, the expression of many chaperones increases due to an elevated demand to refold denatured proteins. This is termed the cytosolic heat-shock response (HSR) and it is induced by the master regulator heat shock transcription factor 1 (HSF1/HSF-1) among other HSFs (Wu, 1995, Morimoto, 1998, McMillan et al., 1998, Anckar and Sistonen, 2007). HSF1 gains DNA binding activity under stress conditions by trimerization after its release from associated chaperones, which keep it in an repressed state (Sarge et al., 1993, Zou et al., 1998, Guisbert et al., 2013, Neef et al., 2014) (Figure 3). The resulting transcriptional response is not restricted to increase in expression of chaperones but also of translational regulators and to ubiquitin, important for protein degradation (Gomez-Pastor et al., 2018).

Proteolytic mechanisms represent another arm of the proteoastasis network. Protein degradation is essential for different reasons: to recycle amino acids for protein synthesis under starvation conditions, to modulate the level of specific proteins in the cell, or to dispose of unfolded, misfolded, and toxic proteins and aggregates. Degradation by the ubiquitin-proteasome system (UPS) and autophagy (greek: self eating) are the two distinct mechanisms that enable breakdown of cellular components (Figure 3).

Autophagy is the process in which cytosolic components, big aggregates, parts of organelles or whole organelles are enclosed by membranes and delivered to lysosomes for their degradation by lysosomal enzymes at low pH (de Duve, 1963, de Duve, 1966). There are distinct types of autophagy:

In chaperone-mediated autophagy (CMA), this recycling process is highly selective for one specific protein at a time. A cytosolic, constitutive chaperone recognizes a pentapeptide motif in the substrate protein, which is consequently bound, targeted to the lysosome and unfolded. With the help of a lysosomal chaperone, the client protein is translocated to the lysosomal lumen, where it is degraded (Chiang et al., 1989, Salvador et al., 2000, Bandyopadhyay et al., 2008, Cuervo and Wong, 2014) Different studies indicate that this form of autophagy is promoting the ability of cells to adapt to unfavorable conditions like long-term starvation or hypoxia (Cuervo et al., 1998, Lv et al., 2011, Ferreira et al., 2013, Kaushik and Cuervo, 2018).

In microautophagy, cytosolic cargo is engulfed by the invaginating lysosomal membrane, which pinches off into the lysosomal lumen where it is directly digested (de Duve 1966, de Duve, 1983, Kunz et al., 2004, Li et al., 2012).

By contrast, substrates of macroautophagy are enclosed by a cytosolic double-membrane structure. Sources of this expanding membrane structure, the phagophore, are reported to include the ER, the Golgi apparatus, mitochondria and even the plasma membrane (Axe et al., 2008, van der Vaart et al., 2010, Ravikumar et al., 2010, Mari et al., 2010, Hailey et al., 2010, Feng et al., 2014, Nascimbeni et al., 2017). Once phagophores are sealed and the cargo is sequestered at their inside, there are termed autophagosomes. The outer membrane of these autophagosomes fuses with lysosomes and, prior to this, may also fuse with endosomes, to enable degradation of cargo and inner autolysosomal membrane (Tooze et al., 1990, Gordon et al., 1992, Liou et al., 1997, Berg et al., 1998) (Figure 3). The disintegrated cargo is shuttled to the cytosol by permeases and efflux carriers, where the monomeric units are then available as building material for nucleic acids, proteins and lipids (Sagné et al., 2001, Yang and Klionsky, 2007).

Lysosomal membrane components can be recycled in a process called autophagic lysosome reformation (Yu et al., 2010, Rong et al., 2011).

In general, all types of autophagy play an important role in biomolecule turnover and in the recycling of limited resources. Beyond this, macroautophagy specifically operates to discard of aggregation-prone proteins and damaged organelles (Riley et al., 2010, Rogov et al., 2014). Thereby, it constantly counteracts the development of diverse severe diseases, particularly neurodegenerative, protein misfolding diseases (Ravikumar et al., 2004, Hara et al., 2006, Komatsu et al., 2007, Levine and Kroemer, 2008).

Likewise, the other system for degradation – the UPS – is absolutely required to assure a healthy proteome and cell survival. In fact, in mammalian cells, the great majority of proteins is broken down by the UPS (Collins and Goldberg, 2017) (Figure 3). To be destined for turnover by this pathway, proteins must be modified by polyubiquitin (polyUb) chains at lysine residues (Finley, 2009). A cascade of three enzymes label the substrate proteins for degradation: the E1 ubiquitin(Ub)-activating enzyme expends ATP to activate Ub by adenylation and then transfers it to the active site of the E2 Ub-conjugating enzyme (Lake et al., 2001, Huang et al., 2007, Lee and Schindelin, 2008, Olsen and Lima, 2013). The E2 enzymes in turn catalyze the Ub transfer directly to the target protein or an E3 Ub ligase as intermediate acceptor (Hamilton et al., 2001, Wu et al., 2003, Pruneda et al., 2011, Dou et al., 2012, Stewart et al., 2016). The substrate specificity of the ubiquitylation process is guaranteed by the E3 Ub ligases. The E3 enzymes recognize the substrate and either mediate the interaction between Ub-loaded E2 enzyme and substrate to aid the final Ub transfer to the substrate or, alternatively, catalyze the Ub ligation to the substrate themselves (Huang et al., 1999, Pruneda et al., 2012, Kamadurai et al., 2013, Maspero et al., 2013, Scott et al., 2014).

The next steps in the degradation process involve the 26S proteasome, which consist of a barrel-shaped 20S core particle (CP), capped on one or both ends by the 19S regulatory particle (RP) (Tomko and Hochstrasser, 2013, Budenholzer et al., 2017). The RP and associated proteins, such as Rpn10 and Rpn13, mediate recognition and binding of the ubiquitinated substrates, recycling of the ubiquitin tag, substrate unfolding and shuttling to the CP (Deveraux et al., 1994, van Nocker et al., 1996, Yao and Cohen, 2002, Verma et al., 2002, Verma et al., 2004, Husnjak et al., 2008, Schreiner et al., 2008, Matyskiela et al., 2013, Śledź et al., 2013). Within the secluded interior of the CP, the three proteolytically active subunits with chymotrypsin-like, trypsin-like and caspase-like activities cleave the substrate protein into small peptides, which are released into the cytosol and further broken down to

free amino acids by cytosolic peptidases (Rivett, 1989, Seemüller et al., 1995, Brannigan et al., 1995, Orlowski et al., 1997, Heinemeyer et al., 1997, Groll et al., 1999, Kisselev et al., 1999, Vabulas and Hartl, 2005).

For protein degradation, the UPS again needs more than a third of the energy required for total protein synthesis. This immense demand of ATP necessitates a high level of control of UPS activity and substrate specificity (Collins and Goldberg, 2017). Indeed, the 26S proteasome is transcriptionally induced after stress and features determining its efficiency like ATPase activity, substrate affinity and proteolytic activity are regulated by diverse post-translational modifications such as phosphorylation, ubiquitination or acetylation at multiple subunits (Meiners et al., 2003, London et al., 2004, Kikuchi et al., 2010, Isasa et al., 2010, Li et al., 2011, Wang et al., 2013, Zhang and Manning, 2015, Lokireddy et al., 2015, Livneh et al., 2016). Furthermore, deubiquitinating enzymes can act fast and selectively and impart an additional level of substrate specificity (Crosas et al., 2006, Lee et al., 2016). This high investment of cells into the UPS is indispensible, considering that dysfunction of the 26S proteasome is strongly associated with multiple proteotoxic diseases like HD, frontotemporal dementia and bovine spongiform encephalitis (Mad Cow Disease) (Holmberg et al., 2004, Bennett et al., 2007, Deriziotis et al., 2011, Myeku et al., 2016).

Chaperones, the lysosomal-autophagosomal system, and the UPS are the three mechanisms of a cell to directly ensure proteostasis by enabling proper protein folding and degradation of terminally misfolded proteins. However, there are further pathways that facilitate and regulate these PQC systems that must be mentioned here:

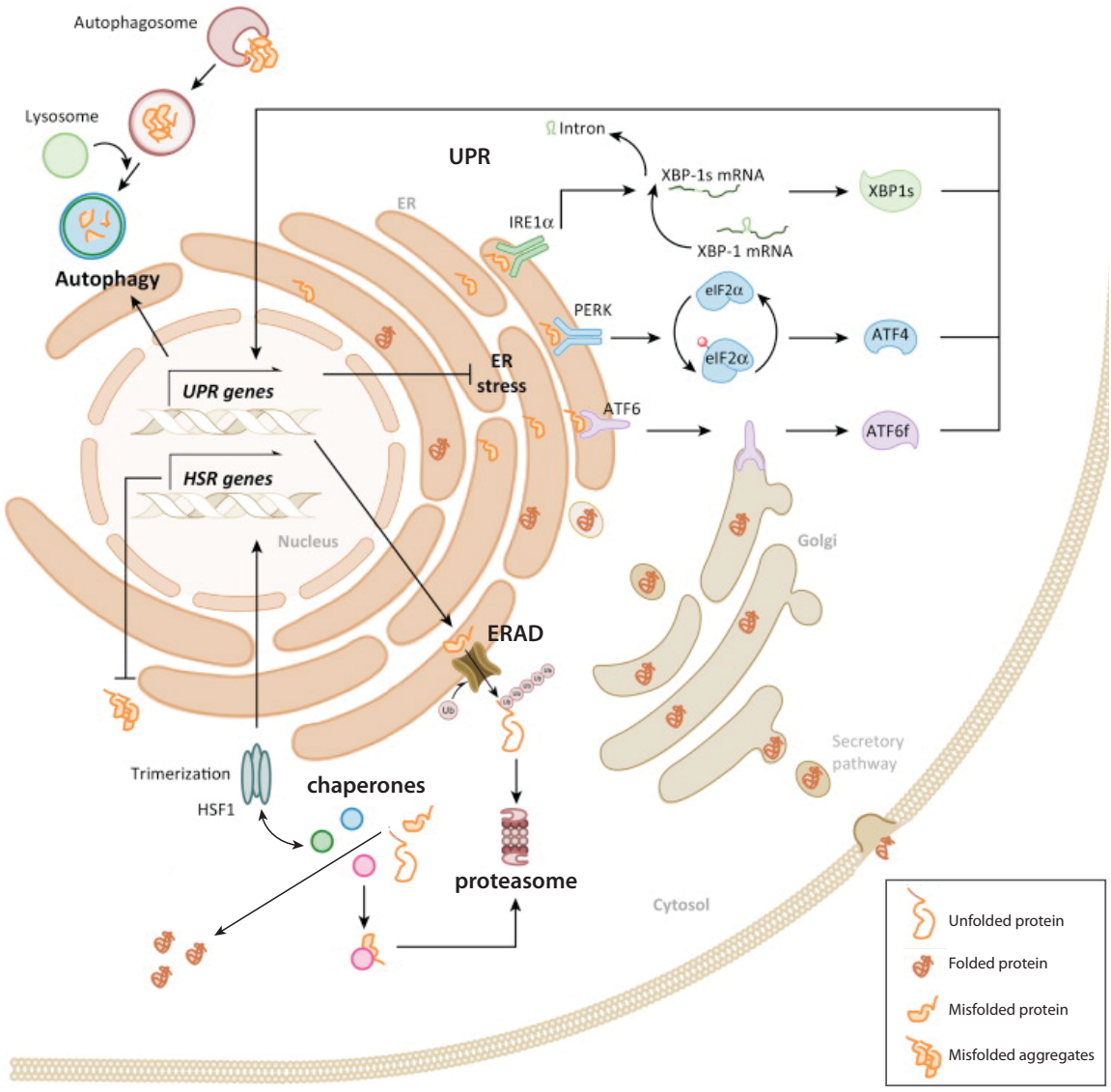


Figure 3. The eukaryotic PN. Schematic representation of the concerted action of chaperones, heat shock response (HSR), UPR, ERAD, UPS, and autophagy to maintain a healthy proteome. Chaperones aid to achieve native structure of unfolded and misfolded proteins. The HSR is activated by heat shock factor-1 (HSF1), which is released of the inhibitory interactions with HSP90 chaperone, trimerizes and is transported to the nucleus to induce heat shock response target genes after detection of misfolded proteins. The IRE1, PERK, and ATF6 branch of the UPR sense unfolded or misfolded proteins within the ER and trigger the expression of UPR target genes and, in case of PERK, halt general translation to counter ER stress and restore protein homeostasis, for example by boosting ERAD and autophagy, or initiate cell death. ERAD describes the process by which misfolded proteins are shuttled from ER to cytosol, followed by their ubiquitination and proteasomal degradation. The UPS targets proteins for degradation by covalent linkage of ubiquitin, followed by proteolytic cleavage by the 26S proteasome. Additionally, protein aggregates and damaged organelles are sequestered and degraded by lysosomal enzymes at low pH via autophagy (modified from Mardones *et al.* 2015).

The ER is a major site of protein synthesis. All proteins destined for the secretory pathway, which amount to about one third of the proteome, are synthesized at the ER by associated ribosomes (rough ER) (Palade, 1975, Chen et al., 2005). The oxidative environment of the ER lumen with its specialized enzymes allows modification of the secretion-bound proteins and membrane proteins by disulfide bonds and – in collaboration with the Golgi apparatus – N-linked and O-linked glycosylation, essential for protein folding and function (Hirschberg and Snider, 1987, Appenzeller-Herzog and Ellgaard, 2008, Braakman and Bulleid, 2011, Stanley, 2011, Aebi, 2013, Oka and Bulleid, 2013). Diverse ER resident chaperones such as GRP94/ENPL-1 or BiP/HSP-3 and HSP-4 of the HSP90 and HSP70 families are employed to manage the folding and re-folding of the immense load of proteins (Braakman and Bulleid, 2011, Behnke et al., 2015, Marzec et al., 2012). However, the ER itself does not accommodate a system to degrade proteins that are either misfolded beyond repair or which have to be downregulated. Instead, it relies on translocation of these proteins back to the cytosol, where they are broken down by the UPS. This process is termed endoplasmic reticulum-associated degradation (ERAD) (Hiller et al., 1996, Avci et al., 2014) (Figure 3). N-linked glycosylation plays an important role in the recognition of ERAD substrates, because specific processing of the sugar moieties can generate a direct signal for destruction. Specialized lectins like OS-9/F48E8.4 can bind the sugar moieties and speed up retrotranslocation and elimination by the proteasome significantly, compared to non-glycosylated ERAD substrates (Aebi et al., 2010, Benitez et al., 2011, Jaenicke et al., 2011). Besides lectins in the ER lumen, chaperones and the HRD ubiquitin ligase complex can perform the task of identifying misfolded proteins within the ER membrane (Taxis et al., 2003, Baldridge and Rapoport, 2016). Further components of the ERAD machinery are in charge of polyubiquitinating and unfolding misfolded proteins, forming channels for their retrotranslocation, generating the force to pull them into the cytosol and, finally, deliver them to the proteasome (Bodnar and Rapoport, 2017, Schulz et al., 2017, Berner et al., 2018).

Another protein quality control system installed in the ER is the unfolded protein response (UPR). Its three parallel branches use different sensors to detect perturbations of ER homeostasis and, as a response, either modify the PN to counteract ER stress, downregulate global protein synthesis or induce cell death. All of the sensor proteins are located to the ER membrane. They have a luminal domain to query the protein folding status within the ER, and a cytosolic domain to transduce signals to the transcriptional and translational machineries (Ron and Walter, 2007, Dufey et al., 2014) (Figure 3).

The most conserved branch of the UPR is activated by dissociation of BiP/HSP-4 from and direct binding of unfolded proteins to the UPR sensor inositol-requiring protein-1 (IRE1/IRE-1) (Cox et al., 1993, Mori et al., 1993, Bertolotti et al., 2000, Okamura et al., 2000, Credle et al., 2005, Mori, 2009, Gardner and Walter, 2011). The consecutive IRE1 oligomerization and trans-autophosphorylation enables activation of its RNAse domain. This in turn leads to regulated IRE1-dependent decay (RIDD) of a subset of mRNAs to specifically stall cell growth and proliferation and even induce cell death. Additionally, the decreased transcript levels might help to alleviate protein folding stress. (Hollien and Weissman, 2006, Kimata et al., 2007, Korennykh et al., 2009, Aragón et al., 2009, Hollien et al., 2009, Han et al., 2009, Li et al., 2010a, Walter and Ron, 2011, Maurel et al., 2014). Arguably the most important target of IRE1 RNAse activity is the *X-box binding protein 1* mRNA (XBP1/XBP-1). Though instead of being degraded, *XBP1* mRNA gets spliced (*XBP1s*) and translated to the potent XBP1s transcriptional activator, following IRE1 activation. XBP1s binds to unfolded protein response elements (UPREs) and thereby induces a set of UPR target genes, such as ER chaperones, ERAD components and glycosylation enzymes, with the aim to counter ER stress and reinstall ER homeostasis (Yoshida et al., 2001, Lee et al., 2002, Calfon et al., 2002, Yoshida et al., 2003, Lee et al., 2003, Yamamoto et al., 2004).

The sensor protein that activates the second branch of the UPR, PKR-like ER kinase (PERK/PEK-1), considerably resembles IRE1 in structure and mode of activation (Liu et al., 2000, Bertolotti et al., 2000). However, after detection of unfolded proteins in the ER, oligomerization and trans-autophosphorylation, the kinase domain of PERK phosphorylates its sole client: the eukaryotic translation initiation factor eIF2α. Phosphorylation of eIF2α inhibits global protein translation while simultaneously stimulating the translation of the transcription factor activating transcription factor 4 (ATF4/ATF-5) (Harding et al., 1999, Scheuner et al., 2001, Jackson et al., 2010). The downstream targets of ATF4 are expressed to antagonize ER stress (Harding et al., 2000b). This branch of the UPR is part of the integrated stress response (ISR).

The third branch of the UPR is operated by the glycoprotein activating transcription factor 6 (ATF6). As an inactive precursor, it is clustered in the ER membrane (Nadanaka et al., 2007). Upon stress, its luminal domain dissociates from BiP/HSP-4 and ATF6 is packaged into vesicles, which are transported to the Golgi apparatus (Shen et al., 2002, Schindler and Schekman, 2009). Here, the luminal and transmembrane portions of ATF6 are clipped by two Golgi resident proteases, site-1 protease and site-2 protease, respectively. The cleavage releases the N-terminal

cytosolic domain of ATF6. This active ATF6 fragment moves to the nucleus and acts as transcription factor at ER stress response elements (ERSEs) upstream of UPR target genes to expand the ER and enhance its folding capacity (Yoshida et al., 1998, Haze et al., 1999, Roy and Lee, 1999, Ye et al., 2000, Okada et al., 2003, Yamamoto et al., 2004, Adachi et al., 2008, Bommiasamy et al., 2009, Mori, 2010).

The ER is the ideal cellular compartment to monitor proper protein folding with the huge proportion of the proteome being modified in and shuttled through this organelle. For example, in human specialized tissues like salivary glands or the pancreas around or above 60% of all transcripts encode for secreted proteins, respectively (Uhlén et al., 2015).

To maintain proteostasis is, however, not only essential within the ER, but also vital for mitochondrial function. Like the ER, mitochondria employ retrograde signaling to the nucleus to induce a specific transcriptional program designed to counter stress. This process is termed the mitochondrial unfolded protein response (UPR^{mt}). The UPR^{mt} is initiated when activating transcription factor associated with stress-1 (ATFS-1) - despite its mitochondrial localization signal - is not properly imported into mitochondria where it is degraded, but instead remains in the cytosol. Stress conditions that impair mitochondrial import, like depletion of mitochondrial DNA, perturbations of the electron transport chain or increased presence of unfolded proteins, trigger this accumulation of ATFS-1 (Martinus et al., 1996, Zhao et al., 2002, Yoneda et al., 2004). As a consequence, the additionally present nuclear localization sequence of ATFS-1 causes its shuttling to the nucleus, where it induces UPR^{mt} targets involved in protein quality control, detoxification, mitochondrial biogenesis, metabolism and innate immunity (Aldridge et al., 2007, Nargund et al., 2012, Haynes et al., 2013, Wu et al., 2014, Schulz and Haynes, 2015).

All of the players and modifiers of the PN mentioned above are important protective mechanisms that are set against the constant insults to a healthy proteome. Unfortunately, as damage accumulates with increasing age, those PQC pathways are progressively failing or become deregulated (Terman 1995, Vittorini et al., 1999, Cuervo and Dice, 2000, Donati et al., 2001, Carrard et al., 2002, Rabek et al., 2003, Ferrington et al., 2005, Balch et al., 2008, Nuss et al., 2008, Ben-Zvi et al., 2009, Hamer et al., 2010, David et al., 2010, Taylor and Dillin, 2013, López-Otín et al., 2013, Jensen and Jasper, 2014, Münch and Harper, 2016, Frakes and Dillin, 2017). Genetic or pharmacological inhibition of parts of the PN can provoke a premature aging phenotype as shown in several studies (Hsu et al., 2003, Simonsen et al., 2008, Zaglia et al., 2014, Higuchi-Sanabria et al., 2018). In *Drosophila*, genetic knockdown of a component of the proteasome lid, led to lifespan reduction and to

premature neurodegeneration in an HD model, to name one example (Tonoki et al., 2009). In another fly study, the administration of the specific proteasome inhibitor PS-341 to adult animals impaired their motor function and also shortened their lifespan in a dose-dependent manner (Tsakiri et al., 2013). Modulation of proteasome activity is relevant in human aging as well: It was for instance observed, that pharmacological, partial proteasome inhibition induced premature senescence in human fibroblasts (Chondrogianni et al., 2003, Chondrogianni and Gonos, 2004). Together, these observations demonstrate the pivotal role of proteostasis during healthy aging.

1.2.4 The integrated stress response

It can be necessary to decrease or shut down cost intensive general translation under various stress conditions like amino acid or glucose starvation, heat shock, anoxia or accumulation of unfolded proteins in the ER. A well studied mechanism to achieve this is the reversible phosphorylation of the α subunit of eIF2 at serine 51 (Ser51), as mentioned above in the context of PERK activation and the UPR. In the unphosphorylated state, GTP-bound eIF2 associates with Met-tRNA^{Met} to form the ternary complex, which is then delivered to the small ribosomal subunit (40S). The resulting 43S pre-initiation complex is essential for translation initiation at the majority of transcripts as it scans the mRNA sequence to deliver methionine to the start codon (Jackson et al., 2010, Shirokikh and Preiss, 2018) (Figure 4A). Phosphorylated eIF2α (P-eIF2α) can still participate in the ternary complex. However, P-eIF2α strongly binds its guanine nucleotide exchange factor (GEF) eIF2B already in the TC, where this association and concomitant release of eIF5 has a destabilizing effect. After the hydrolysis of the bound GTP, and before the 80S initiation complex is assembled, P-eIF2-GDP is liberated from the TC but remains tightly bound to eIF2B (Unbehaun et al., 2004, Kapp and Lorsch, 2004, Jennings et al., 2017). This stable interaction abrogates the eIF2B GEF function and eIF2-GTP is not available to form TCs anew. Thereby, P-eIF2 is its own competitive inhibitor. It lowers the amount of free eIF2B and hence the amount of its own active form, eIF2-GTP (Sudhakar et al., 2000, Sonenberg and Hinnebusch, 2009, Kashiwagi et al., 2016, Bogorad et al., 2017) (Figure 4B). Consequently, phosphorylation of the α subunit of eIF2 attenuates translation initiation at mRNAs that are translated by the cap-dependent mechanism, which is the vast majority.

Simultaneous to the attenuation of global translation, the expression of a distinctive set of stress-related genes is elevated by the phosphorylation of eIF2α. The underlying cause for this particular expression signature is the specific translation of genes with at least two upstream open reading frames (uORFs), such as ATF4 (ATF-5) (Figure 4B). Under control conditions, the level of the P-eIF2α is low and translation initiation can proceed quickly. In this case, the ribosome transitions from the positive-acting 5´ proximal uORF (uORF1) in the ATF4 mRNA directly to the inhibitory second uORF (uORF2), where translation is reinitiated. As uORF2 overlaps with the ATF4 coding region, it is repressive and ATF4 is only expressed at a very low basal level. With rising P-eIF2α levels, reinitiation becomes delayed and ribosomes can skip uORF2. Instead there is increasing translation initiation at the coding region for ATF4, delivering an active transcription factor (Mueller and Hinnebusch, 1986, Mueller et al., 1987, Vattem and Wek, 2004, Lu et al., 2004, Zhou et al., 2008, Jackson et al., 2010). The broad transcriptional program activated by ATF4 and other factors that are regulated in a similar manner, like ATF5, is designed to re-establish cellular homeostasis or induce apoptosis (Harding et al., 2000a, Natarajan et al., 2001, Hinnebusch and Natarajan, 2002, Harding et al., 2003, Lu et al., 2004, Jiang et al., 2004).

The integration of many different signals, their convergence on phosphorylation of eIF2α, and the resulting translation attenuation and expression of specific stress relevant genes was termed the integrated stress response (ISR) (Harding et al., 2003). In mammals, four protein kinases achieve this integration of distinct stimuli by targeting eIF2α at Ser51: Protein kinase R (PKR) is activated by double-stranded RNA (dsRNA) and plays a role in antiviral immune defense (Levin et al., 1980, Meurs et al., 1990). Heme-regulated inhibitor (HRI) kinase is activated by heme deprivation in erythroid cells (Chen et al., 1991, Chen and London, 1995). PERK (PEK-1) detects and is activated by misfolded proteins in the ER lumen. It is part of the UPR^{ER} and helps to counter ER stress (Harding et al., 1999, Bertolotti et al., 2000). General control non-depressible 2 (GCN2/GCN-2) is activated by uncharged tRNAs under amino acid depletion, but further roles were described (Dever et al., 1992, Zhang et al., 2002, Jackson et al., 2010, Taniuchi et al., 2016): In *C. elegans* for example, GCN-2 is activated by hypertonic stress and is required to survive those conditions (Lee and Strange, 2012). Furthermore, it was shown that GCN-2 is necessary for lifespan extension caused by dietary restriction or inhibition of target of rapamycin (TOR) (Rousakis et al., 2013, Ferraz et al., 2016).

In vivo, GCN2 requires assistance by additional proteins to phosphorylate eIF2α and activate the ISR. GCN1 (GCN-1) is a direct interaction partner of GCN2 as

well as the ribosome, and while it is dispensable for GCN2 kinase activity, it is essential for GCN2 activation by uncharged tRNAs (Ramirez et al., 1992, Marton et al., 1993, Garcia-Barrio et al., 2000, Kubota et al., 2000, Sattlegger and Hinnebusch, 2000, Qiu et al., 2002, Pereira et al., 2005, Cambiaghi et al., 2014). GCN1 binds to the ribosome, where uncharged tRNAs are likely to occur as they inadvertently enter the ribosome acceptor site (A-site) and get stuck at the stalling translational machinery. Here, GCN1 is proposed to facilitate the liberation of the uncharged tRNA and its transfer onto GCN2 (Ramirez et al., 1991, Marton et al., 1997, Sattlegger and Hinnebusch, 2000, Sattlegger and Hinnebusch, 2005, Waller et al., 2012, Roffé et al., 2013). The yeast GCN20 (ABCF3/ ABCF-3) protein forms a complex with GCN1 and was found to modulate the crucial ribosome binding of GCN1. Despite its own weak affinity to ribosomes, GCN20 can greatly enhance the polysome-associated fraction of GCN1/GCN20 complex. This activity is dependent on the energy status of a cell, which can be sensed by two domains within GCN20 that are highly similar to nucleotide binding domains in ATP binding cassette (ABC) transporters. In the presence of ATP the portion of GCN1 at polysomes is strongly promoted by GCN20 (Vazquez de Aldana et al., 1995, Marton et al., 1997, Kerr, 2004). Interestingly, a residual activating function of GCN20 on GCN2 activity remains even if the ABC containing region is deleted (Marton et al., 1997). To conclude, while GCN1 is absolutely required for the activation of GCN2 under diverse stress conditions like amino acid, purine or glucose starvation or hypertonic stress, GCN20 is not essential under most conditions but can greatly enhance ribosome association of GCN1 and GCN2 and thereby tune the ISR (Rolfes and Hinnebusch, 1993, Marton et al., 1997, Yang et al., 2000a, Goossens et al., 2001, Narasimhan et al., 2004, Castilho et al., 2014). Consistently, GCN1 is well conserved and has a clear homolog in mammals (www.uniprot.org/uniprot/Q92616). For GCN20 the situation is less clear, but ABCF1 (also ABC50) is considered a functional homolog: Within the critical N-terminus that enables interaction with GCN1, human ABCF1 has 20% sequence identity and 30% sequence similarity to yeast GCN20. Although ABCF1 cannot substitute for GCN20 in a yeast deletion strain, it does associate with GCN2 and ribosomes and fulfills functions in regulation of mRNA translation as well as start codon selection (Tyzack et al., 2000, Paytubi et al., 2008, Paytubi et al., 2009, Castilho et al., 2014, Stewart et al., 2015).

The ISR and many of its players also exist in *C. elegans*: GCN-2 and PEK-1 have been identified as homologs of the eIF2α kinases GCN2 and PERK, respectively. Furthermore, GCN-1 is conserved in the nematode and ABCF-3 is

expressed as the functional homolog of yeast GCN20 (Zhao et al., 2007, Hirose and Horvitz, 2014).

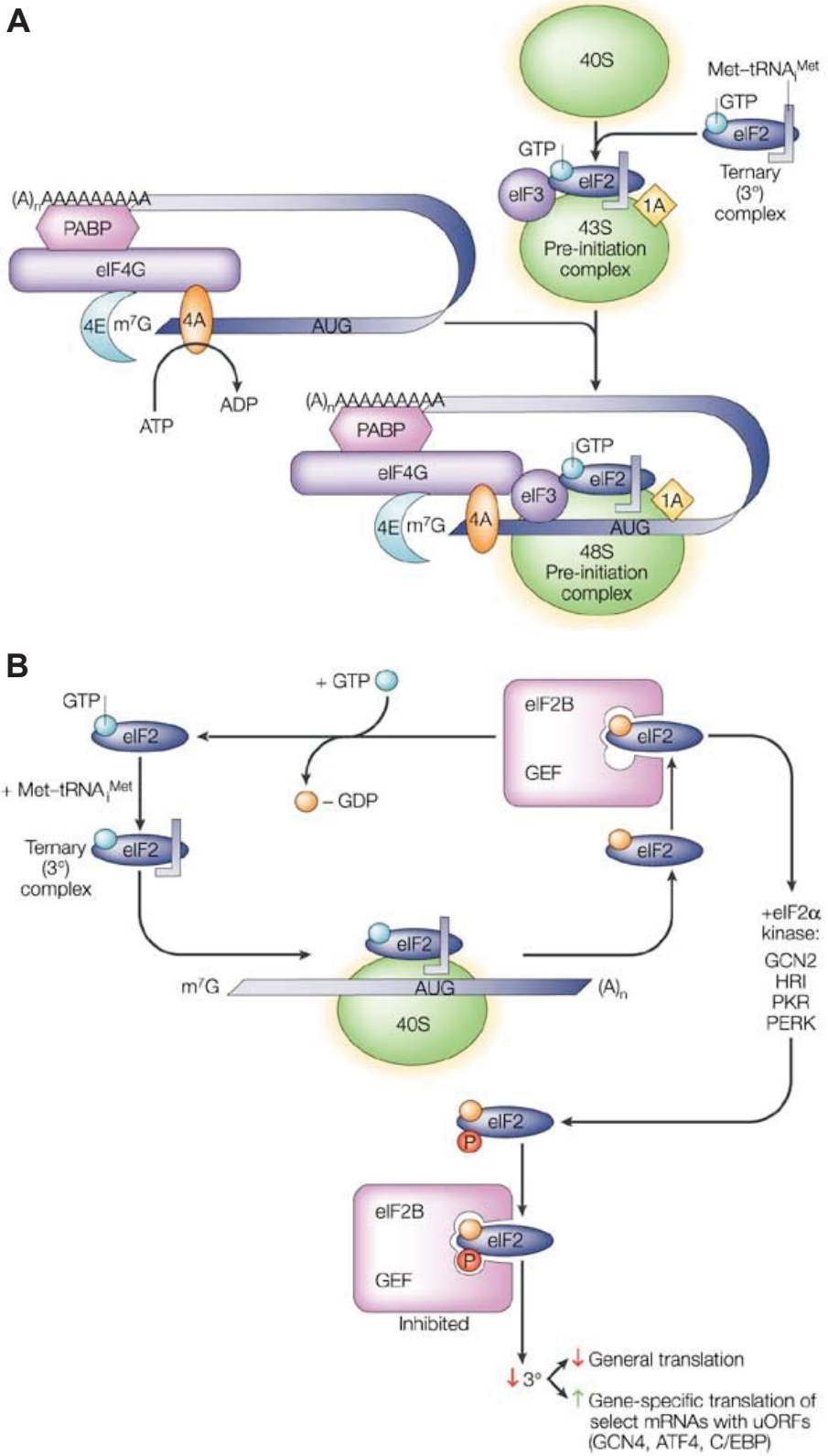


Figure 4. Simplified schemes of the regulatory role of eIF2 on mRNA translation. A. eIF2, GTP and Met-tRNA^{Met} form the ternary complex, which associates with the 40S ribosomal subunit, supported by additional factors, such as eIF3 and eIF1A (1A). The resulting 43S pre-initiation complex binds to mRNA, which is circularized and primed by the 7-methyl-GTP (m7GTP) cap structure-binding complex, consisting of eIF4E (4E), eIF4G and eIF4A (4A), and by the poly(A)-binding protein (PABP). The assembled 48S pre-initiation complex can start the scanning process to identify the AUG start codon, followed by binding of the large, 60S ribosomal subunit and mRNA translation**. B.** eIF2-GTP binary complex binds to Met-tRNA^{Met} to form the ternary complex, which then associates with the 40S ribosomal subunit to initiate mRNA translation. After start-codon identification, GTP is hydrolyzed and eIF2-GDP is released. The GEF eIF2B converts inactive eIF2-GDP to active eIF2-GTP. This process is inhibited by phosphorylation (P) of the α-subunit of eIF2 catalyzed by four eIF2α kinases. P-eIF2-GDP is a competitive inhibitor of eIF2B, and thereby lowers the level of active eIF2B and ternary complexes. This, in turn, reduces general translation but increases translation of mRNAs containing uORFs, such as GCN4, ATF4 and CCAAT/enhancer-binding protein (C/EBP) (adapted from Klann and Denver 2004).

1.2.5 Improved protein quality control at the basis of longevity

The progressive failure of PQC mechanisms and resulting loss of proteostasis is a hallmark of aging and the accumulation and aggregation of misfolded proteins is a common feature of many neurodegenerative diseases like AD, PD and HD (López-Otín et al., 2013). Intriguingly, a study of related species with great differences in lifespan, such as house mouse and naked mole rat, revealed differences in the capacities of their proteostasis mechanisms. The long-lived species showed higher expression of chaperones and elevated levels of autophagy and proteasome activity compared to their shorter-lived relatives (Salway et al., 2011, Pride et al., 2015, Rodriguez et al., 2016). With this in mind, it is interesting to ask whether improved proteostasis is causative and sufficient for longevity and healthy aging and whether it can decrease the incidence of neurodegenerative and other age-associated diseases. Many of the studies addressing this question are carried out in *C. elegans* with its easy genetics and high degree of conservation of important signaling pathways and PQC mechanisms:

First, induction of the heat shock response by overexpression of HSF-1 or short, hormetic heat treatment promotes stress resistance and longevity and delays the accumulation of protein aggregates in *C. elegans* disease models (Hsu et al., 2003, Baird et al., 2014, Kumsta et al., 2017). Additionally, increased abundance of key players of the chaperone network such as the chaperonin-containing T-complex

can extend nematode lifespan (Noormohammadi et al., 2016). Second, the overexpression of the key autophagy regulator helix loop helix 30 (HLH-30), the nematode homolog of the mammalian transcription factor EB (TFEB), as well as its pharmacological activation prolong *C. elegans* lifespan (Lapierre et al., 2013, Wang et al., 2017a). Third, enhanced proteasome abundance or activity can confer resistance to proteotoxic stress and extend the life of nematodes (Hassan et al., 2009, Vilchez et al., 2012, Chondrogianni et al., 2015). Furthermore, an enhancement of a combination of protein quality control pathways was achieved by activation of the hexosamine pathway, which produces an amino sugar essential for protein glycosylation. The moderate increase in autophagy, proteasome activity and ERAD in combination, lead to improved proteostasis and longevity (Denzel et al., 2014). Lastly, also the manipulation of modifiers of the PN can have beneficial effects on the health and lifespan of organisms. For example, the activation of the IRE-1 branch of the UPR^{ER} by neuronal expression of constitutively active spliced XBP-1 leads to stress resistance and longevity in the nematode (Taylor and Dillin, 2013).

Many of the health- and lifespan promoting effects of these pathways are conserved to mammals. To give a few examples, the genetic or pharmacological activation of HSF1 can decrease cytotoxicity in cell culture and extend health- and lifespan in protein folding disease mouse models (Kieran et al., 2004), (Fujimoto et al., 2005, Neef et al., 2010, Liangliang et al., 2010, Pierce et al., 2010, Calamini et al., 2011). Similarly, the activation of autophagy by overexpression of Atg5, a protein involved in autophagic vesicle formation, prolongs health- and lifespan in mice and elevates stress resistance (Pyo et al., 2013). Beyond this, analysis of samples from healthy human centenarians revealed an elevated abundance and activity of the proteasome compared to other elderly subjects and increased levels of the autophagy regulator BECLIN 1 (Chondrogianni et al., 2000, Emanuele et al., 2014).

It is important to note that longevity usually comes at a price. In *C. elegans,* several distinct longevity models exist and many display obvious trade-offs in accordance with the antagonistic pleiotropy theory of aging (Williams, 1957): For instance, slowed down development, small body size or reduced reproductive fitness are phenotypes that can be observed in stress-resistant and long-lived genetic variants with modified insulin signaling (e.g. *daf-2*), food intake (e.g. *eat-2*) and mitochondrial function (e.g. *isp-1*) among others (Gems et al., 1998, Jenkins et al., 2004, Chen et al., 2007b, Hughes et al., 2007, Chen et al., 2007a). In contrast to this, there are longevity models based on improved protein quality control which lack obvious detrimental effects (Denzel et al., 2014). It could be interesting to perform more systematic studies of mutants with elevated PQC regarding their life history

traits, as targeting the PN could turn out to be a very sensible therapeutic way to improve human health span. However, it will be necessary to transiently induce proteostasis mechanisms or fine tune their activation, because chronic and overshooting responses of PQC mechanism can be pro-apoptotic as well as auxiliary to tumor growth (Upton et al., 2012, Chen et al., 2014, Mariño et al., 2014, Dai and Sampson, 2016, Levy et al., 2017, Higuchi-Sanabria et al., 2018).

1.3 The hexosamine pathway

1.3.1 The hexosamine pathway as metabolic hub

The hexosamine pathway (HP) is a major anabolic pathway that produces uridine diphosphate-N-Acetylglucosamine (UDP-GlcNAc). This aminosugar is an essential precursor for protein N- and mucin-type O-glycosylation, O-GlcNAcylation, and the synthesis of biopolymers such as hyaluronic acid and chitin, and of GPI anchored proteins and proteoglycans among other things.

UDP-GlcNAc is produced from Fructose 6-phosphate (Fruc-6-P) in four consecutive steps by the designated enzymes of the HP (Figure 5). Around 2 to 5 % of cellular glucose enter the HP as Fruc-6-P, an intermediate product of glycolysis (Marshall et al., 1991). Glutamine-fructose 6-phosphate aminotransferase (GFAT-1), the first enzyme of the HP, uses Glutamine (Gln) as nitrogen donor to convert Fruc-6-P to Glucosamine 6-phosphate (GlcN-6-P) (Figure 5). This is the rate-limiting step of the HP and, consequently, GFAT-1 activity controls the consumption of Fruc-6-P and production of UDP-GlcNAc. The second enzymatic step of the HP is executed by Glucosamine 6-phosphate N-acetyltransferase, which requires Acetyl-Coenzyme A (Acetyl-CoA) to form N-acetylglucosamine 6-phosphate (GlcNAc-6-P) from GlcN-6-P. The third enzyme, Phosphoacetylglucosamine mutase, converts GlcNAc-6-P to its reactive isomer N-acetylglucosamine 1-phosphate (GlcNAc-1-P). Finally, uridine triphosphate (UTP) is utilized by UDP-N-acetylglucosamine pyrophosphorylase to produce UDP-GlcNAc (Ghosh et al. 1960) (Figure 5).

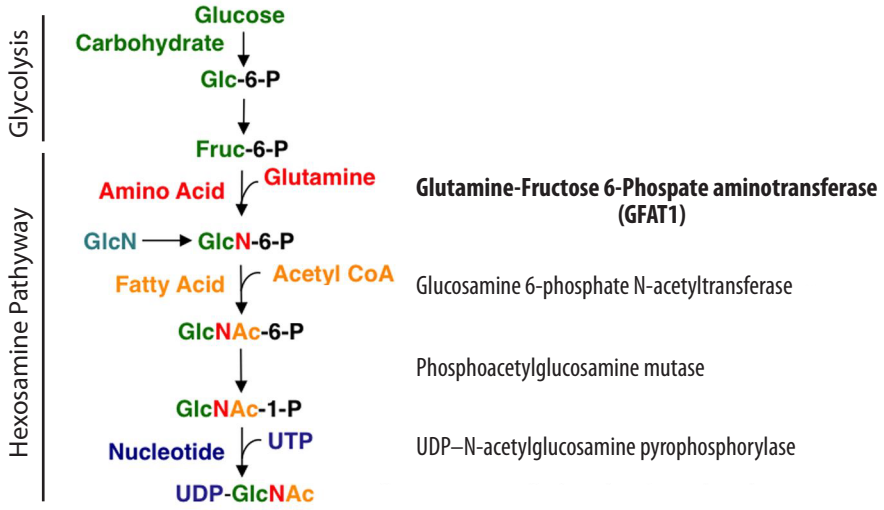


Figure 5. Production of the high energy metabolite UDP-GlcNAc. The HP uses products from carbohydrate (green), amino acid (red), fatty acid (orange), and nucleotide metabolism (blue) to produce UDP-GlcNAc. The first enzymatic reaction of the HP, conversion of Fruc-6-P to GlcN-6-P under Glutamine expenditure, is catalyzed by GFAT1 and is rate-determining. This limitation can be bypassed by providing GlcN (turquoise) (modified from Tan et al., 2017).

Taken together, the production of one high energy molecule UDP-GlcNAc depends on Fruc-6-P from glycolysis, Gln from amino acid metabolism, Acetyl-CoA from fatty acid oxidation and UTP from nucleotide metabolism. Hence, the HP is ideally positioned to sense the energy status of a cell and to integrate signals from multiple metabolic pathways. Indeed, flux through the HP corresponds to Glucose availability (Wells et al., 2003, Marshall et al., 2004, Nakajima et al., 2010, Abdel Rahman et al., 2013). Furthermore, it was reported that the reversible attachment of single UDP-GlcNAc molecules to peptides at serine or threonine residues by the enzyme O-GlcNAc transferase (OGT, discussed in detail below) is a way to signal energy availability and metabolic state (Torres and Hart, 1984, Kreppel et al., 1997, Lubas et al., 1997). Studies that support this idea show a low affinity of OGT to UDP-GlcNAc and, *in vitro*, a strong responsiveness of the enzyme to an exceptionally wide range of UDP-GlcNAc concentrations. Additionally, the affinity of OGT to peptide substrates seems to be tuned by UDP-GlcNAc concentrations. On the one hand this allows successful O-GlcNAc modification even at low metabolite levels. On the other hand the presence of nutrients and resulting UDP-GlcNAc production can directly be coupled to alterations in post-translational protein modifications (Haltiwanger et al., 1992, Kreppel and Hart, 1999, Walgren et al., 2003,

Bond and Hanover, 2013). Consistently, changes in HP activity and O-GlcNAc signatures were implicated in diseases that either lead to or require altered glucose metabolism, such as diabetes and cancer (Marshall et al., 1991, Patti et al., 1999, Yang et al., 2008, Caldwell et al., 2010, Slawson et al., 2010, Lynch et al., 2012, Taparra et al., 2016).

It is important to note that the generation of UDP-GlcNAc does not increase proportionally with available resources, but that GFAT-1 and thereby the whole HP is regulated more intricately. The best described mode of regulation of GFAT-1 is its strict feedback inhibition by its own product GlcN-6-P and the pathway's endproduct UDP-GlcNAc (Kornfeld, 1967, DeHaven et al., 2001, Niimi et al., 2001, Broschat et al., 2002). Further modulations of GFAT-1 activity for example by phosphorylation are less well understood and detailed later.

1.3.2 Diverse functions of the HP products

The HP consumes high energy intermediates and products of several metabolic pathways to produce UDP-GlcNAc. This aminosugar can be interconverted into its isomer UDP-N-Acetyl galactosamine (UDP-GalNAc) in a reversible enzymatic step catalyzed by UDP-galactose-4-epimerase (GALE) (Thoden et al., 2001, Holden et al., 2003). Both molecules are essential for the assembly of diverse biomolecules as well as co- and posttranslational sugar modifications of proteins (Moussian, 2008, Corfield and Berry, 2015). Especially GlcNAc is highly abundant in nature. It is the sole building block of chitin, the most common natural polymer second only to cellulose. Chitin is a prevailing component of the cell wall of fungi, the exoskeleton of arthropods and the egg shell of nematodes like *C. elegans* (Rudall and Kenchington 1973, Zhang et al., 2005, Ruiz-Herrera, 2016). Furthermore, GlcNAc accounts for half of the sugar molecules used to construct the peptidoglycan layer of bacterial cell walls (Leyh-Bouille et al., 1966, Osborn, 1969, Bugg and Walsh, 1992).

In eukaryotes, aminosugars are found in a layer surrounding single cells as well: the extracellular matrix (ECM). It contains fibrous proteins like the highly abundant collagens or elastins and fibronectins. Moreover, the ECM contains glycosaminoglycans (GAGs), which are long unbranched polysaccharide chains comprising acetylated aminosugars such as UDP-GlcNAc and UDP-GalNAc. These GAGs are typically linked to a core protein to form proteogylcans like heparan sulphate, heparin, or keratan sulphate, or, in the case of hyaluronic acid, exist freely

in the ECM (Frantz et al., 2010, Hynes and Naba, 2012) (Figure 6). Obviously, the ECM presents an extracellular scaffold and has a structural and mechanical role in tissue architecture. Beyond that, it interacts with cell surface receptors, growth factors and pathogens and thus fulfills manifold functions including roles in cell adhesion, proliferation, migration, differentiation, and survival (Panayotou et al., 1989, Meredith et al., 1993, Engler et al., 2006, Wang et al., 2008a, Rozario and DeSimone, 2010, Plotnikov et al., 2012, Lee-Sayer et al., 2015, Mecham and Ramirez, 2018).

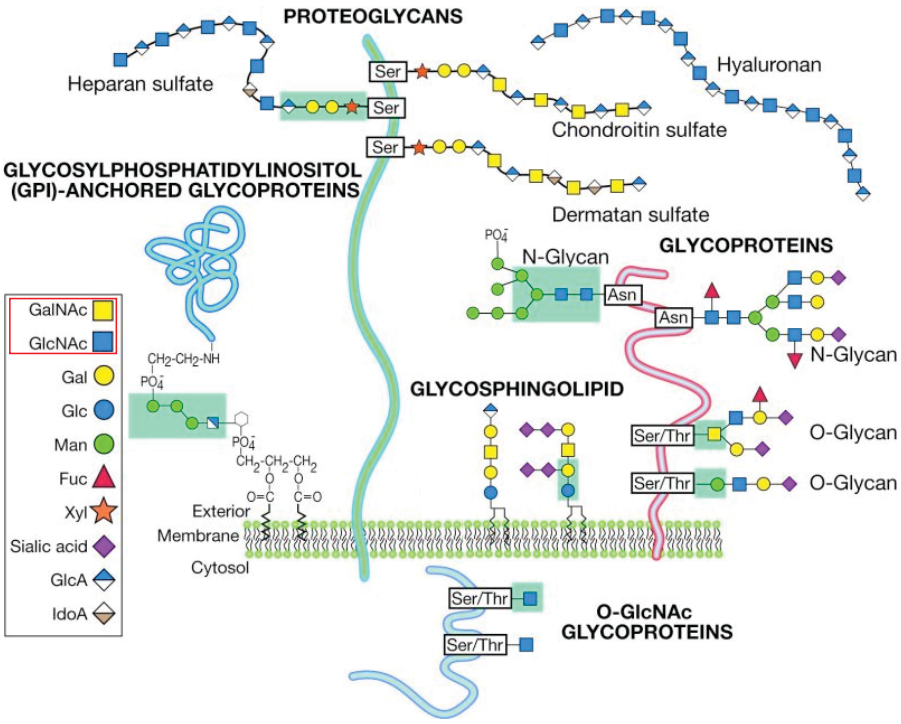


Figure 6. Schematic representation of major glycan types and the prevalence of GalNAc and GlcNAc. Glycosaminoglycans are common structures of the extracellular matrix. The long carbohydrate chains can, in case of Hyaluronan, exist freely or be linked to proteins to form proteoglycans. Another class of glycans are glycosphingolipids, which consist of a membrane-anchored ceramide modified by oligosaccharides. Different posttranslational modifications involve the attachment of carbohydrate structures: Glycosylphosphatidylinositol (GPI) anchors are glycolipid moieties that allow membrane tethering of proteins. Exceptionally complex, branched oligosaccharides are found on secreted and membrane-bound N-glycans and O-glycans, where they modulate protein stability, interactions and biophysical properties. The transient modification of intracellular O-GlcNAc glycoproteins by single GlcNAc molecules fulfills important signaling functions. GalNAc, N-Acetylgalactosamine; GlcNAc, N-Acetylglucosamine; Gal, Galactose; Glc, Glucose; Man, Mannose; Fuc, Fucose; Xyl, Xylose; GlcA, Glucuronic acid; IdoA, Iduronic acid (modified from Varki et al., 2009a).

Furthermore, GlcNAc and GalNAc are part of integral components of biological membranes. They are precursors for the diverse group of glycosphingolipids, which typically constitute a transmembrane glucosyl or galactosyl ceramide core modified by an extracellular oligosaccharide chain (Sandhoff and Kolter, 2003, D'Angelo et al., 2013) (Figure 6). In general, glucosylceramide-derived glycosphingolipids are essential for embryonic development in mammals, but also *C. elegans* (Yamashita et al., 1999, Marza et al., 2009, Jennemann et al., 2012, Boland et al., 2017). Depending on the composition of the saccharide chain they are involved in numerous fundamental biological processes like intracellular trafficking, apoptosis, nerve myelination, immunity and fertility, just to name a few (Pasquini et al., 1989, Yamashita et al., 1999, Sprong et al., 2001, Sillence et al., 2002, Jennemann et al., 2005, Watanabe et al., 2010, Kuan et al., 2010, D'Angelo et al., 2013, Allende and Proia, 2014)

Additionally, UDP-GlcNAc and UDP-GalNAc are essential building blocks for different types of protein modifications: UDP-GlcNAc is a key molecule for the synthesis of glycosylphosphatidylinositol (GPI) anchors (Figure 6). This complex PTM is characterized by the addition of a widely conserved glycolipid structure to select proteins in the ER. GPI anchors allow the tethering of target proteins to membranes, in particular the outer sheet of the cell membrane (Homans et al., 1988, Miyata et al., 1993). The inhibition of enzymes required for GPI anchor biogenesis leads to severe defects in several biological processes, including blood coagulation, neurological function and fertility (Nozaki et al., 1999, Almeida et al., 2006, Ueda et al., 2007, Fujita and Kinoshita, 2012).

Importantly, UDP-GlcNAc and UDP-GalNAc are also required for protein glycosylation, highly abundant modifications that are conserved between all three domains of life (Van den Steen et al., 1998, Apweiler et al., 1999, Schwarz and Aebi, 2011, Zielinska et al., 2012). There are three distinct types of protein glycosylation: protein N-glycosylation, mucin-type O-glycosylation and O-linked attachment of a single GlcNAc molecule termed O-GlcNAcylation (Corfield and Berry, 2015) (Figure 6).

N-glycosylation occurs in the ER and is thus limited to secreted and membrane-associated proteins. It is essential and universal, with all living cells being coated with a dense layer of glycans, the glycocalyx (Varki, 2011). In N-glycosylation, specialized glycosyltransferases preassemble a core oligosaccharide on an ER membrane integral lipid carrier, namely dolichyl-pyrophosphate in most eukaryotes (Kornfeld and Kornfeld, 1985, Abeijon and Hirschberg, 1992, Burda et al., 1999). Certain assembly steps of the glycan structure occur in the cytosol, others in the ER

lumen. Hence, the glycan biosynthesis requires translocation of sugar molecules across the ER membrane and flipping of initial sugar structures from cytosol to ER lumen (Snider et al., 1980, Hanover and Lennarz, 1982, Snider and Rogers, 1984, Kean, 1991, Bickel et al., 2005, Chantret et al., 2005, Gao et al., 2005, Sanyal and Menon, 2010) (Figure 7A). After complete construction on dolichyl-pyrophosphate, the $Glc_3Man_9GlcNAc_2$ core glycan is transferred on bloc to asparagine residues, characterized by the asparagine-X-serine/threonine (N-X-S/T) consensus sequence (in place of X there may be any amino acid except proline), in unfolded peptide chains (Marshall, 1972, Welply et al., 1983, Wieland et al., 1985). This reaction is catalyzed in the ER by the key enzyme oligosaccharyltransferase (OST) and can occur co-translationally or post-translationally (Chen et al., 1995, Karaoglu et al., 2001, Ruiz-Canada et al., 2009, Lizak et al., 2011). After attachment to the protein substrates, the core oligosaccharides are modified in the ER and Golgi apparatus to generate mature glycoproteins (Figure 7B). At first, the early glycoprotein undergoes stepwise removal of glucose residues in the ER (Hubbard and Ivatt, 1981, Peyrieras et al., 1983, Parodi, 2000, Kalz-Füller et al., 1995). “Trimming” and transient re-glycosylation is necessary so that the lectin chaperones calnexin and calreticulin recognize the carbohydrate structure and assist in the folding of the glycoprotein into its native conformation (Trombetta et al., 1991, Sousa and Parodi, 1995, Hebert et al., 1997, Schrag et al., 2001, Kapoor et al., 2003, Trombetta and Parodi, 2003). If misfolded, the glycoprotein is, at first, retained within the positive folding environment of the ER by the chaperones. After prolonged failure to fold, it is delivered to the cytosol for degradation after further glycan trimming by ER mannosidases, (Su et al., 1993, Hammond et al., 1994, Ermonval et al., 2001, Frenkel et al., 2003, Deprez et al., 2005, Avezov et al., 2008). Thereby, early modification of the attached glycan can generate a signal for binding of the carrier protein by chaperones, transport to the cytosol for degradation or anterograde trafficking to further pass through the secretory pathway. Then, while the proteins traverse the sub-compartments of the Golgi apparatus, their sugar moieties undergo an organism-specific and even cell type-specific series of additional trimming and extension steps carried out by a multitude of glycan processing enzymes (Rabouille et al., 1995, Igdoura et al., 1999, Papanikou and Glick, 2009, Moremen et al., 2012). The resulting mature glycoproteins exhibit elaborate N-oligosaccharides of immense variety, which can be clustered into three distinct groups according to their composition and branching: oligomannose, complex and hybrid type (Varki et al., 2009b, Wang et al., 2017b). In the *Deuterostome* lineage, including vertebrates, an exceptional degree of glycoprotein diversity is achieved by refined modification of sialic acid (not present in

C. elegans), the sugar molecule that caps most glycan branches (Harduin-Lepers et al., 2005, Cummings et al., 2009).

The great investment in and complicated logistics of N-glycan assembly and maturation are justified by its important and versatile functions: The attachment of N-glycans can accelerate and aid folding, monitor the folding status and stabilize the native state of proteins thereby preventing aggregation and serving as protein quality control mechanism (Kern et al., 1992, Helenius, 1994, Wang et al., 1996, Ellgaard et al., 1999, Hanson et al., 2009, Roth et al., 2010). Consequently, preventing protein glycosylation causes ER stress, and genes involved in N-glycan production are induced by the UPR^{ER} in order to alleviate protein folding stress (Doerrler and Lehrman, 1999, Travers et al., 2000, Ng et al., 2000, Shang et al., 2002). Beyond that, N-glycosylation is involved in nutrient uptake, metabolism, cell proliferation and differentiation (Lau et al., 2007, Abdel Rahman et al., 2015). Furthermore, N-glycans are strongly implicated in cell adhesion, cell signaling, cell-cell interactions and immunity (Dennis et al., 1987, Hakomori, 1996, Partridge et al., 2004, Dennis et al., 2009, Kariya et al., 2010, Kariya and Gu, 2011). Indeed, the immense diversity of glycan structures, presented by proteins at the cell surface, is best understood in light of co-evolution between host and pathogens. While invading microbes require an attachment structure on the host cell and invest in avoiding its defenses, the host cell has a sustained interest in prohibiting close contact. In this regard, N-glycans can be seen as a protective sheath that can be greatly variegated to not be recognized by pathogens anymore but simultaneously leaving the original protein function unaltered (Varki, 2006, Varki, 2011, Le Pendu et al., 2014, Cohen, 2015).

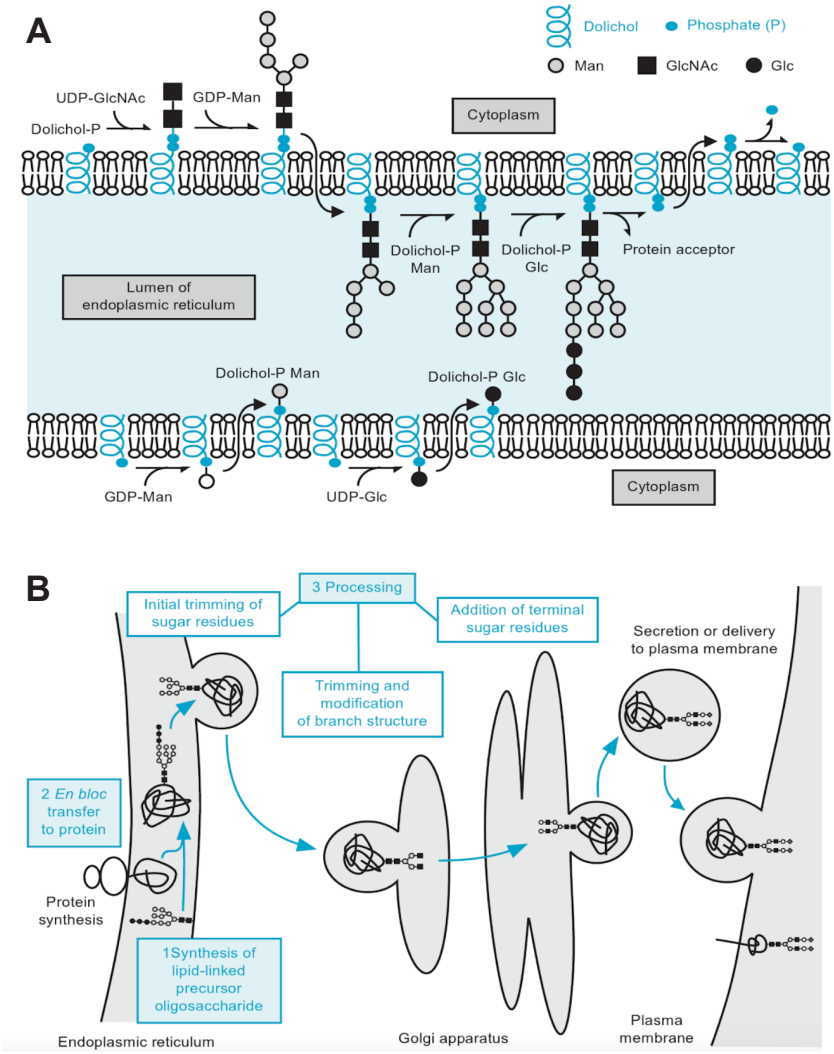


Figure 7. N-glycoprotein synthesis and processing in ER and Golgi. A. The assembly of the N-glycan core oligosaccharide is initiated on the lipid carrier dolichyl-pyrophosphate (Dolichol-P) at the cytoplasmic side of the ER membrane. Flipping of Dolichol-P allows the translocation of single sugar molecules and the preassembled carbohydrate structure, consisting of GlcNAc and mannose, to the ER lumen. Here, further mannose and glucose residues are attached, before the $Glc_3Man_9GlcNAc_2$ core glycan is attached *en bloc* to the protein acceptor. **B.** The biosynthesis of N-glycoproteins involves collaboration between different cellular compartments. The lipid-linked core glycan is assembled in cytoplasm and ER (1) and is transferred *en bloc* to nascent polypeptides within the ER (2). The glycoprotein is, after initial glycan trimming (3) and protein folding within the ER, shuttled to the Golgi apparatus. It traverses the organelle where it is further processed by trimming steps, modification of the glycan branch structure and addition of further intermediate and terminal sugar residues (3). Finally, native glycoproteins are secreted to the extracellular space or delivered to the plasma membrane, where they fulfill their functions (adapted from Taylor and Drickamer 2011).

Mucin-type O-glycosylation is a posttranslational protein modification that is mainly executed within the Golgi apparatus (Watkins, 1966, Hanover et al., 1982, Gill et al., 2011). A family of highly conserved and partially redundant polypeptide N-acetyl-α-galactosaminyltransferases (ppGalNAcTs) catalyzes multiple of the consecutive biosynthesis steps (Sugiura et al., 1982, Sørensen et al., 1995, Wandall et al., 1997, Hagen and Nehrke, 1998, Schwientek et al., 2002, Hagen et al., 2003, Bennett et al., 2012, Las Rivas et al., 2018). The initial reaction is the α-glycosidic linkage of GalNAc to the hydroxyl group of Serine or Threonine residues (McGuire and Roseman, 1967, Weissmann and Hinrichsen, 1969, Coltart et al., 2002). The attached single GalNAc represents the inner most core common to all mucin-type O-glycans and is their simplest example. Then again, along with further Golgi-resident glycosyltransferases, the ppGalNAcTs can generate carbohydrate trees of extraordinary complexity that exceed the size, degree of branching and heterogeneity of N-glycans by far (Shogren et al., 1989, Carraway and Hull, 1991, McMaster et al., 1999, Hanisch, 2001, Perdivara et al., 2009, Jin et al., 2017). Due to their elaborate structure, mucin-type O-glycans are difficult to study and many of their key characteristics like their biological role, the regulation of their production and the identities of the modified proteins still remain elusive (Jensen et al., 2010, Hurtado-Guerrero, 2016). Accordingly, it is not known, whether a consensus sequence defines the Ser/Thr residues targeted by ppGalNAcTs. Rather, the amino acid composition and already existing PTMs in the vicinity of the glycosylation site as well as secondary and tertiary structure of proteins entering the Golgi were shown to be decisive factors (Gooley and Williams, 1994, Nehrke et al., 1996, Nehrke et al., 1997, Gerken et al., 1997, Müller et al., 1997, de Haan et al., 1998, Kirnarsky et al., 1998, Hanisch et al., 1999, Asada et al., 1999, Elhammer et al., 1999, Hanisch et al., 2001, Takeuchi et al., 2002, Gerken et al., 2002, Steentoft et al., 2013). Once a protein is modified by O-glycans, it becomes protected from proteolytic cleavage and, hence, is more stable (Kozarsky et al., 1988, Garner et al., 2001). Their biophysical properties furthermore facilitate the hydration, lubrication and shielding of cellular surfaces (Gendler and Spicer, 1995, Tabak et al., 1982). Moreover, the expression of O-glycans is strongly implicated in cell adhesion and polarity, underscored by their essential role in embryonic development and involvement in cancer formation (Bleil and Wassarman, 1980, Fukuda, 2002, Xia et al., 2004, Alexander et al., 2006, Ten Hagen et al. 2009, Zhang et al., 2010, Gill et al., 2013, Hussain et al., 2016, Nguyen et al., 2017). Additional functions in protein secretion and cellular trafficking have been suggested (Huet et al., 1998, Alfalah et al., 1999, Altschuler et al., 2000, Jablonka-Shariff et al., 2002, Liebl and Featherstone, 2005, Zhang and Hagen,

2010). Like N-glycans, mucin-type O-glycans fulfill crucial roles in host-pathogen interaction and the immune system (Yang et al., 2000b, Tenno et al., 2007, Morozov et al., 2018, Dias et al., 2018). For instance, they can interact with pathogens and either prohibit or foster infection, serve as antigens and modulate the inflammatory response (Schroten et al., 1992, Sieling et al., 1995, Ellies et al., 1998, Vlad et al., 2002, Bäcklund et al., 2002, Hang and Bertozzi, 2005, Parsons et al., 2014, Everest-Dass et al., 2017).

O-GlcNAcylation describes the post-translational modification of cytosolic and nuclear proteins by single β-GlcNAc molecules (Torres and Hart, 1984, Love et al., 2003, Hanover et al., 2003, Banerjee et al., 2015). Hereby, cellular signals are generated as a function of the metabolic state of a cell (Chatham and Marchase, 2010, Bond and Hanover, 2015). OGT is the sole enzyme catalyzing the O-linked bond formation between the sugar donor UDP-GlcNAc and the hydroxyl group of serine and threonine residues (Torres and Hart, 1984, Haltiwanger et al., 1992, Kreppel et al., 1997, Cummings et al., 2009). Recently, it was discovered that GlcNAc can, in rare cases, also be linked to tyrosine and cysteine residues (Jank et al., 2015, Maynard et al., 2016). Only one additional enzyme is relevant for O-GlcNAc cycling: the glycoside hydrolase O-GlcNAcase (OGA) can remove the attached sugar from proteins and directly counteract OGT, which makes O-GlcNAc a highly dynamic PTM (Braidman et al., 1974, Gao et al., 2001). The importance of O-GlcNAc cycling is reflected in the fact that OGT and OGA are highly conserved, ubiquitous and essential (Lubas et al., 1997, Shafi et al., 2000, Wells et al., 2002, O'Donnell et al., 2004, Yang et al., 2012).

Three different splice variants are produced from the single OGT gene in humans. While the catalytic domain is identical, the isoforms comprise distinct numbers of N-terminal tetratricopeptide repeats (Kreppel et al., 1997, Lazarus et al., 2006). The encoded antiparallel α-helix pairs mediate protein-protein interactions and thereby shape substrate specificity (Blatch and Lässle, 1999, Jínek et al., 2004, Martinez-Fleites et al., 2008, Lazarus et al., 2011, Levine et al., 2018). Furthermore, the isoforms differ in their subcellular localization. The long and short isoform are distributed throughout cytosol and nucleus, whereas the OGT isoform of intermediate length contains a mitochondrial targeting sequence and also acts within the organelle (Lazarus et al., 2006). The enzymatic activity, expression level and substrate selection of OGT are additionally influenced by multiple factors such as local UDP-GlcNAc availability as well as input from other metabolic pathways, the aa sequence in proximity of the glycosylation site within substrates, PTMs of OGT itself and its substrates, stress stimuli and interacting proteins (Haltiwanger et al., 1992,

Kreppel et al., 1997, Wang et al., 1998, Iyer et al., 2003, Zachara et al., 2004, Whisenhunt et al., 2006, Cheung and Hart, 2008, Ryu and Do, 2011, Shen et al., 2012, Rafie et al., 2017). Interestingly, in *Drosophila* OGT was originally identified as a member of the conserved polycomb transcriptional repressor family, which have a pivotal role in development. Mutations in OGT resulted in pupal lethality and transformations of multiple body segments caused by misexpression of multiple homeotic genes. These observations identified OGT as potent modulator of gene expression (Gambetta et al., 2009).

Like OGT, OGA is expressed as splice variants with differential localization. Although the glycoside hydrolase domain at the N-terminus is identical between both isoforms, the long isoform of primarily cytoplasmic expression shows a much higher catalytic efficiency than the short isoform, which is mainly located to nucleus and was suggested to also be found at the surface of lipid droplets (Comtesse et al., 2001, Gao et al., 2001, Wells et al., 2002, Kim et al., 2006, Macauley and Vocadlo, 2009, Li et al., 2010b, Keembiyehetty et al., 2011). Consequently, the C-terminus, a histone acetyltransferase like domain, exclusively expressed in the long isoform, seems to be relevant for enzymatic activity, proper folding or binding of co-factors (Schultz and Pils, 2002, Toleman et al., 2004, Toleman et al., 2006, Butkinaree et al., 2008). OGA recognizes its substrates by forming hydrogen bonds with the protein-bound GlcNAc moiety itself. The amino acid composition of the substrate protein can hinder the interaction with OGA and thus modulates its specificity (Shen et al., 2012, Schimpl et al., 2012, Li et al., 2017a, Elsen et al., 2017, Roth et al., 2017). Most of the selectivity of O-GlcNAc is, however, achieved by the intricate regulation of OGT activity.

O-GlcNAcylation resembles protein phosphorylation with regards to being a rapidly and transiently occurring PTM. There is extensive crosstalk between both modifications and they even compete for a proportion of their molecular targets. Hereby, O-GlcNAc modification can prohibit phosphorylation at consensus sites, but O-GlcNAc also modulates phosphorylation at adjacent sites and vice versa (Comer and Hart, 2001, Kamemura et al., 2002, Wang et al., 2008b, Ande et al., 2009, Zeidan and Hart, 2010, Hart et al., 2011, Kaasik et al., 2013, Zhong et al., 2015, Tian et al., 2016). This is one of the features of O-GlcNAc cycling that help to understand how this PTM can be involved in the regulation of an enormous number of biological processes. These include chromatin remodeling, regulation of gene transcription and immune responses, protein solubility, stability and degradation, just to name a few (Han and Kudlow, 1997, Zhang et al., 2003, Wang et al., 2008b, Srikanth et al., 2010, Ranuncolo et al., 2012, Yuzwa et al., 2012, Hanover et al., 2012, Guo et al., 2017, Leturcq et al., 2017, Li et al., 2017b, de Jesus et al., 2018).

1.3.3 The enzyme Glutamine-fructose 6-phosphate aminotransferase (GFAT)

The structure of bacterial as well as eukaryotic GFAT-1 is divided into two domains connected by a hinge region. The glutaminase domain catalyzes the hydrolysis of glutamine to glutamate and, via an ammonia channel, provides ammonia as nitrogen source to the isomerase domain. Here, Fruc-6-P is transformed into its isomer Glucose 6-phosphate (Glc-6-P) and ammonia is transferred to Glc-6-P to generate GlcN-6-P (Denisot et al., 1991, Teplyakov et al., 2001, Olchowy et al., 2007, Durand et al., 2008). In the bacterial GFAT homolog glucosamine-6-phosphate synthase (GlmS), dimerization of and cooperation between two GlmS molecules is required for enzymatic activity, while eukaryotic GFAT-1 is an active enzyme in a homotetrameric state (Kornfeld, 1967, Raczynska et al., 2007, Richez et al., 2007).

Two isoforms of *gfat-1*, which are products of alternative splicing, are expressed. Both isoforms merely differ in the length of the hinge region between isomerase and glutaminase domain and their expression pattern. In mice, the shorter splice variant lacking amino acids 229-246 (in *C. elegans* lacking amino acids 226-238) is ubiquitously und uniformly expressed, while the long isoform is predominantly expressed in skeletal muscle (DeHaven et al., 2001, Niimi et al., 2001). As the HP product UDP-GlcNAc is an essential building block for diverse biopolymers and distinct posttranslational protein modifications, it is plausible that it is well conserved between species. In fact, *gfat-1* exhibits 99% sequence homology between humans and mice and still 60% sequence homology between humans and the distant relative *C. elegans*. Residues with important functions, such as formation of a channel to transport ammonia between the two enzyme domains, generation of binding pockets for educts, shielding reaction space from solvent, or catalytic activity itself are almost identical even between *E. coli* and these species (McKnight et al., 1992, Sayeski et al., 1994, Teplyakov et al., 2001, DeHaven et al., 2001, Mouilleron et al., 2006).

Additionally, a second gene with around 75% amino acid sequence homology to *gfat-1* was identified in mouse and human and termed *gfat-2* (Oki et al., 1999). Like *gfat-1*, *gfat-2* is well conserved between organisms with known homologs in *C. elegans*, flies, yeast and even plants (www.ncbi.nlm.nih.gov/homologene/68439). While *gfat-1* and *gfat-2* are clearly distinct genes, their protein products both fulfill the same function: catalyzing the first enzymatic reaction of the HP. In contrast to the ubiquitous expression of GFAT-1, GFAT-2 shows a more limited expression pattern in mice and humans, mainly being detected in the central nervous system (Zhou et al., 1995, Oki et al., 1999, De Haven et al., 2001, Zhang et al., 2004). Whether

GFAT-1 and GFAT-2 fulfill gene specific functions besides producing GlcN-6-P is not yet known, but both GFAT-1 splice variants and GFAT-2 exhibit distinct enzyme kinetics. Differences in affinities for educts and products, which serve as feedback inhibitors, likely reflect the specific needs of the tissues, where the distinct GFAT variants are expressed (Kornfeld, 1967, De Haven et al., 2001, Niimi et al., 2001, Broschat et al., 2002).

To additionally adapt UDP-GlcNAc production to transiently high or low requirements and general energy availability, GFAT harbors multiple sites for posttranslational modifications (PTMs) (www.phosphosite.org). To which extent and in which context these PTMs influence GFAT stability or activity is, however, largely still unknown. Few studies exist that describe the phosphorylation of Ser205 and Ser235 by protein kinase A (PKA) (Zhou et al., 1998, Chang et al., 2000, Hu et al., 2004). At rising concentrations of the second messenger cyclic adenosine monophosphate (cAMP) (triggered by hormones and neurotransmitters but also regulated by glucose, intracellular pH, ATP, bicarbonate and calcium) cAMP molecules bind to PKA regulatory subunits. As a consequence, the catalytic subunits become liberated and are able to phosphorylate PKA target proteins (Kamenetsky et al., 2006, Tamaki, 2007, Kim et al., 2007, Ramos et al., 2008, Sjoberg et al., 2010, Zippin et al., 2013, Smith et al., 2013). The effects of GFAT phosphorylation by PKA remain still unclear as they are described inconsistently by different research groups: While phosphorylation at Ser235 does not result in a measurable change of enzymatic activity, phosphorylation at Ser205 was characterized in one publication to be diminishing and in another publication to be boosting GFAT activity (Zhou et al., 1998, Chang et al., 2000). A third study found phosphorylation at Ser205 in GFAT-1 to be decreasing and the corresponding phosphorylation at Ser202 in GFAT-2 to be increasing enzymatic activity (Hu et al., 2004).

Apart from PKA, adenosine monophosphate (AMP)-activated protein kinase (AMPK) is able to phosphorylate GFAT (Li et al., 2007, Eguchi et al., 2009, Zibrova et al., 2017). AMPK, as central mediator of cellular energy homeostasis, senses rising AMP/ATP and adenosine diphosphate (ADP)/ATP ratios and becomes active at low energy availability. Thereupon, AMPK phosphorylates numerous substrates, including mechanistic target of rapamycin complex 1 (mTORC1). Thus, it decreases cap-dependent protein synthesis and increases autophagy, among other things (Gwinn et al., 2008, Kim et al., 2011, Egan et al., 2011, Hardie et al., 2012, Xu et al., 2012, Burgos et al., 2013). The consequences of AMPK-mediated Ser243 phosphorylation in GFAT are only poorly understood. While Li and colleagues reported an increase in GFAT enzymatic activity upon phosphorylation, two different

research groups described an inhibitory effect (Li et al., 2007, Eguchi et al., 2009, Zibrova et al., 2017). Interestingly, mTOR complex 2 was recently also identified as modulator of GFAT-1 Ser243 phosphorylation and a promoting effect of phosphorylation on HP flux was reported (Moloughney et al., 2018).

Besides phosphorylation sites, multiple studies could identify ubiquitination sites in GFAT (www.phosphosite.org). Thus, GFAT degradation might well be mediated by the proteasome and GFAT stability could be another way to control HP flux.

Lastly, several sites in the GFAT peptide sequence are predicted to be modified by O-GlcNAc (www.cbs.dtu.dk/services/YinOYang/). This can potentially add another layer to the feedback regulation of the HP, by involving OGT and OGA in the tuning of GFAT activity. As the HP is critical to cellular metabolism and organismal integrity is important to gain a more complete insight into the complex regulation of GFAT-1 activity as well as its abundance.

1.3.4 Intersection of the hexosamine pathway with protein quality control

The HP produces the essential molecule UDP-GlcNAc, which is a building block for a large variety of biomolecules and PTMs that play a central role in numerous signaling pathways. Hence, it is conceivable that there are various points of contact between the HP and the complex network of PQC mechanisms.

Multiple studies describe the influence of protein O-GlcNAcylation as a function of cellular energy status on autophagy. Elevated O-GlcNAc attachment was shown to enhance autophagic flux in certain conditions by manipulating activity of FOXO (DAF-16) transcription factor and by directly targeting components of the autophagy core machinery (Housley et al., 2008, Housley et al., 2009, Wang and Hanover, 2013, Zhu et al., 2018). In the majority of reports however, a decrease in O-GlcNAc promoted the induction of autophagy and the maturation of autophagic vesicles (Marsh et al., 2013, Guo et al., 2014, Park et al., 2015, Fahie and Zachara, 2016, Wani et al., 2017, Zhang et al., 2018). These controversial observations emphasize the complex participation of O-GlcNAc, which is only one of the outputs of the metabolic HP, in the regulation of different steps of autophagic recycling.

O-GlcNAc signaling is also implicated in distinct steps of protein degradation via the UPS, ranging from regulation of gene expression of proteasomal subunits, over crosstalk with ubiquitination at shared substrates, to direct modification of

components of the proteasome and consequently modulation of proteolytic capacity (Zhang et al., 2003, Sümegi et al., 2003, Guinez et al., 2008, Overath et al., 2012, Sekine et al., 2018). Again, the effects of increased O-GlcNAc were complex. They included not only inhibition of proteolytic cleavage by the proteasome, but also positive correlation with proteasome abundance (Zhang et al., 2003, Keembiyehetty et al., 2011, Sekine et al., 2018).

In the context of aging and disease, the tau protein, a binding partner of tubulin that is essential for the formation of microtubules, is one well-characterized example for the influence of O-GlcNAcylation on protein solubility versus aggregation (Weingarten et al., 1975). Hyperphosphorylated tau is aggregation prone and a major constituent of neurofibrillary tangles, a hallmark of AD (Kosik et al., 1986, Wood et al., 1986, Grundke-Iqbal et al., 1986, Bancher et al., 1989). Increasing O-GlcNAc modifications on tau prevents tau hyperphosphorylation and directly counteracts its aggregation, which results in measurable cognitive benefits and generally improved health in mouse models of AD (Liu et al., 2004, Yuzwa et al., 2012, Borghgraef et al., 2013, Yuzwa et al., 2014b, Graham et al., 2014, Yuzwa et al., 2014a, Lim et al., 2015, Hastings et al., 2017, Frenkel-Pinter et al., 2018). One conflicting study could, however, not identify extensively O-GlcNAc modified endogenous tau *in vivo* (Morris et al., 2015).

Beyond affecting the physical properties of specific proteins, a very broad protective and stabilizing role was suggested for co-translationally occurring O-GlcNAc at nucleocytoplasmic nascent polypeptide chains. Co-translational O-GlcNAc modifications were found to exert their protective role through modulating ubiquitination and by preventing premature proteasomal degradation (Zhu et al., 2015). For protein N-glycosylation a general protective role, assistance in protein folding and monitoring of protein conformation by modification with intricate carbohydrate structures is already well established for proteins of the secretory pathway (Joao and Dwek, 1993, Rudd et al., 1994, Mer et al., 1996, Sinha and Surolia, 2007, Hanson et al., 2009, Glozman et al., 2009, Jayaprakash and Surolia, 2017). Due to its prevalence and positioning at the nexus of aminosugar and protein biosynthesis, N-glycosylation is, furthermore, ideally suited to integrate energy availability and expenditure as well as different stress cues. Hence, improper protein glycosylation can potently induce the UPR to restore cellular homeostasis (Doerrler and Lehrman, 1999, Travers et al., 2000, Ng et al., 2000, Shang et al., 2002, Wang et al., 2015, McCaffrey and Braakman, 2016).

But although O-GlcNAcylation and protein N-glycosylation affect a huge pool of proteins in multiple biological processes and signaling pathways, they are only

singular outputs of the HP. Only in recent years, it became clear that tuning the activity of the HP directly would also shape PQC mechanisms. Wang and colleagues worked with ischemia/reperfusion (I/R) in the mouse heart, which causes calcium imbalance and a burst of mitochondrial reactive oxygen species production, resulting in protein misfolding. They demonstrated that I/R elevated XBP1s levels by activation of the IRE1 branch of the UPR. This in turn led to induction of HP enzyme expression, confirming GFAT1 as direct target of the UPR. Concomitantly, there was an overall increase in O-GlcNAc protein modifications *in vitro* and *in vivo*. Elevated O-GlcNAcylation was also observed after exposing cells to distinct stress conditions, suggesting a general role for HP and OGT activation downstream of the UPR. In case of I/R, XBP1s overexpression could protect from I/R injury and cell death. Importantly, this effect was dependent on GFAT-1 and GFAT-1 overexpression by itself was cardioprotective (Wang et al., 2014).

Denzel and co-workers in the Antebi/host laboratory showed a beneficial effect of HP activation on multiple proteostasis mechanisms culminating in longevity in the model organism *C. elegans*. Initially, they identified gain-of-function (*gof*) mutations in GFAT-1 to confer resistance to the drug tunicamycin, which triggers ER stress by interfering with protein N-glycosylation. When they characterized these mutants, they observed an increase in lifespan and a decrease in aggregation and toxicity of aggregation-prone proteins. The benefits were likewise achieved in wildtype worms by feeding GlcNAc, a UDP-GlcNAc precursor. The positive effects on lifespan and PQC were dependent on UPR, ERAD and autophagy and *gfat-1 gof* animals exhibited slightly elevated ERAD, autophagy and proteasome activity (Denzel et al., 2014).

In summary, these findings indicate that the ubiquitous and essential HP, which is entangled in a myriad of biological processes, has an especially tight connection to PQC pathways. Not only does the HP seem to mediate responses to protein folding stress, but also it participates in the control of distinct proteostasis and protein recycling mechanisms under basal conditions. The coordinated upregulation of multiple PQC mechanisms achieved by HP activation could be particular effective in countering proteotoxicity. To exploit this for potential treatment strategies of protein aggregation diseases and age-dependent failure of the PN it is critical to better understand the regulation and diverse functions of GFAT-1. It will be essential to identify downstream effectors of GFAT-1 responsible for the improved PQC and also to determine tissue-specificity and possible negative effects.

1.4 Aims of this study

Evidently, multiple complex processes ensuring proper protein synthesis, folding, maintenance and degradation join forces to preserve PQC during the lifetime of an organism. With rising age, the proteostasis machinery increasingly fails to meet the demanding challenge of sustaining a healthy proteome with severe consequences including the growing incidence of protein folding diseases. As longevity and extended healthspan can be linked to improved PQC a better understanding of the regulation and integration of proteostasis mechanisms is critical in our efforts to reduce the burden of age-associated diseases.

The metabolic HP is implicated in the folding process of glycoproteins within the ER by producing the essential precursor molecule UDP-GlcNAc. It was shown only recently that the connection of the HP and PQC is, in fact, far deeper and more intricate than previously expected. GFAT-1 is a target of the UPR and, at the same time, GFAT-1 activation by *gof* mutations causes longevity by mildly inducing multiple pathways of the PN (Wang et al., 2014, Denzel et al., 2014). Whether GFAT-1 fulfills a specific role under stress conditions remains unknown. Furthermore, knowledge about the processes and downstream effectors mediating between GFAT-1 and PQC mechanisms is missing. Hence, I addressed the following three major aims and questions during my doctoral study:

Aim 1: Is GFAT-1 expression regulated by environmental stress and does GFAT-1 overexpression (OE) and HP activation influence stress resistance?

Aim 2: What role might tissue-specific GFAT-1 OE play in *C. elegans* cell autonomous and cell non-autonomous PQC?

Aim 3: What are the downstream effectors of GFAT-1 activation/OE responsible for improvement of PQC?

2. RESULTS

2.1 Regulation of GFAT-1 under stress

As an essential component of a cell´s metabolism, the HP is constantly active to produce UDP-GlcNAc. Additionally, recent studies provided first evidence of HP regulation by ER stress and nutrient shortage (Wang et al., 2014, Chaveroux et al., 2016, Moloughney et al., 2016). Up to now, there are, however, no comprehensive studies on the regulation of *gfat-1* in response to diverse environmental challenges.

In order to address this gap in knowledge, we exposed worms to different stress conditions and assessed *gfat-1* mRNA and protein levels. Exposure to 30°C heat for 8 hours led to clearly elevated *xbp-1* and *xbp-1s* mRNA levels, which served as marker for an activated UPR^{ER}. However, this treatment did not influence *gfat-1* mRNA levels. Likewise, induction of protein folding stress in the ER by tunicamycin slightly upregulated *xbp-1* and *xbp-1s* but had no effect on *gfat-1* transcript (Figure 8A). Furthermore, *gfat-1* levels remained unchanged during starvation or high glucose treatment, although these conditions alter the availability of the *gfat-1* substrate fructose-6 phosphate. Moreover, induction of oxidative stress by paraquat or perturbations of the ER calcium homeostasis by thapsigargin did not influence *gfat-1* mRNA amount (Figure 8B). Included in the assay are *gst-4* as oxidative stress responsive gene and *cdc-42* as second housekeeping gene and both serve as additional controls (Tawe et al., 1998, Leiers et al., 2003, Hoogewijs et al., 2008, Zhang et al., 2012) (Figure 8B).

As no stress-induced changes in *gfat-1* mRNA level were detected, we analyzed GFAT-1 protein expression using a translational reporter strain. Under standard growth conditions, GFAT-1 was mainly expressed in pharynx and seam cells of *C. elegans* and showed a smooth distribution pattern. Interestingly, ER stress caused by tunicamycin and thapsigargin led to a strong elevation of GFAT-1 protein, specifically in the worm intestine. An upregulation of GFAT-1 was also observed after heat, paraquat and high glucose treatment. In this case, pharynx and seam cells remained the tissues of highest expression, but a shift from smooth to a more granular and punctate expression pattern was observed (Figure 8C and 8D).

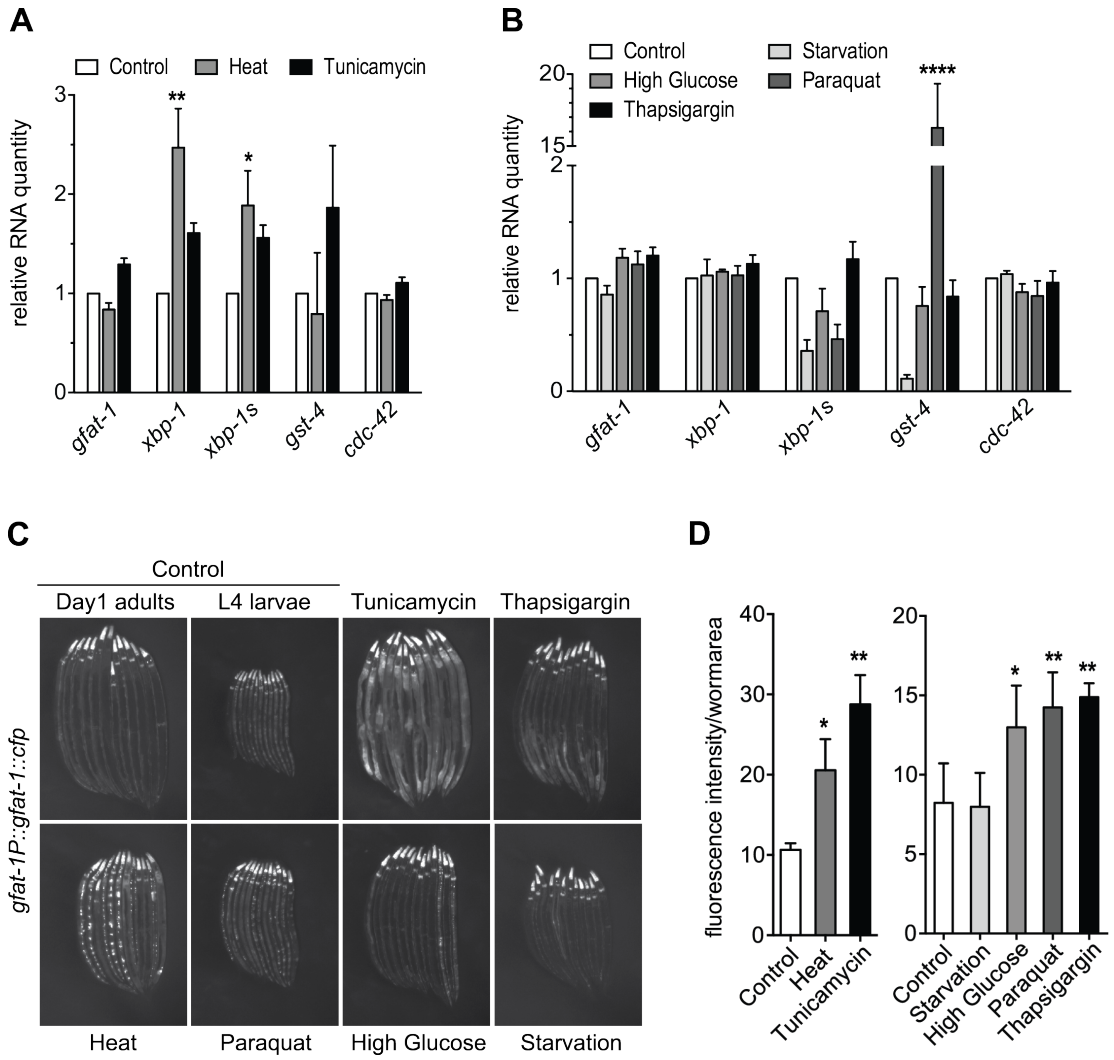


Figure 8. Regulation of protein level in response to stress. A. and **B.** Relative mRNA amount of worms exposed to stress conditions on NGM plates and OP50 for 8 h after culture on NGM plates with OP50 to late L4 stage, measured by qRT-PCR (**A**) or exposed to stress conditions in liquid culture with OP50 (**B**), shown is mean +SEM, expression normalized to *ama-1*, N=3, statistical analysis 2way ANOVA, Dunnett´s multiple comparisons test, *, p<0.05; **, p<0.01; ****, p<0.0001. **C.** Representative fluorescence images, *gfat-1P::Flag::HA::CFP::gfat-1* worms cultured on NGM and OP50 to late L4 stage, images after 14h stress exposure, worms cultured on NGM plates with OP50 without being submitted to stress served as control. **D.** Quantification of absolute pixel intensity (CFP signal) per worm area of *gfat-1P::Flag::HA::CFP::gfat-1* strain, 10 worms per experiment, shown mean +SEM, N=3, *, p<0.05; **, p<0.01. Stress conditions were as follows: Heat 30°C, Tunicamycin 10 µg/ml, starvation in M9, Paraquat 100mM, Thapsigargin 5 µM, Glucose 2%.

In mice, it was reported before that *gfat-1* transcript levels respond to ER stress and *xbp-1* splicing as consequence of UPR^{ER} activation (Wang et al., 2014). Although we could not detect a regulation of *gfat-1* mRNA level by ER stress in *C. elegans*, we further investigated the link between UPR signaling and HP flux.

There is a specific UPR element (UPRE) encoded in the mammalian *gfat-1* promoter, which is missing in *C. elegans*. However, we could identify sequences similar to the ER stress response element (ERSE) and UPRE in the region upstream of the *gfat-1* open reading frame (ORF) (Yoshida et al., 1998, Roy and Lee, 1999, Wang et al., 2000, Yamamoto et al., 2007) (Figure 9A). This observation could argue for a transcriptional regulation of *gfat-1* by ER stress. In order to examine functional induction of the HP by the UPR^{ER} we made use of a genetic model overexpressing *xbp-1s* in nematode neurons. It was previously established that this tissue-specific OE is sufficient for a systemic UPR activation (Taylor and Dillin, 2013). Although we could measure an elevation of *xbp-1s* mRNA levels, we did, also in this system, not detect any change in *gfat-1* mRNA expression consistent with our previous results (Figure 9B and 8A). Interestingly, neuron-specific *xbp-1s* OE resulted in a small but distinct accumulation of the HP end product UDP-GlcNAc in worms (Figure 9C). Altogether these observations suggest that GFAT-1 expression and tissue-distribution may be regulated mainly through post-transcriptional mechanisms.

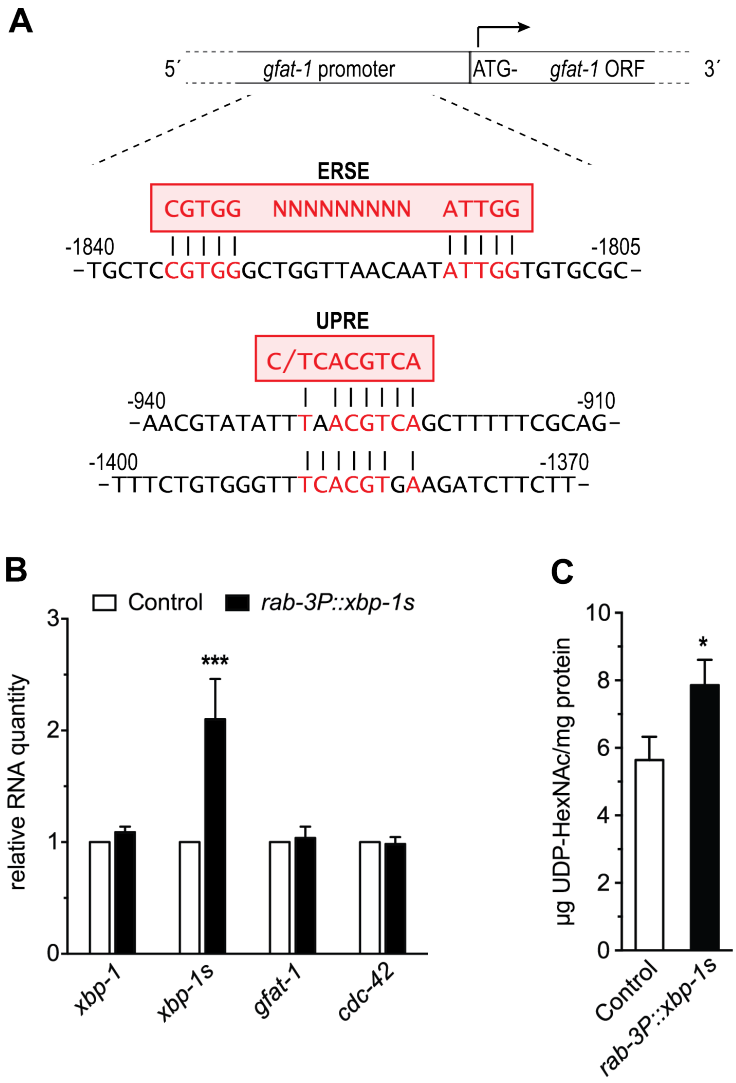


Figure 9. Activation of the HP as response to ER stress. A. Sequences resembling the ER stress response element (ERSE) consensus sequence CCAAT-N9-CCACG and the UPR element (UPRE) consensus sequence TGACGTGG/A upstream of *C. elegans gfat-1* ORF. **B.** Relative mRNA quantity in late L4 larvae of N2 control and *rap-3P::xbp-1s* strain as measured by qRT-PCR, shown is mean +SEM, expression normalized to *ama-1*, N=3, 2way ANOVA, Sidak´s multiple comparisons test, ***, p<0.001 **C.** LC-MS analysis of UDP-HexNAc levels, shown is mean +SEM, N=9, unpaired t-test, *, p<0.05.

The observation that GFAT-1 expression and HP activity is induced by stress conditions led us to ask whether GFAT-1 fulfills a function in stress responses. Hence, we assessed mRNA levels of various detoxifying genes, namely superoxide dismutases (*sod-1*, *sod-2*, *sod-3*), glutathione-S transferase (*gst-4*) and catalases (*ctl-1*, *ctl-2*). These genes reportedly counteract oxidative stress and are, beyond that, implicated in modulating lifespan (Zhou et al., 2011). Increased HP flux by

gfat-1 gof did not result in an increased transcript level of any of the tested genes, with the exception of a slight elevation of *gst-4* mRNA in *gfat-1 gof (dh784)*, the strain with particularly high UDP-GlcNAc levels (Denzel et al., 2014) (Figure 10A). In agreement with the overall unchanged expression of genes counteracting oxidative stress, we did not observe a heightened resistance of *gfat-1 gof* worms against acute paraquat treatment. As positive control *daf-2* mutants showed an increased survivorship in this assay (Figure 10B). We, furthermore, induced protein unfolding, misfolding and aggregation in *C. elegans* by heat stress and scored the worms ability to recover. While *daf-2* mutants did, again, exhibit augmented stress resistance, *gfat-1 gof* mutants were not distinguishable from the wildtype controls (Figure 10C).

Tunicamycin prevents N-linked protein glycosylation in the ER and thereby causes protein folding stress (Elbein, 1987). Originally, *gfat-1 gof* mutations were identified in a forward genetic screen selecting for worms resistant to this drug (Denzel et al., 2014). Here, we confirmed the exceptional resistance to tunicamycin with one representative *gfat-1 gof* line over a broad range of drug concentrations. The striking difference between controls and *gfat-1 gof* worms becomes especially apparent at 10 µg/ml tunicamycin: while no control animal developed from egg to L4 larvae, *C. elegans* harboring the *gfat-1 gof* mutation were completely resistant and grew from egg into adults within 4 days (Figure 10D).

From the described experiments we concluded that the amount of GFAT-1 protein is upregulated in *C. elegans* by a multitude of distinct stress conditions. Despite the responsiveness of the HP to stress, elevated UDP-GlcNAc levels do not seem to be involved in detoxification and general stress resistance, but specifically protect from tunicamycin toxicity.

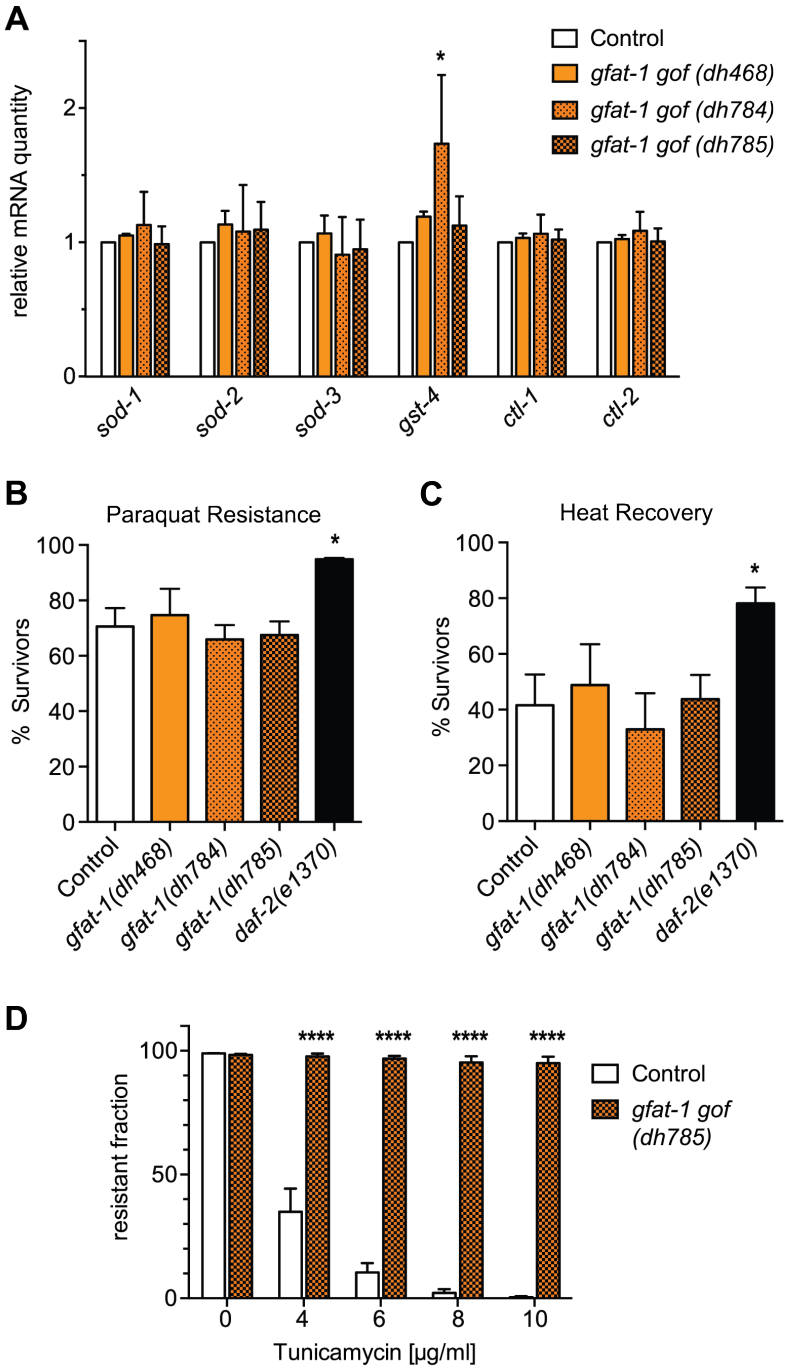


Figure 10. Tunicamycin resistance, but not general stress resistance conferred by *gfat-1 gof*. **A.** qRT-PCR of late L4 larvae of N2 control and *gfat-1 gof* strains, shown is mean +SEM, expression normalized to *ama-1*, N=3, 2way ANOVA, Dunnett´s multiple comparisons test, *, p<0.05 **B.** Oxidative stress/ Paraquat resistance, late L4 larvae were transferred to NGM plates with OP50 containing 50mM Paraquat, scored was survival after 24h, 60 worms per strain, shown is mean +SEM, N=3, unpaired t-test, *, p<0.05 **C.** Heat Recovery, worms switched to 33°C at day1 adult stage for 6h, rescued to 20°C, and survival scored after 48h, 75 worms per strain, N≥3, unpaired t-test, *, p<0.05 **D.** Development of eggs to L4 or adult stage after 4 days on 0, 4, 6, 8 or 10 µg/ml Tunicamycin, shown is mean +SEM, N=3, 2way ANOVA, Sidak´s multiple comparisons test, ****, p<0.0001.

2.2 Transcriptome and proteome of *gfat-1 gof* mutants

The increased production of UDP-GlcNAc by *gfat-1 gof* mutations induces PQC pathways and prolongs life in *C. elegans* (Denzel et al., 2014). The molecular mechanism that lead to these benefits are, however, still completely unknown. To get an unbiased view of potential changes resulting from HP activation and to address this open question we performed transcriptomic and proteomic analyses in *gfat-1 gof* worms. Surprisingly, there were only very few genes with mildly changed transcriptional profile between *gfat-1 gof* and wildtype control (Figure 11A). The upregulation in mRNA expression of these few candidate genes was confirmed by quantitative reverse transcription PCR (qRT-PCR) (done by Martin Denzel, data not shown) but was not detectable on protein level. Stable isotope labeling with amino acids in (cell) culture (SILAC) was used for direct proteomic comparison of *gfat-1 gof* mutants with wildtype worms. With this technique we did not identify any protein that was expressed differentially at a significant level (Figure 11B). We performed forward and reverse labeling growing *gfat-1 gof* and control worms on bacteria incubated with heavy $^{13}C6/^{15}N2$ or light lysine and pooled cross-labeled samples for analysis. The compiled analysis of all reverse labeling experiments did not generate any candidate responsible for the *gfat-1 gof* induced benefits, as not a single protein was significantly ($p<0.05$) enriched or decreased in either *gfat-1 gof* strain compared to controls (Figure 11B). The same observation was made analyzing all forward labeling experiments and even combining forward and reverse labeling experiments (data not shown), which increases statistical power by elevating the number of independent repeats and eliminates effects of the labeling itself.

A

gene ID	gene name	description	fold change	p value	adjusted p value
C29F7.5	*fkh-4*	ForKHead transcription factor family member	0.40	2.83E-14	2.25E-10
C29F7.4	*fkh-3*	ForKHead transcription factor family member	0.53	4.26E-09	1.69E-05
F42G8.3	*pmk-2*	mitogen-activated protein kinase (MAPK)	1.99	1.56E-07	4.66E-04
B0218.3	*pmk-1*	mitogen-activated protein kinase (MAPK)	1.57	2.08E-05	4.97E-02
T25G12.7	*dhs-30*	short-chain dehydrogenase/reductase	0.40	1.22E-20	2.9E-16
T28F2.5	*ccb-1*	subunit of a voltage-gated calcium channel	2.51	1.50E-18	1.78E-14
C55C3.3			4.88	9.16E-14	5.46E-10
T10D4.6			2.10	6.46E-11	3.08E-07
C55C3.6			7.71	1.19E-07	4.06E-04
T28F2.1			9.85	1.76E-06	4.66E-03

B

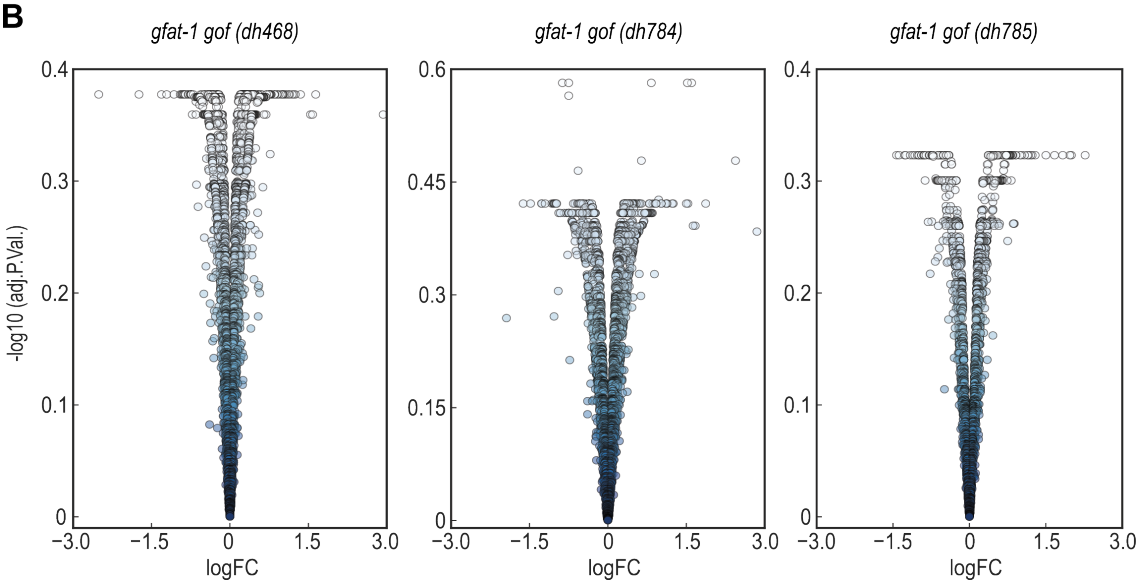


Figure 11. No major transcriptomic or proteomic changes induced by *gfat-1 gof* mutations. **A**. Regulated genes in day 1 adult *gfat-1 gof (dh468)* relative to wildtype control identified by RNAsequencing, experiment performed by **Martin Denzel**. **B.** No significant changes in protein expression detected by SILAC and MS analysis between *gfat-1 gof* mutants and wildtype controls at day 1 of adulthood, shown are compiled results of independent biological replicates of reverse labeling (control strain grown on bacteria that incorporated heavy $^{13}C6/^{15}N2$ lysine), N=3 for *gfat-1 gof (dh468)* and *gfat-1 gof (dh784)*, N=2 for *gfat-1 gof (dh785)*, logFC, log(fold change) of mutant/control.

Among the very few genes that were transcriptionally mildly induced in *gfat-1 gof* mutants, we found the MAP kinase *pmk-1* to be the most interesting candidate. If worms are challenged by oxidative stress or pathogens, *pmk-1* becomes activated by phosphorylation and in turn promotes *skn-1* (Nrf2 in mammals) nuclear localization (Inoue et al., 2005). As a result, *skn-1* mounts defense mechanisms, induces the UPR^{ER}, and promotes longevity (An and Blackwell, 2003, Tullet et al., 2008, Hoeven et al., 2011, Glover-Cutter et al., 2013). These downstream effects of *pmk-1*

activation partially overlap with the observed phenotype of *gfat-1 gof* mutants and we wondered whether the *pmk-1–skn-1* signaling axis is hyperactive in *gfat-1 gof* worms and might be responsible for the improved proteostasis and longevity (Denzel et al., 2014).

To test this hypothesis, we assessed PMK-1 phosphorylation as proxy for PMK-1 activity in *gfat-1 gof* worms compared to controls. Interestingly, we observed a considerable accumulation of phosphorylated PMK-1 (P-PMK-1) in two of the three analyzed *gfat-1 gof* mutants (Figure 12A). However, there was a strong variability between biological replicates and despite the strong increment in P-PMK-1 in some of the experiments, the increase did not reach significance (Figure 12B). The expression pattern and signal strength of total PMK-1 was comparable between wildtype control and *gfat-1 gof* strains (Figure 12C and 12D). As a second readout for PMK-1 activation and its downstream signaling, we measured the transcript levels of the putative immune factors *C17H12.8*, *K08D8.5* and *T24B8.5*. These peptides are known to be robustly elevated by the innate immune response and active *pmk-1* (Troemel et al., 2006, Shivers et al., 2010). We found their mRNA level to be unaltered by *gfat-1 gof* mutations (Figure 12E).

To test the implication of PMK-1 in *gfat-1 gof* induced downstream benefits by another method, we studied the effect of complete *pmk-1* deletion on the lifespan of *gfat-1 gof* worms. Loss of *pmk-1* decreased the lifespan of *gfat-1 gof (dh468)* and the wildtype control in a similar manner (Figure 12F). However, this experiment was performed only once and should be repeated to represent conclusive evidence. Nevertheless, it is likely that *pmk-1* deletion leads to a reduced general fitness of *C. elegans* and there are no indications that this is an effect specific for *gfat-1 gof* mutants. Accordingly, no conclusions can be drawn to whether the *gfat-1 gof* induced lifespan extension is dependent on *pmk-1*.

Overall, we established that the level of PMK-1 phosphorylation is increased in the presence of *gfat-1 gof* mutations but is enormously variable between different *gfat-1 gof* lines and even biological replicates. Beyond that, PMK-1 activation does not correlate with HP activation and the amount of accumulated UDP-GlcNAc (highest level measured in *gfat-1 gof (dh784)* (Denzel et al., 2014)). We concluded that PMK-1 is unlikely to mediate the benefits of HP activation on PQC and lifespan.

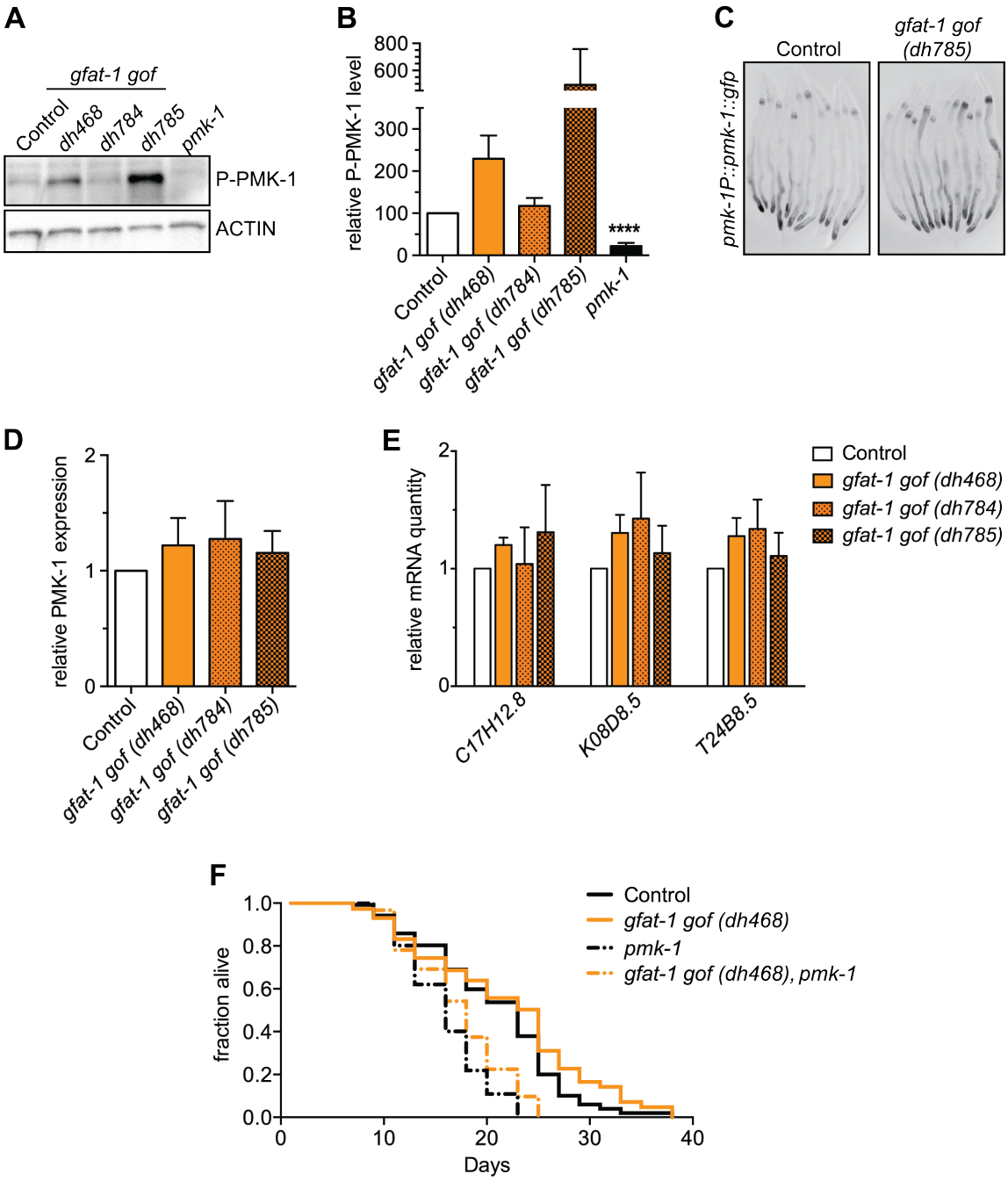


Figure 12. Role of MAPK PMK-1 signaling downstream of *gfat-1 gof*. A. Representative Western blot from young adult worms. **B.** Expression of P-PMK-1 relative to Actin and normalized to control animals. Quantification of Western Blots from young adults (like in **A.**), N=4, unpaired t-test, ****, p<0.0001 **C.** Representative images of PMK-1::GFP expression in day 1 adult *pmk-1P::pmk-1::gfp* and *pmk-1P::pmk-1::gfp, gfat-1 gof (dh785)* worms **D.** Expression of total PMK-1 in *gfat-1 gof* relative to control animals. Quantification from images of *pmk-1P::pmk-1::gfp* strain (like in **C.**). Shown is mean +SEM, N≥3, 1way ANOVA, Dunnett´s multiple comparisons test, no significant difference. **E.** Relative mRNA quantity in late L4 larvae of N2 control and *gfat-1 gof* strains as measured by qRT-PCR, shown is mean +SEM, expression normalized to *ama-1*, N=3, 2way ANOVA, Dunnett´s multiple comparisons test, no significant difference. **E.** Kaplan-Meier survival curves of controls and *gfat-1 gof (dh468)* worms, and the influence of *pmk-1* deletion in both backgrounds, N=1.

2.3 Tissue-specific *gfat-1* OE

To better understand how modulating *gfat-1* in *C. elegans* leads to improved protein homeostasis we set out to dissect the role of *gfat-1* in a tissue-specific manner and to address the question of cell autonomy and cell non-autonomy. For that purpose, we molecularly cloned vectors enabling the overexpression of *gfat-1* under the control of the *gfat-1* endogenous promoter (done by Martin Denzel (Denzel et al., 2014)), a muscle-specific promoter (*myo-3P*), a pan-neuronal promoter (*rgef-1P*) and an intestine-specific promoter (*ges-1*). We inserted a Flag- and HA-tag as well as a cyan fluorescent protein (CFP) at the 5´end of the *gfat-1* ORF to be able to detect the transgene in biochemical assays and under the microscope (Figure 13A). Under its endogenous promoter GFAT-1 was observed to be most highly expressed in pharynx and seem cells. The transgenic lines *myo-3P::gfat-1* and *rgef-1P::gfat-1* showed the expected expression pattern: the GFAT-1::CFP signal was detected throughout the body wall muscle in the *myo-3P::gfat-1* lines and in head-and tail ganglia as well as ventral and dorsal nerve chords in the *rgef-1P::gfat-1* lines (Figure 13B). Unfortunately, the multiple transgenic *C. elegans* lines generated by microinjection of the *ges-1P::gfat-1* OE construct all exhibited only patchy CFP expression, had a very low plasmid transmission rate (<10%), showed slowed development and decreased body size (data not shown). Overall, worms with intestinal GFAT-1 OE appeared unfit and were excluded from all further experiments. For the remaining transgenic lines, the expression of GFAT-1 fusion protein was confirmed by Western blot analysis. The level of expression differed strongly between the lines with relatively low expression in both neuronal OE lines and considerably strong expression in *myo-3P::gfat-1* (2) and particularly *gfat-1P::gfat-1* (Figure 13C). In the two strains with highest GFAT-1 transgene expression we measured UDP-HexNAc levels to compare their degree of HP activation to wildtype controls and *gfat-1 gof* worms. Interestingly, the UDP-HexNAc level was strongly elevated by the *gfat-1 gof* mutation but remained completely unaltered by GFAT-1 OE (Figure 13D). This suggests that wildtype GFAT-1 is efficiently feedback-inhibited even if its amount is strongly increased.

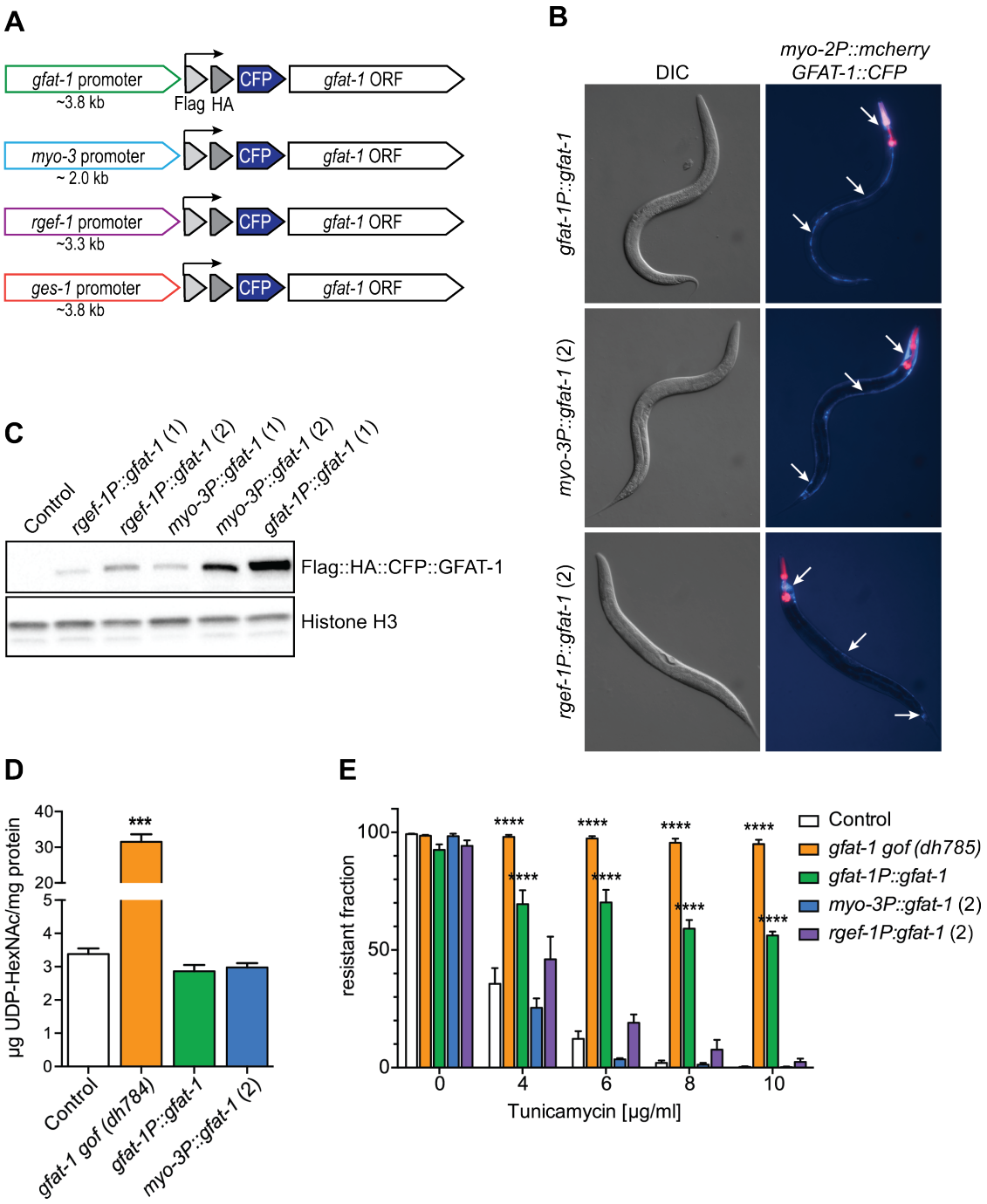


Figure 13. Tissue-specific overexpression of *gfat-1*. A. Cloning scheme of *gfat-1* OE constructs under tissue-specific promoters; *gfat-1P* for endogenous OE, *myo-3P* for muscle-specific OE, *rgef-1P* for pan-neuronal OE, *ges-1P* for intestine-specific OE. **B.** Representative images of different *gfat-1* OE strains at late L4 larval stage, DIC image (left panel) and overlay of TexasRed (expression of pharyngeal co-injection marker) and CFP channel (GFAT-1 expression) (right panel) are shown, arrows indicate tissue of transgenic GFAT-1 expression **C.** Representative Western blot from L4 larvae shows transgene expression at expected molecular weight. **D.** LC-MS analysis of UDP-HexNAc, shown is mean +SEM, N=5, 1way ANOVA, Tukey´s multiple comparisons test, ****, p<0.0001. **E.** Development of *C. elegans* on 0, 4, 6, 8 or 10 µg/ml Tunicamycin, animals that reach L4 larval or adult stage from egg after 4 days were designated the resistant fraction, shown is mean +SEM, N=4, 2way ANOVA, Dunnett´s multiple comparisons test, ****, p<0.0001.

As an alternative readout for functional HP activation, we used the same *C. elegans* lines to assess tunicamycin resistance, which directly corresponds to UDP-GlcNAc production (Denzel et al., 2014). While, in wildtype controls, the ability to develop from eggs into mature worms is reduced by roughly 60% at the lowest tested tunicamycin concentration and entirely abolished at higher concentrations, *gfat-1 gof* mutants are completely protected (Denzel et al., 2014). Overexpression of *gfat-1* under its endogenous promoter also rendered animals strikingly resistant to tunicamycin, which demonstrates that our transgene is a functional enzyme although we did not detect changes in steady state UDP-GlcNAc levels. Contrarily, in this assay, neither neuronal nor muscular *gfat-1* OE strains were protected from tunicamycin, which argues that these are likely not the relevant tissues to confer resistance (Figure 13E).

Given the functionality of the transgene, we asked whether *gfat-1* OE could stimulate PQC similar to *gfat-1 gof.* To this end, we measured the autophagic flux in a transgenic reporter strain expressing GFP-tagged LGG-1. LGG-1::GFP associates with inner and outer membrane of the forming phagophore. Upon membrane closure, the autophagosomes fuses with lysosomes to form the autolysosome. While the LGG-1::GFP in the outer membrane is recycled to the cytosol for further use, the LGG-1 attached to the inner membrane leaflet is quickly degraded by the lysosomal proteases. GFP is more resistant to proteolytic cleavage and is only degraded at a low pH (Figure 14A). Hence, it stays intact for an extended period before it is broken down as well. The ratio of free GFP (after cleavage and degradation of LGG-1) to the sum of total GFP (free GFP and LGG-1::GFP) can be assessed by Western Blot analysis and be considered to indicate the autophagic activity or the autophagic flux (Ni et al., 2011, Klionsky et al., 2016). When we used this method, we noted an increase in the intensity of the free GFP band in *gfat-1 gof* as well as *gfat-1* OE worms compared to controls, suggesting an enhanced autophagic flux in the transgenic strains (Figure 14B). This trend was observed in multiple experiments and the boost of autophagic flux by *gfat-1 gof* was, in its extent, comparable to the one induced by neuronal as well as muscular *gfat-1* OE (Figure 14C). These results suggest that modulating GFAT-1 activity and abundance might be separable processes: *gfat-1 gof* probably enhances PQC mechanisms through the overall enhanced production of UDP-GlcNAc, while the abundance of the GFAT-1 protein might serve a distinct function as no accumulation of the metabolite was measured in muscular *gfat-1* OE animals under standard growth conditions. We set out to tackle the interesting question whether the presence of GFAT-1 protein itself could affect

PQC without inducing accumulation of the HP end product and to better understand the molecular mechanism underlying the elevation of PQC by *gfat-1* OE.

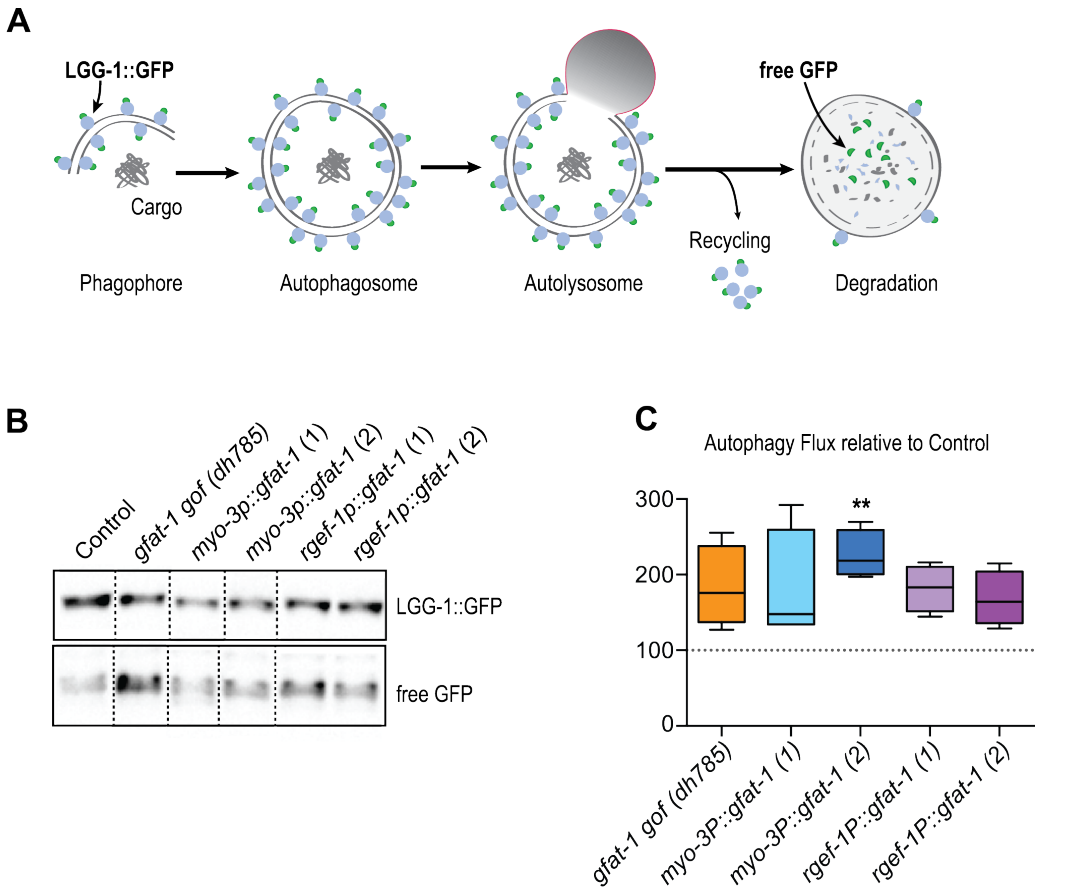


Figure 14. Induction of autophagy by *gfat-1 gof* and OE. A. Scheme of autophagic degradation of LGG-1::GFP along with cargo (based on Djeddi et al., 2012). LGG-1::GFP binds to inner and outer membrane leaflet of the nascent phagophore, which closes to form an autophagosomes. After fusion with lysosomes, LGG-1::GFP attached to the outer membrane of the autolysosome is recycled for further use, while LGG-1::GFP linked to the inner membrane is degraded by lysosomal proteases. GFP is stable for a short period of time before it is degraded at low pH. **B.** Representative Western blot from young adult worms. **C.** Quantification of autophagic flux measured as free GFP/ total GFP (LGG-1::GFP + free GFP) in day 1 adults, normalized to control (indicated by dotted line at 100%), Box extends from 25th to 75th percentile with the line indicating the median, whiskers extend from the minimal to maximal measured values, N=4, 1way ANOVA, Dunnett´s multiple comparisons test, **, p<0.01.

To identify genes that would mediate protein homeostasis downstream of GFAT-1, we aimed to establish an assay with a strong and very clear readout for the positive effect of *gfat-1* OE on PQC. This would allow us to test the requirement of multiple candidate genes in the *gfat-1* OE induced PQC improvement. A well-

established model for proteotoxicity in *C. elegans* is the expression of a fluorophore fused to stretches of polyglutamine repeats (polyQ) of distinct length and in different tissues (Morley et al., 2002, Brignull et al., 2006, Mohri-Shiomi and Garsin, 2008). The abnormal extension of polyQ repeats within certain proteins was identified as cause of several severe human diseases, such as spinocerebellar ataxia and HD (La Spada et al., 1991, Di Prospero and Fischbeck, 2005). The *C. elegans* disease model strains reflect certain characteristics of the human protein folding diseases like accumulation of protein aggregates and progressive paralysis. We therefore analyzed the effect of *gfat-1* OE on disease phenotypes in some of these model strains.

When stretches of 44 glutamines fused to YFP were expressed in the nematode´s intestine, the signal was smoothly distributed in young worms but incrementally aggregated from mid-age onward. Surprisingly, we noted an appearance of polyQ aggregates already at an earlier stage in *gfat-1* OE strains (Figure 15A). While the OE of *gfat-1* under it´s endogenous promoter does not affect the polyQ aggregation in the intestine compared to controls, we detected a slight trend for enhanced polyQ aggregation in neuronal *gfat-1* OE strains and significantly increased polyQ aggregation in muscular *gfat-1* OE worms at day 8 of adulthood (Figure 15B). This might indicate that *gfat-1* OE could, indeed, affect PQC mechanisms in a tissue-specific manner. While it might be overall positive and beneficial in certain tissues, it could be detrimental in other tissues. Alternatively, the formation of aggregates from disease proteins might be harmful in some tissues but could be protective under specific circumstances as well (Ross and Poirier, 2005, Carija et al., 2017). In any case, the observed induction of polyQ aggregate accumulation in the worm gut by OE of *gfat-1* in muscle suggests cross-tissue communication and a potential compensatory mechanism.

To investigate the influence of *gfat-1* OE on the general health of worms with neuronal polyQ expression model we monitored the nematodes´ lifespan. Interestingly, the OE of *gfat-1* also seems to be mildly harmful in this proteotoxicity model, both, in a cell autonomous manner as well as in a cell non-autonomous manner: The lifespan was equally shortened by *gfat-1* OE under the *gfat-1* endogenous promoter, a muscle-specific and a pan-neuronal promoter (Figure 15C). Again, we must conclude that *gfat-1* OE does not confer a general resistance to proteotoxic diseases and, to the contrary, can even exacerbate polyQ toxicity.

These findings are in contrast with the expectation that *gfat-1* OE, like *gfat-1 gof*, would improve PQC and decrease the harmful effects of aggregation prone proteins. However, it was our goal to establish an assay, in which *gfat-1* OE was

distinguishable from controls by a large margin to understand the downstream actions of *gfat-1* OE leading to amelioration or deterioration of proteostasis. Hence, we did not further analyze the phenotype of these models but focused on the identification of a suitable model.

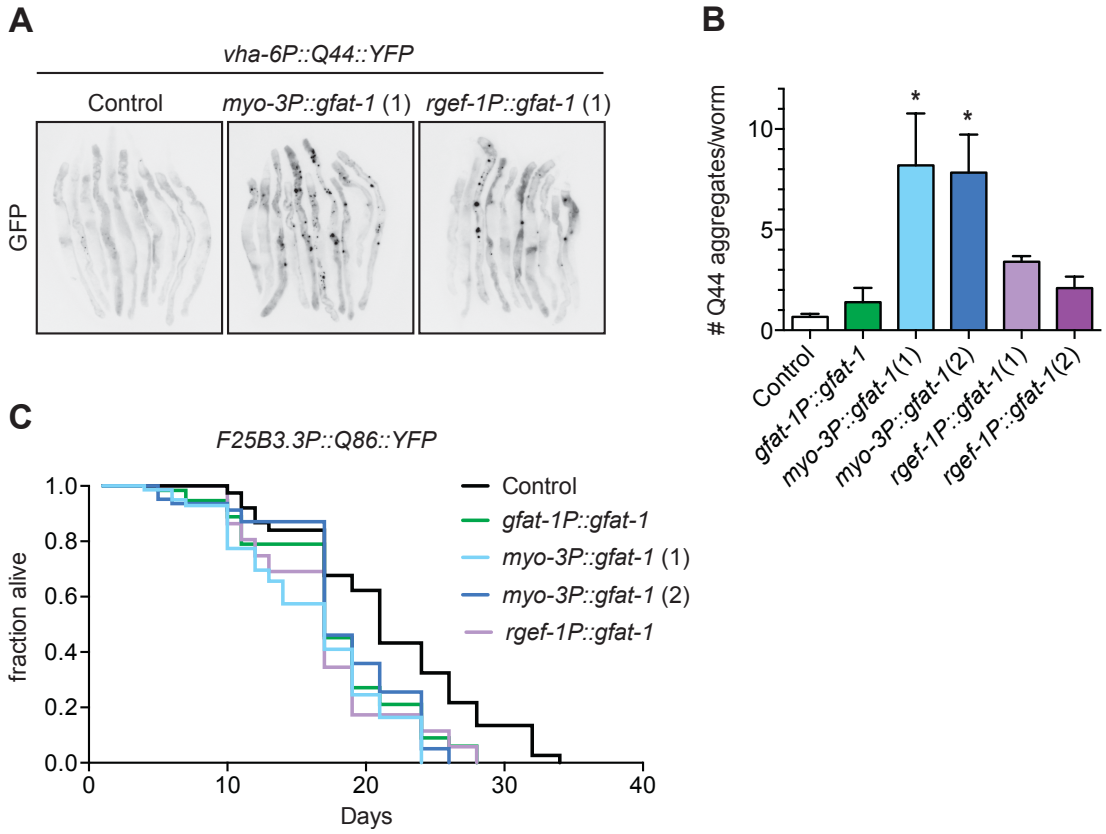


Figure 15. Adverse effects of *gfat-1* OE in models of intestinal and neuronal proteotoxicity. A. Representative images of intestinal polyQ model strain (*vha-6P::Q44::YFP*) at day 8 of adulthood in presence and absence of *gfat-1* OE. **B.** Quantification of images (as in **A.**) taken of *vha-6P::Q44::YFP* strain at day 8 of adulthood, N≥2, 10 to 30 worms per experiment, 1way ANOVA, Dunnett´s multiple comparisons test, *, p<0.05. **C.** Lifespan analysis of neuronal polyQ model strain (*F25B3.3P::Q86::YFP*) in presence and absence of *gfat-1* OE, 75 worms/ strain, N=1.

2.4 Cell-autonomous benefit of muscle-specific *gfat-1* OE on PQC

Although we did not detect any benefit of tissue-specific *gfat-1* OE on proteostasis and general health in models of protein folding stress and aggregation in intestine and neurons, we continued to investigate the role of *gfat-1* in muscle integrity. Muscle polyQ expression leads to polyQ aggregation and progressive paralysis of worms with age (Morley et al., 2002). As this phenotype is specific for

muscle function, easily measurable, and physiologically relevant, the model provides an ideal readout for polyQ toxicity.

First, we studied the effect of *gfat-1* OE on visible polyQ aggregation in a disease strain with a stretch of 40 glutamines fused to YFP expressed under the control of the muscle-specific *unc-54* promoter. In this strain, the transgene rapidly formed aggregates already in larval stages. This process was slowed down considerably with concurrent muscular *gfat-1* OE (Figure 16A). By contrast, the OE of *gfat-1* under the *gfat-1* or a neuronal promoter was not beneficial in this assay, leaving the load of polyQ aggregates unchanged compared to controls (Figure 16B). This observation could be corroborated by a biochemical approach. We extracted protein from day 1 adult worms and separated the polyQ::YFP fusion protein in SDS soluble and SDS insoluble fraction. It is commonly believed that the toxic form of many aggregation prone proteins like polyQ containing proteins and amyloid species is the SDS resistant oligomer (Walsh et al., 2002, Takahashi et al., 2008, Weiss et al., 2008, Wong et al., 2008). Indeed, we detected significantly less SDS resistant Q40::YFP when expression of GFAT-1 in muscle was high. The amount of non-toxic, SDS soluble monomers and small oligomers of Q40 was found to be much more abundant than the SDS insoluble fraction in all tested conditions. Additionally, *gfat-1* OE in general seemed to slightly increase the amount of soluble polyQ fusion protein (Figure 16C).

As the toxicity of the transgene in the *unc-54P::Q40::YFP* strain set in already in *C. elegans* larval stages, we chose to use a model which expressed a shorter polyQ stretch in muscle tissue for further analysis. Using the *unc-54P::Q35::YFP* transgenic line, we expected a delayed onset and slowed down progression of toxicity, which would better reflect the hallmarks of actual polyQ diseases and would hopefully allow the observation of more subtle changes of aggregation and disease phenotype.

Indeed, multiple and clear polyQ aggregates were observed in the *unc-54P::Q35::YFP* model starting only at day 4 of adulthood. Like in the Q40 model, the measurable benefit of *gfat-1* OE was strictly cell autonomous: OE of *gfat-1* in muscle massively decreased the load of aggregates, while OE of *gfat-1* under its endogenous or a pan-neuronal promoter did not ameliorate polyQ aggregation (Figure 17A and 17B).

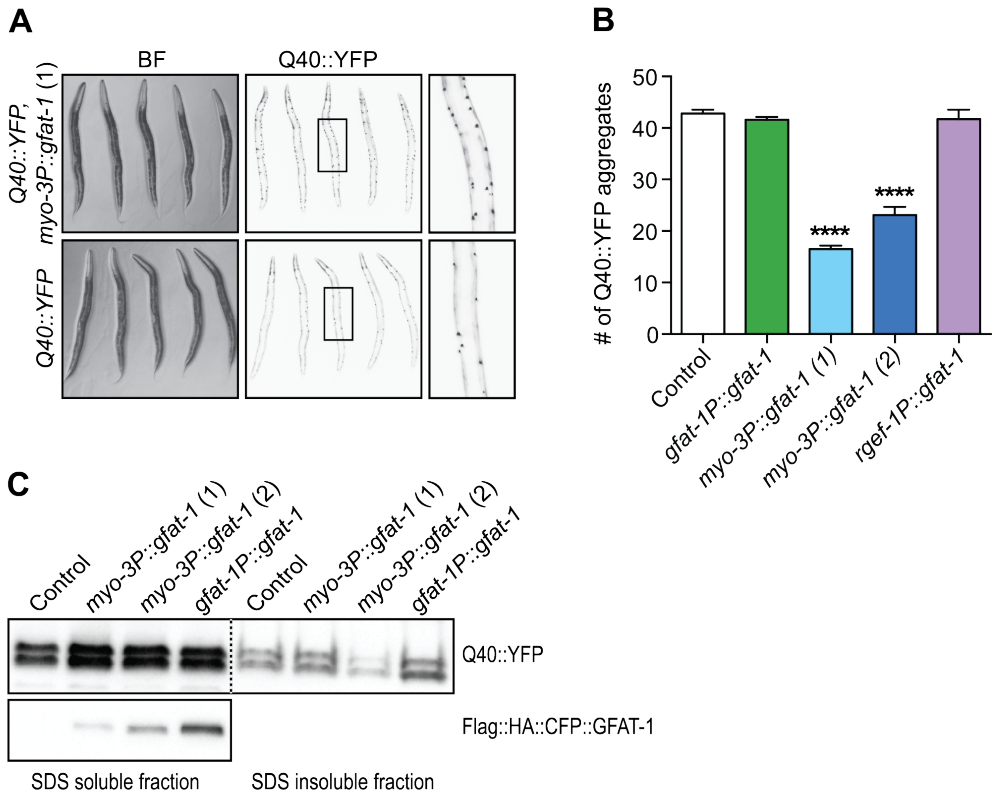


Figure 16. Decreased polyQ protein aggregation in muscle by muscular *gfat-1* OE. A. Representative images of late L4 larvae of the *unc-54P::Q40::YFP* strain. **B.** Quantification of Q40::YFP aggregates at late L4 stage in *unc-54P::Q40::YFP* background and *gfat-1* OE under endogenous (*gfat-1P*), muscle-specific (*myo-3P*) (as in **A.**) and pan-neuronal promoter (*rgef-1P*), shown is the compiled mean +SEM of N=3 experiments, 10 worms per condition, 1way ANOVA, ****, p<0.0001. **C.** Representative Western blot of SDS-soluble and SDS-insoluble Q40::YFP fractions of day 1 adult *unc-54P::Q40::YFP* animals in presence and absence of tissue-specific *gfat*-1 OE, background, N=2.

Importantly, we detected a clear mitigation of a further major disease characteristic by *gfat-1* OE: While, in a swimming assay, Q35 expressing control animals were completely paralyzed at day 10 of adulthood, simultaneous *gfat-1* OE in muscle greatly preserved the worms´ motility. Interestingly, also the *gfat-1* OE under its endogenous promoter was mildly beneficial in this assay, although there was no significant influence on visible polyQ aggregation (Figure 17B and 17C).

To rule out the possibility that the improved muscle function merely stems from a decreased disease transgene expression, we measured the total expression of Q35 in presence and absence of *gfat-1* OE as important control. As we did not observe a reduction in overall Q35 expression by increasing GFAT-1 levels, we concluded that *gfat-1* OE is indeed counteracting polyQ toxicity in muscle. This effect was particularly large when polyQ and *gfat-1* were both expressed alongside in the

body wall muscle and the amount of muscular GFAT-1 positively correlated with the extent of motility improvement (Figure 17D and 13C).

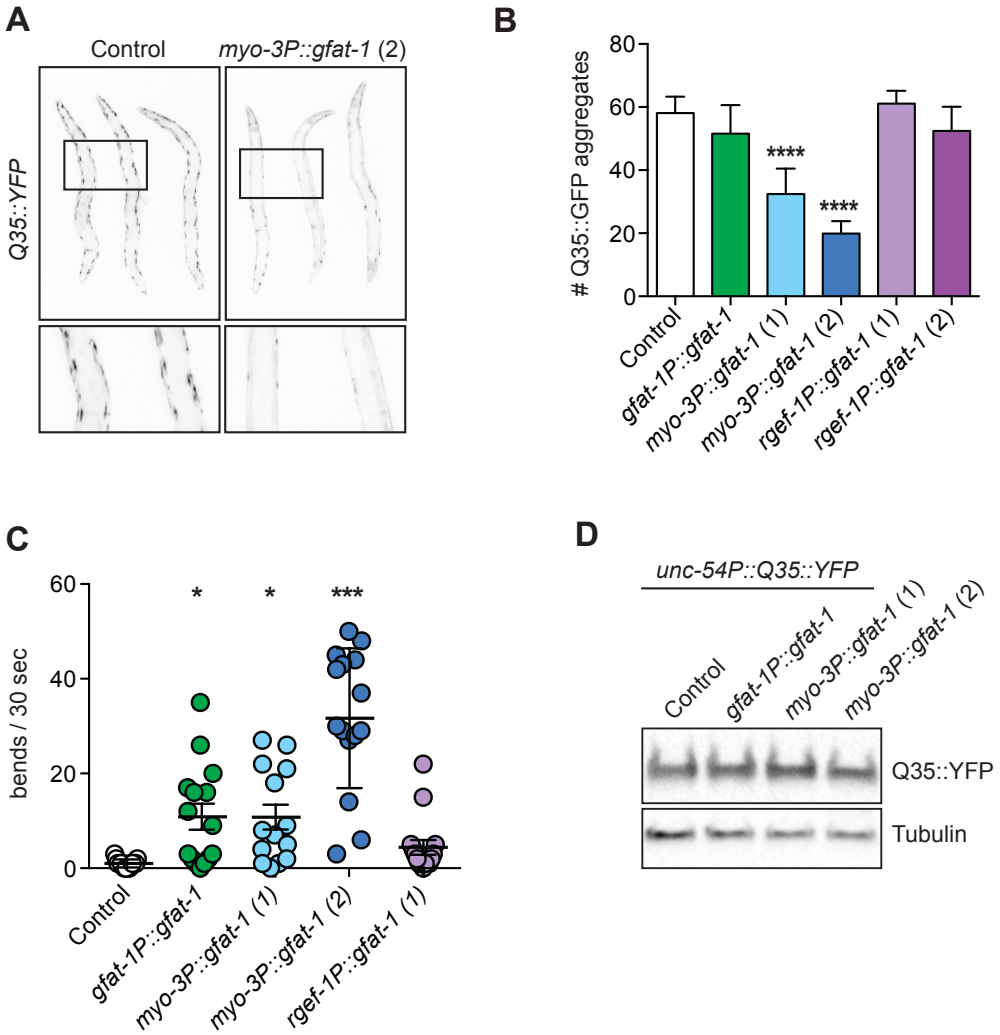


Figure 17. Cell-autonomous improvement of polyQ muscle toxicity by *gfat-1* OE. A. Representative images of *unc-54P::Q35::YFP* animals in presence and absence of muscular *gfat-1* OE at day 4 of adulthood**. B.** Quantification of Q35::YFP aggregates at day 4 of adulthood (like in **A**.), shown is mean +SD of 10 worms per condition from representative experiment, , N≥3 for *myo-3P::gfat-1* and control, N=1 for *gfat-1P::gfat-1* and *rgef-1P::gfat-1*, 1way ANOVA, Dunnett´s multiple comparisons test, ****, p<0.0001**. C.** Motility of *gfat-1* OE strains in *unc-54P::Q35::YFP* background at day 10 of adulthood as measured by swimming assay, 15 worms per condition, line and error bars indicate mean ± SD of one representative experiment, N≥3, only for *rgef-1P::gfat-1*, N=1, 1way ANOVA, Tukey´s multiple comparisons test, *, p<0.05, ***, p<0.001. **D.** Representative Western blot of total Q35::YFP in day 1 adults, N=2.

We, furthermore, wanted to exclude the possibility that the observed benefit on polyQ disease by *gfat-1* OE is due to unspecific side effects of expressing any transgene in the worm muscle. To this end we knocked down *gfat-1* expression by RNAi feeding levels in C. elegans with muscular *gfat-1* OE and evaluated motility in a swimming assay. Reducing *gfat-1* levels led to an apparent reduction in motility compared to controls treated with *luciferase* RNAi. Although *gfat-1* RNAi did not reverse the improved swimming ability of *gfat-1* OE worms to complete paralysis, it is safe to assume that PQC improvement is specific to *gfat-1* OE and no experimental artifact (Figure 18A).

Next, we wondered whether the benefit of *gfat-1* OE on the health of polyQ expressing worms was dependent on activity of the HP and production of UDP-GlcNAc. To address this, we, in this assay, evaluated the consequences of RNAi against *gna-2*, the second enzyme of the HP. Similar to *gfat-1* knock down, *gna-2* knock down markedly reduced the motility of muscular *gfat-1* OE strains (Figure 18B). To check the knock down efficiency and to clarify whether *gna-2* knock down would directly impact on GFAT-1 levels and thereby show its strong effect, we measured *gna-2* transcript levels and GFAT-1 protein amount in response to RNAi. Feeding of *gna-2* RNAi dramatically lowered the mRNA level of *gna-2* to about 20% of the *luciferase* RNAi reference in control and *gfat-1 gof* strain, whereas *gfat-1* mRNA levels were not affected. The second housekeeping gene *cdc-42* was included as an additional control of the experiment (Figure 18C). We monitored the knock down efficiency of *gfat-1* RNAi at the protein level as well by using our transgenic *gfat-1* OE strains. Feeding of *gfat-1* RNAi resulted in a mild reduction of GFAT-1 protein. The RNAi knock down of *gna-2* did not decrease GFAT-1 protein (Figure 18D and 18E). In conclusion, *gna-2* knock down does not seem to destabilize GFAT-1 protein, nor to induce a compensatory upregulation of GFAT-1.

The observation that *gfat-1* as well as *gna-2* knock down led to a reduction of the *gfat-1* OE induced motility improvement in polyQ transgenic worms indicates that the flux through the HP and the production of UDP-GlcNAc plays a central role in decreasing proteotoxicity. Hence, we measured the effect of *luciferase*, *gfat-1* or *gna-2* RNAi on the level of UDP-GlcNAc by LC-MS. We included control worms as well as *gfat-1 gof* worm strain in the analysis. UDP-HexNAc levels are elevated more than tenfold in *gfat-1 gof (dh784*) compared to controls, which is clearly above the concentrations essential for survival. Furthermore, the detection method was very sensitive in the range of UDP-HexNAc present in samples prepared from the *gfat-1 gof* strain and consequently, we expected to even identify small changes in HP product levels. Surprisingly, neither *gfat-1*, nor *gna-2* knock down decreased the

steady-state concentration of UDP-HexNAc in wildtype controls or *gfat-1 gof* worms (Figure 18F).

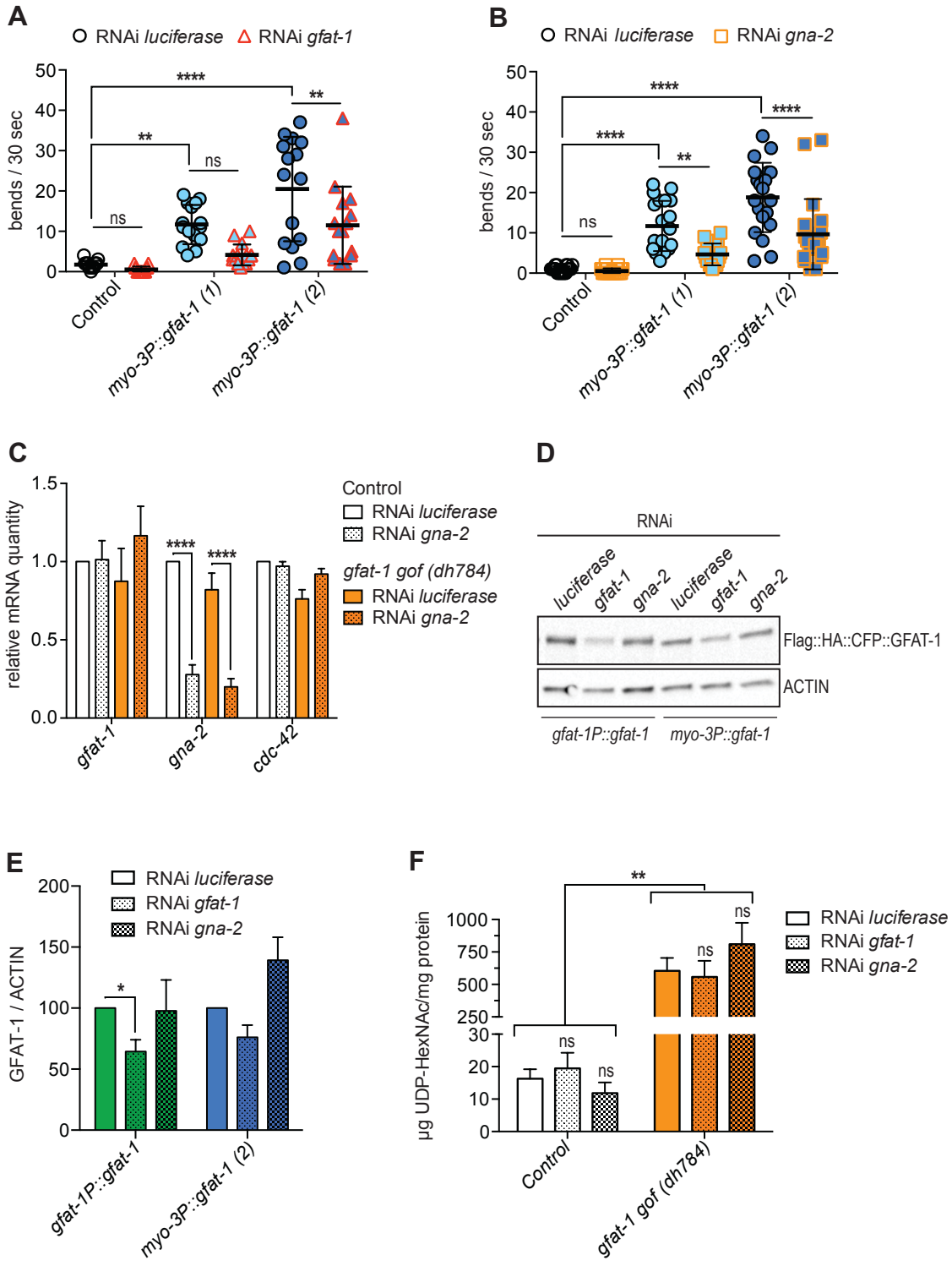


Figure 18. Improved motility in polyQ model by *gfat-1* OE dependent on HP enzyme levels but not HP product. A. Motility of muscular *gfat-1* OE in *unc-54P::Q35::YFP* background at day 10 of adulthood when grown on *luciferase* or *gfat-1* RNAi from early L4 stage onward, 15 worms per condition, line and error bars indicate mean ± SD, N=2, same trend was observed at day 8 of adulthood, 1way ANOVA, Bonferroni´s multiple comparisons test, **, p<0.01, ****, p<0.0001. **B.** as in (**A.**) but 20 worms per condition, line and error bars indicate mean ± SD of one representative experiment, N=2, same trend was observed at day 8 of adulthood)**,** 1way ANOVA, Bonferroni´s multiple comparisons test, **, p<0.01, ****, p<0.0001. **C.** Relative mRNA amount in day 1 adult worms, grown on RNAi from egg onwards as measured by qRT-PCR, shown is mean +SEM, values normalized to *ama-1*, N=3, 2way ANOVA, Tukey´s multiple comparisons test, ****, p<0.0001. **D.** Representative Western blot of GFAT-1::CFP and actin loading control in day 1 adult *gfat-1 OE* strains after RNAi treatment from egg onward. **E.** Quantification of GFAT-1::CFP expression in day 1 adult *gfat-1 OE* strains after *gfat-1* or *gna-2* RNAi treatment (as in **D.**), shown is mean +SEM, N=3, 2way ANOVA, Tukey´s multiple comparisons test, *, p<0.05. **F.** LC-MS analysis of UDP-HexNAc levels in day 1 adult *gfat-1 gof* and control animals after *gfat-1* or *gna-2* RNAi treatment from egg onward, shown is mean +SEM, N=4, 2way ANOVA, Tukey´s multiple comparisons test, **, p<0.01.

These findings led us to believe that the mechanism through which *gfat-1* OE amended polyQ toxicity in muscle was not only tracing back to altered UDP-GlcNAc levels but they suggested, again, that there might be a secondary function of GFAT-1 protein affecting PQC.

To test this hypothesis, we generated *C. elegans* strains that express an enzymatic dead version of GFAT-1 under a muscle-specific promoter (strains generated and experiments performed by Vignesh Karthikaisamy under my supervision). For this purpose we introduced a mutation in the isomerase domain of the protein, which transformed lysine (K) 720 of *C. elegans gfat-1* to an arginine (R). It is known that the corresponding mutation of the conserved residue in *E. coli* and human *gfat-1* leads to only negligible enzymatic activity (Mouilleron et al., 2011, Sabine Ruegenberg, personal communication) (Figure 19A). The intimate interaction of the lysine residue with the GFAT-1 substrate Fruc-6-P becomes obvious when studying the structure of the enzyme dimer with associated substrate. Even the conservative exchange of positively charged lysine (K) with equally charged, but bulkier arginine (R) seems likely to strongly interfere with Fruc-6-P binding, while simultaneously leaving the protein structure intact (Figure 19B). As the amounts of transgenic wildtype GFAT-1 and enzymatic dead GFAT-1 expressed in our different muscle-specific OE lines was very similar, we could directly compare these *C. elegans* lines in further experiments (Figure 19C). We analyzed the functionality of transgenic GFAT-1 in the *gfat-1K720R* strains by testing tunicamycin resistance, which can directly be correlated to UDP-GlcNAc production (Denzel et al., 2014).

Control animals were included as sensitive reference and animals with *gfat-1* OE under the endogenous promoter as resistant reference. While muscle-specific OE of wildtype *gfat-1* rendered animals mildly resistant to low levels of tunicamycin, C. elegans overexpressing enzymatic dead *gfat-1* in muscle were undistinguishable from sensitive controls (Figure 19D). This observation confirmed the *myo-3P::gfat-1K720R* strains to be unable to produce higher UDP-GlcNAc levels as controls and consequently proved GFAT-1K720R as catalytically inactive enzyme. Strikingly, *C. elegans* strains that overexpress this enzymatic dead version of GFAT-1 under a muscle-specific promoter are still somewhat protected from the detrimental effects of polyQ aggregation. When we tested the motility of aged animals in a swimming assay, *myo-3P::gfat-1K720R* worms performed significantly better than controls. Although animals with OE of functional GFAT-1 enzyme in muscle were still clearly healthier and more motile, with about three times more swimming motions observed, we conclude that GFAT-1 protein itself counteracts polyQ toxicity in muscle (Figure 19E). And while the enzymatic function of GFAT-1 is definitively involved in improving PQC, GFAT-1 seems to, indeed, fulfill a dual role in this context.

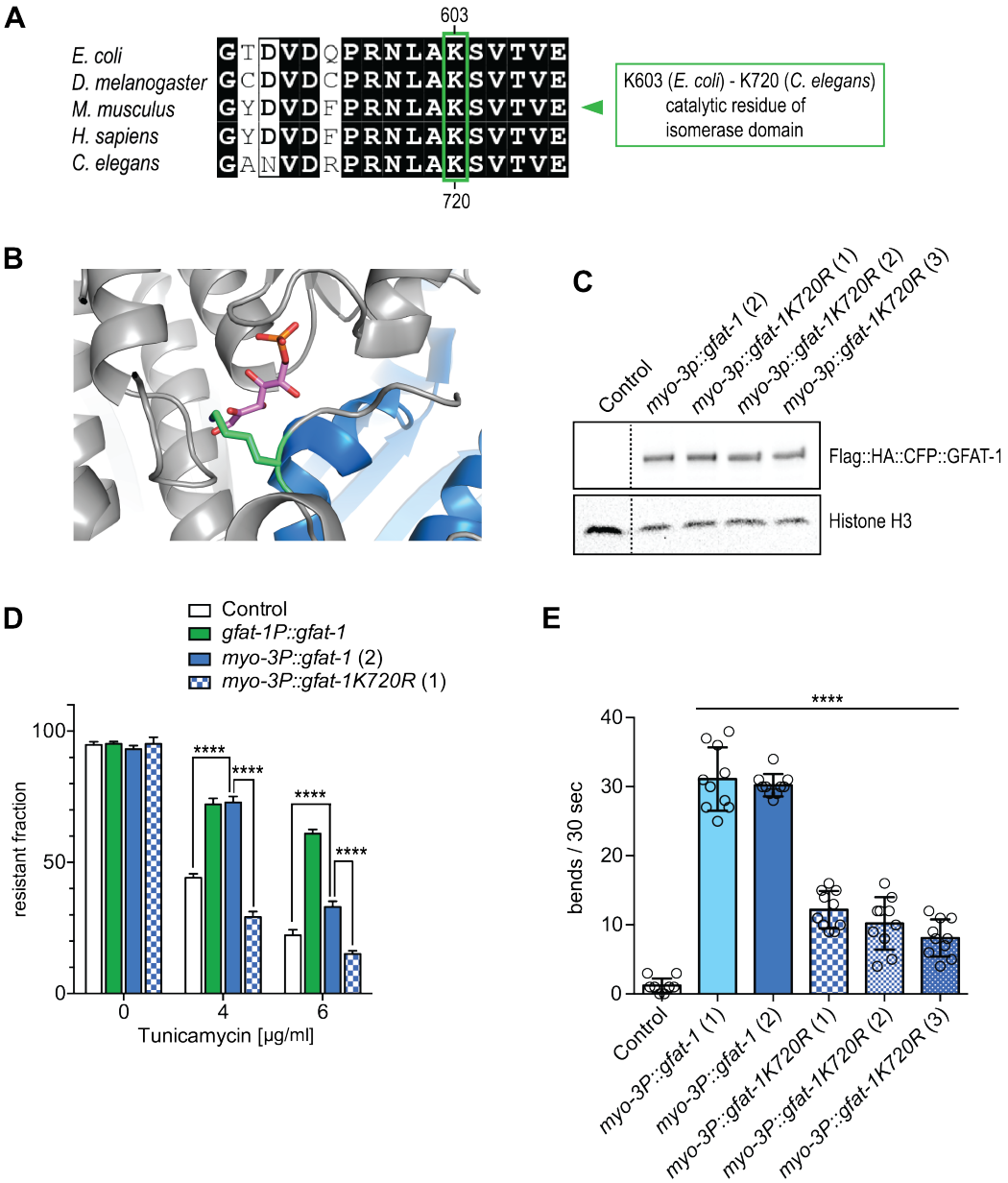
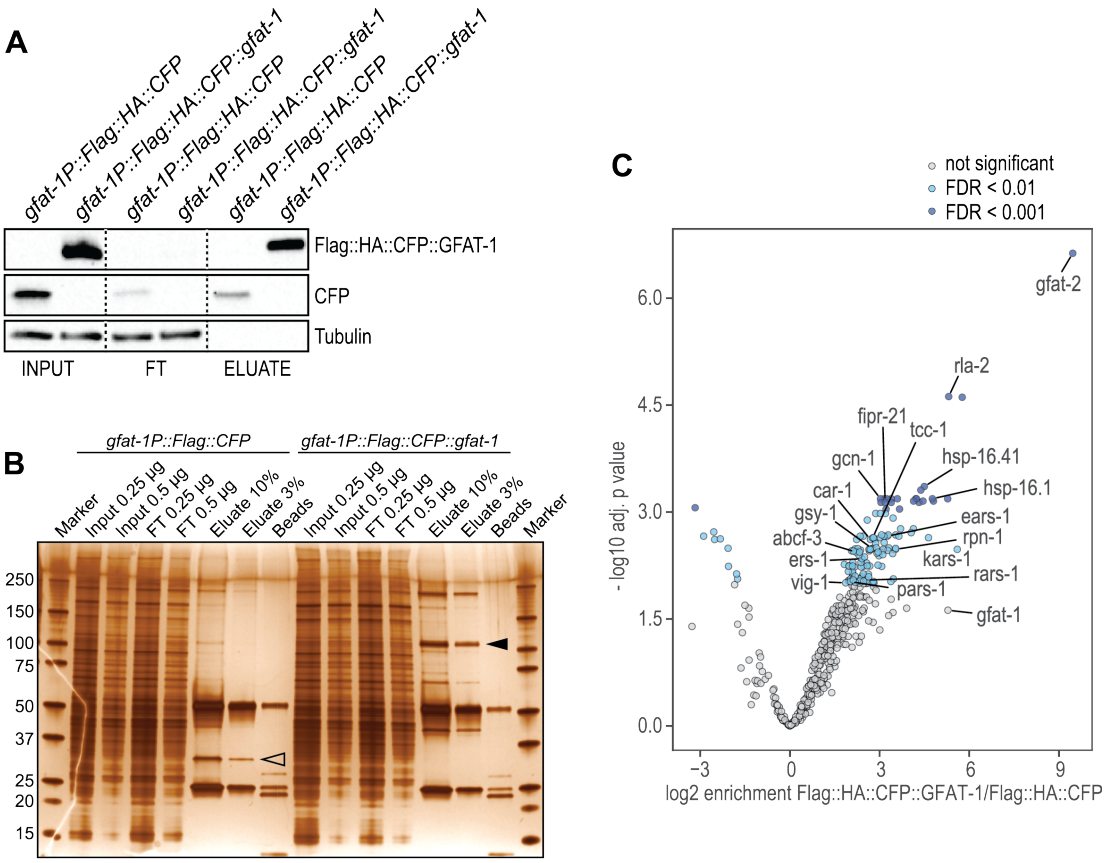


Figure 19. Mildly improved motility in polyQ model by OE of enzymatic dead *gfat-1*. A. Alignment of the *gfat-1* C-terminus from different species with conserved catalytic lysine residue *E. coli* K603, corresponding to *C. elegans* K720, alignment produced using ENDscript 2 {Robert:2014fy}. **B.** Molecular structure of *E. coli* GFAT-1 homodimer (monomers in grey and blue) with bound substrate Fruc-6-P (pink), highlighted is lysine 603 (green), figure constructed with PyMOL using pdb code 4AMV. **C.** Representative Western blot of day 1 adult controls and worms expressing functional and enzymatic dead GFAT-1 (*gfat-1K720R*) under a muscle-specific promoter, N=3 **D.** Development of *C. elegans* strains on 0, 4, or 6 µg/ml Tunicamycin, animals that reach L4 larval or adult stage from egg after 4 days were designated the resistant fraction, Control animals were included as sensitive strain and *gfat-1P::gfat-1* as resistant control, shown is mean +SEM, N=3, 2way ANOVA, Dunnett´s multiple comparisons test, ****, p<0.0001. **E.** Motility of muscular *gfat-1* and *gfat-1K720R* OE strains in *unc-54P::Q35::YFP* background at day 8 of adulthood as measured by swimming assay, 10 worms per condition, line and error bars indicate mean ± SD of one representative experiment, N=3, 1way ANOVA, Tukey´s multiple comparisons test, ****, p<0.0001. All experiments for Figure **19 C**, **19 D** and **19 E** were performed by **Vignesh Karthikaisamy** under my supervision.

2.5 Physical interaction partners of GFAT-1

GFAT-1 functions as an enzyme in the HP to generate the essential molecule UDP-GlcNAc. However, our results suggest that, additionally, GFAT-1 can ameliorate proteotoxicity in muscle independently of UDP-GlcNAc production. To gain a full picture of the possible mode of action by which *gfat-1* OE leads to improved PQC, we set out to identify physical interaction partners of GFAT-1 in *C. elegans*. We generated all *gfat-1* OE strains with Flag and HA peptide as well as CFP at the protein´s N-terminus. This allowed co-immunoprecipitation (IP) of GFAT-1 with associated proteins from worm lysates. To minimize false positive interaction partners, we included the *gfat-1P::Flag::HA::CFP* strain lacking the *gfat-1* ORF as reference. Both transgenic proteins were strongly bound by Flag antibody and only a very small fraction of the apparently less firmly associated Flag::HA::CFP control protein was detected in the flow-trough (FT) fraction. Incubation with Flag peptide effectively replaced GFAT-1 fusion protein control protein in antibody binding and there was a high abundance of both proteins in the eluate fraction, while no contamination with tubulin was observed (Figure 20A). The unspecific identification of proteins in the different IP fractions by silver staining confirmed the feasibility of the GFAT-1 co-IP: the majority of proteins was successfully depleted after the IP and only a limited number of bands was visible in the eluate fraction from lysate of *gfat-1* OE and control worms. As this number was higher after pull down with GFAT-1 fusion protein, it suggested that specific interaction partners of GFAT-1 could be identified by our approach (Figure 20B).

MS analysis of the eluate and comparison between both strains could, indeed, determine a set of GFAT-1 binding partners with false discovery rates (FDRs) of 0.001 or 0.01. Although GFAT-1 itself was clearly enriched in the IP eluate from the *gfat-1* OE strain, it was also present and relatively abundant in the eluate from the control strain, which rendered its enrichment not significant (Figure 20C). Study of the complete list of GFAT-1 binding partners did not reveal a clustering of the interacting proteins within a biological pathway or process. Of note, however, was the identification of multiple heat shock proteins (F44E5.4, HSP-16.1, HSP-16.41, HSP-1, HSP-16.48) and proteins involved in mRNA translation like ribosomal subunits (RLA-2, RPL-31, RLA-1, RPL-23, RPL-8, RPS-14, RPS-27, RPS-3, RPS-30), tRNA synthetases (MRS-1, EARS-1, ERS-1;QARS-1, KARS-1;KRS-1, RARS-1;RRT-1) and translation initiation and elongation factors (EEF-1B.2, EEF-1G, EGL-45, EIF-3.B, EIF-3.I, Y39G10AR.8) (Figure 20D).



D

GFAT-1 interactors									
anc-1	*mrs-1*	*abcf-3*	*ddx-17*	*eif-3.B*	*icl-1*	*misc-1*	*rars-1;rrt-1*	*rps-3*	*vbh-1*
atad-3	*ola-1*	*acaa-2*	*dhc-1*	*eif-3.I*	*immp-2*	*nol-5*	*ril-1*	*rps-30*	*vig-1*
cey-2	*pab-2*	*B0303.3*	*dnc-1*	*ergo-1*	*immt-2*	*npp-5*	*rla-1*	*rpt-5*	*vit-1*
cgh-1	*plp-1*	*C05G5.4*	*dnj-12*	*ers-1;qars-1*	*irs-1*	*nra-2*	*rpac-40*	*sip-1*	*vit-2*
F44E5.5	*pyr-1*	*C13B9.3*	*dpm-1*	*F08F3.4*	*K01C8.1*	*nst-1*	*rpl-23*	*T07A9.9*	*vit-4*
fasn-1	*rla-2*	*C28H8.3*	*dyn-1*	*F47B10.1*	*K02F3.2*	*pab-1*	*rpl-8*	*T07D3.9*	*vit-5*
fipr-21	*rpl-31*	*car-1*	*ears-1*	*fib-1*	*K07C5.4*	*pars-1*	*rpn-1*	*tba-7*	*vit-6*
fln-1	*tba-1*	*cdk-1*	*ech-1.2*	*gdh-1*	*K11H3.3*	*pck-2*	*rpn-2*	*tbb-2*	*vrs-2*
gcn-1	*ucr-2.2*	*cey-3*	*eef-1B.2*	*glh-1*	*kars-1;krs-1*	*pgl-1*	*rpn-3*	*tcc-1*	*Y39G10AR.8*
gfat-2	*vgln-2*	*clu-1*	*eef-1G*	*gsy-1*	*laf-1*	*pod-2*	*rpoa-2*	*tsn-1*	*Y69A2AR.18*
hsp-16.1	*Y38F1A.6*	*copa-1*	*egl-4*	*hsp-1*	*mccc-1*	*pyc-1*	*rps-14*	*ucr-2.3*	*Y71F9AL.9*
hsp-16.41		*csr-1*	*egl-45*	*hsp-16.48*	*metr-1*	*R02D3.1*	*rps-27*	*unc-43*	*ZK669.4*
FDR < 0.001		FDR < 0.01							

Figure 20. Identification of physical interaction partners of GFAT-1. A. Representative Western blot of Flag IP fractions from lysates of mixed population of *gfat-1P::Flag::HA::CFP::gfat-1* and *gfat-1P::Flag::HA::CFP* as control, elution with Flag peptide, N=3, FT=low-trough. **B.** Representative silver gel, samples as in (**A.**), Elute=beads before trypsin digest, Beads=beads after 24 h trypsin digest, arrow head indicates Flag::HA::CFP, filled arrowhead indicates Flag::HA::CFP::GFAT-1, N=2. **C.** Volcano plot of GFAT-1 interaction partners identified by Flag IP with lysates of mixed population of *gfat-1P::Flag::HA::CFP::gfat-1* and *gfat-1P::Flag::HA::CFP* as control, only select binding partners of GFAT-1 with FDR≤0.01 and GFAT-1 itself are labeled, peptide digest from beads with trypsin, peptide purification and MS-analysis, N=4, FDR, false discovery rate. **D.** List of all physical interaction partners of GFAT-1 with FDR of 0.001 or 0.01 (as in **C.**).

2.6 Screen for essential factors in *gfat-1* OE induced PQC improvement

It was one of my overarching aims to identify the molecular mechanism underlying the *gfat-1* OE induced improvement of PQC mechanisms. To narrow down the list of potential candidates and processes, we used our muscular *gfat-1* OE, polyQ model to set up a selective, multi-step RNAi based suppressor screen for motility (Figure 21A). We generated a set of 227 RNAi clones targeting identified physical interactors of GFAT-1, PMK-1, factors involved in glycosylation and PQC mechanisms, amongst others. In a first screening round we roughly determined the effect of each RNAi clone on the motility of *unc-54P::Q35, myo-3P::gfat-1* worms. For this purpose we visually assessed the ability of aged worms to leave a marked area on culture plates within one minute. Nematodes exclusively overexpressing polyQ in muscle and grown on *luciferase* control RNAi were completely paralyzed at day 12 of adulthood and not a single animal was able to leave the marked area within our set time interval and even longer time periods. By contrast, polyQ expressing worms grown on *luciferase* RNAi with additional muscular *gfat-1* OE were still very motile and all worms left the marked spot within one minute. Feeding *gfat-1* and *gna-2* RNAi in this background served as our reference for hits in this assay, as it led to an intermediate phenotype. The nematodes could still move sluggishly but most of them were not able to crawl out of the marked area within one minute (Figure 21B). All RNAi clones that caused a comparable phenotype were considered primary hits and taken to the second screening round in which three to five worms per condition were subjected to a quantitative motility assay in liquid (Figure 21A). We quantified swimming motions within an interval of 30 seconds. If the mean motility of the nematodes on specific candidate RNAi clones was below the standard error of the *luciferase* RNAi control and comparable to the performance of worms on *gna-2* RNAi, they were taken to the next step of the screening process (Figure 21C). This step was designed to exclude RNAi clones that aggravate the polyQ phenotype and decrease the worms´ motility independently of *gfat-1* OE. To address this, we performed a counter screen in polyQ background without *gfat-1* OE. As complete paralysis sets in very early in this model, the quantitative motility assay in liquid had to be performed at a younger age (Figure 21A). In this case, the cut off for candidates was set within the range of the standard error of polyQ worms grown on *luciferase* RNAi or *gna-2* RNAi, which had no detrimental effects in this setup. If the mean swimming ability of animals grown on specific RNAi clones was below the cut off value, the decreased motility was considered a sign of toxicity in polyQ animals and independent of *gfat-1* OE. These clones were excluded from further analyses

(Figure 21D). After the first three steps of the screening process, the remaining candidates were confirmed with a more extensive swimming assay, comprising a higher number of worms for each condition. For this assay, *unc-54P::Q35, myo-3P::gfat-1* worms were cultured without Fluorodeoxyuridine (FUDR), a DNA synthesis inhibitor, and motility was evaluated already at day 10 of adulthood to allow the detection of subtle differences (Figure 21A and 22A) . The cut off for candidates was, as before, determined by the motility of *unc-54P::Q35, myo-3P::gfat-1* animals on *luciferase* RNAi as negative control. Every RNAi clone that caused a decrease of mean motility below the standard error of this control and that was well in the range of *unc-54P::Q35, myo-3P::gfat-1* animals on *gna-2* RNAi as positive control was classified a true hit (Figure 22A). Again, a counter screen was carried out to determine whether single RNAi hits reduced motility in polyQ background, independently of *gfat-1* OE. Here, RNAi treatment that led to a swimming capacity within the standard error of polyQ worms on *luciferase*, *gfat-1* and *gna-2* RNAi as controls was considered a true hit and was included in our shortlist of potential *gfat-1* downstream effectors (Figure 22B and 22D).

180 out of the 227 tested RNAi clones could already be excluded from further analyses in the initial motility screen on plates and consecutive small swimming assay in liquid. Another 11.5% were censored after the first counter screen in polyQ background. Further 2.6% of candidates could not be confirmed in the more extensive fourth step of our screen, which left us with 6.6% true hits corresponding to15 candidate genes (Figure 22C). These genes do not cluster in a single pathway or biological process and most genes are not associated to PQC mechanisms. However, we realized that three of the candidates were involved in protein translation: *abcf-3*, the ortholog of *S. cervisiae gcn20*, works in a complex to modulate phosphorylation of eIF2α and is thereby implicated in translation initiation and regulation of *atf-5* transcription (Vazquez de Aldana et al., 1995, Marton et al., 1997, Garcia-Barrio et al., 2000, Hirose and Horvitz, 2014); *rars-1* codes for the arginyl-tRNA synthetase and is, as such, necessary for protein translation (Ibba and Soll, 2000); *rla-2* is encoding an acidic protein in the 60S ribosomal subunit that is essential for protein synthesis (Rich and Steitz, 1987) (Figure 22D). We found this novel link between the energy sensor GFAT-1 and protein synthesis highly intriguing and decided to further investigate along these lines.

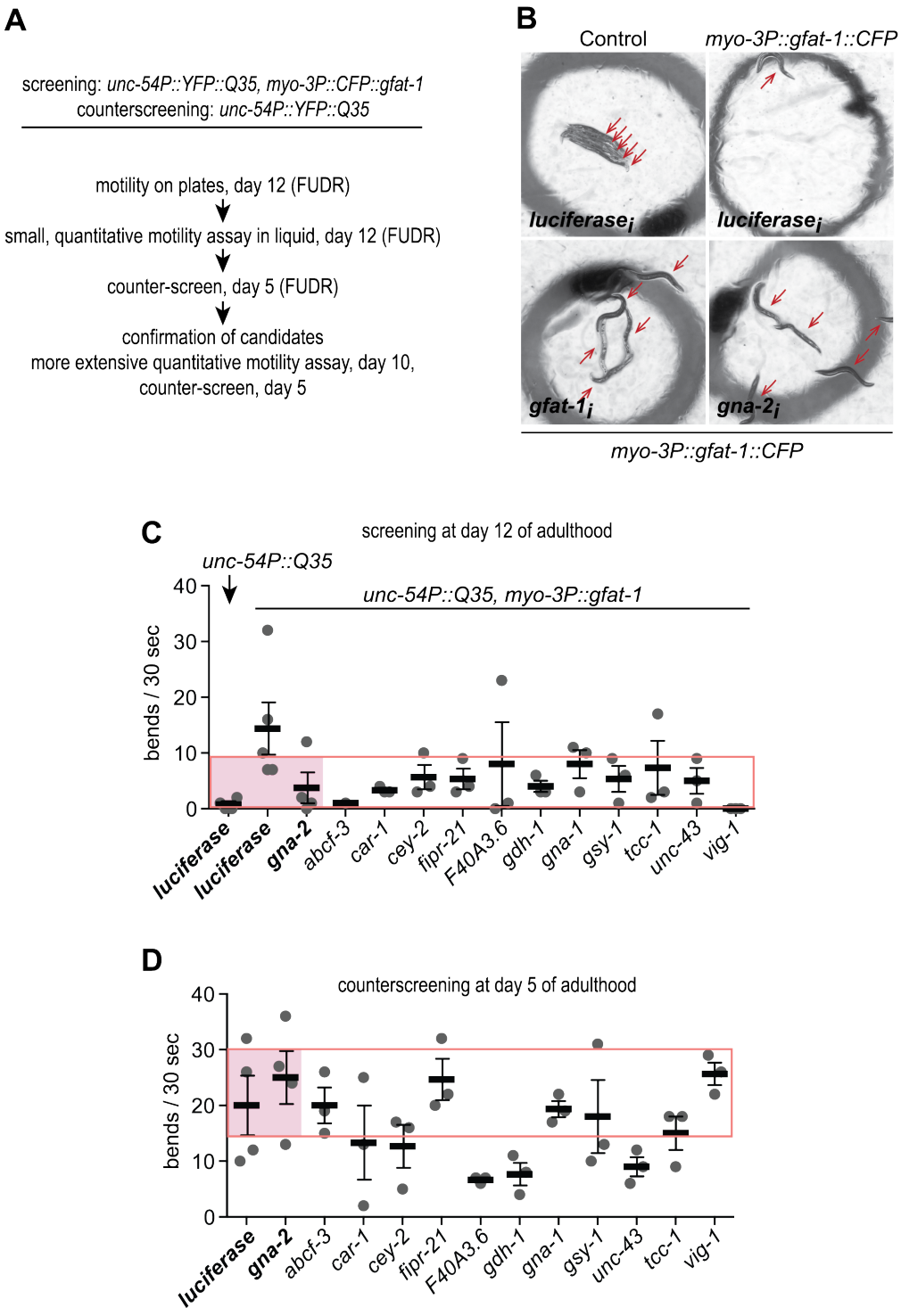


Figure 21. Screen for downstream effectors of *gfat-1* OE induced motility improvement in polyQ background. A. Outline of multi-step RNAi based suppressor screen. **B.** Representative image of control animals in first screening round (motility on plates), ability to leave marked area on culture plates within one minute. **C**. and **D**. Only a subset of screened genes is shown, red box indicates cut off for potential candidates, filled red area shows controls, RNAi clones with mean motility within red box are considered hits, red background signifies controls, line and error bars indicate mean ±SEM. **C.** Small quantitative assay in liquid in *unc-54P::Q35::YFP, myo-3P::Flag::HA::cfp::gfat-1* background at day 12 of adulthood, in the depicted subset all candidates were taken to next screening step. **D.** Counter-screen in *unc-54P::Q40::YFP* background at day 5 of adulthood, in the depicted subset 5 genes were excluded from further analyses. FUDR, Fluorodeoxyuridine.

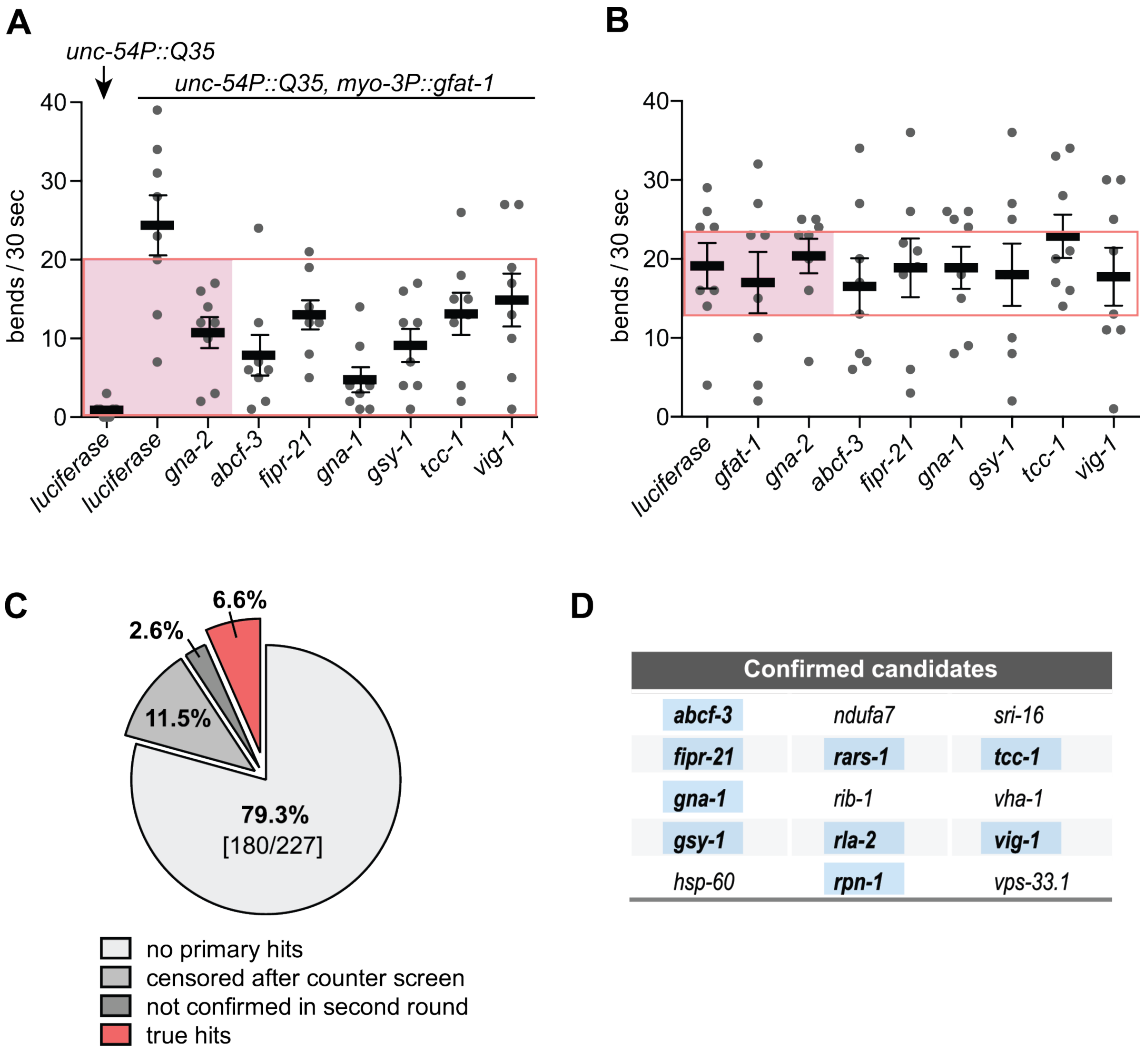


Figure 22. Confirmation of *gfat-1* downstream effectors causing motility improvement in polyQ background. A. and **B.** Only a subset of screened genes is shown, red box indicates cut off for potential candidates, filled red area shows controls, RNAi clones with mean motility within red box are considered hits, red background signifies controls, line and error bars indicate mean ±SEM. **A.** Confirmation of candidates in quantitative swimming assay in *unc-54P::Q40::YFP, myo-3P::Flag::HA::cfp::gfat-1* (2) background at day 10 of adulthood**. B.** Confirmation of candidates in counter screen, quantitative swimming assay in *unc-54P::Q40::YFP* background at day 5 of adulthood. **C.** Pie chart of RNAi clone distribution within screen categories; 180 out of 227 (79.3%) screened RNAi clones were no primary hits, 11.5% aggravated the polyQ phenotype independent of *gfat-1* OE and were censored after counter screening, 2.6% of the remaining candidates were not confirmed in more extensive second screening round, 6.6% of screened RNAi clones remained as true hits. **D.** List of confirmed candidates from completed RNAi based suppressor screen to identify effectors of *gfat-1* OE induced PQC improvement (15 hits out of 227 screened genes), GFAT-1 interacting partners are marked by blue boxes.

2.7 Effects of *gfat-1* OE on protein translation

Protein synthesis is one of the most energy intensive processes in the cell and is, therefore, tightly controlled. Activation of the integrated stress response (ISR) is one method to stall global translation in response to various insults. Four widely conserved serine/threonine kinases are known in mammals to target the key protein of the ISR, the translation initiation factor eIF2. The kinases sense stress conditions, namely viral infection, heme depletion, amino acid deprivation or protein folding stress in the ER and, as a response, phosphorylate and thereby inactivate the α subunit of eIF2 (Berry et al., 1985, Chen and London, 1995, Harding et al., 1999, Hao et al., 2005). This, in turn, reduces global mRNA translation but simultaneously leads to the expression of the ATF4 (yeast GCN4, *C. elegans* ATF-5) transcription factor, which is usually repressed by translation initiation at upstream open reading frames (uORFs) (Mueller and Hinnebusch, 1986, Hershey, 1991, Hinnebusch, 1994, Vattem and Wek, 2004, Castilho et al., 2014). ATF4 downstream targets comprise genes involved in amino acid synthesis and transport, metabolism, the UPR^{ER} and induction of apoptosis, to name few processes. Thereby, ATF4 signaling assists in opposing the cellular stress that caused the attenuation of protein translation in the first place (Hai et al., 1989, Ron, 2002, Harding et al., 2003, Pakos-Zebrucka et al., 2016) (Figure 23A).

To gain first insights into whether protein synthesis might be affected by *gfat-1* OE we determined the amount of phosphorylated eIF2α by Western blot analysis. As negative control we included a strain that lacks the only two known eIF2α kinases in *C. elegans*, *gcn-2* (GCN2) and *pek-1* (PERK) (Baker et al., 2012). Indeed, no P-eIF2α could be detected in lysates of these worms. As positive control we induced protein folding stress in wildtype worms by exposure to tunicamycin. This intervention resulted in a roughly twofold increase in P-eIF2α level compared to the untreated controls. Interestingly, we noted an elevation of P-eIF2α of about 1.5 fold in the *gfat-1P::gfat-1* strain relative to Histone H3 compared to controls. A tendency for elevated P-eIF2α is additionally present in the muscular *gfat-1* OE strain with high levels for transgenic GFAT-1. *C. elegans* lines with comparably low GFAT-1 expression, *myo-3P::gfat-1* (1) and neuronal *gfat-1* OE worms, showed no measurable increase in P-eIF2α (Figure 23B and 23C).

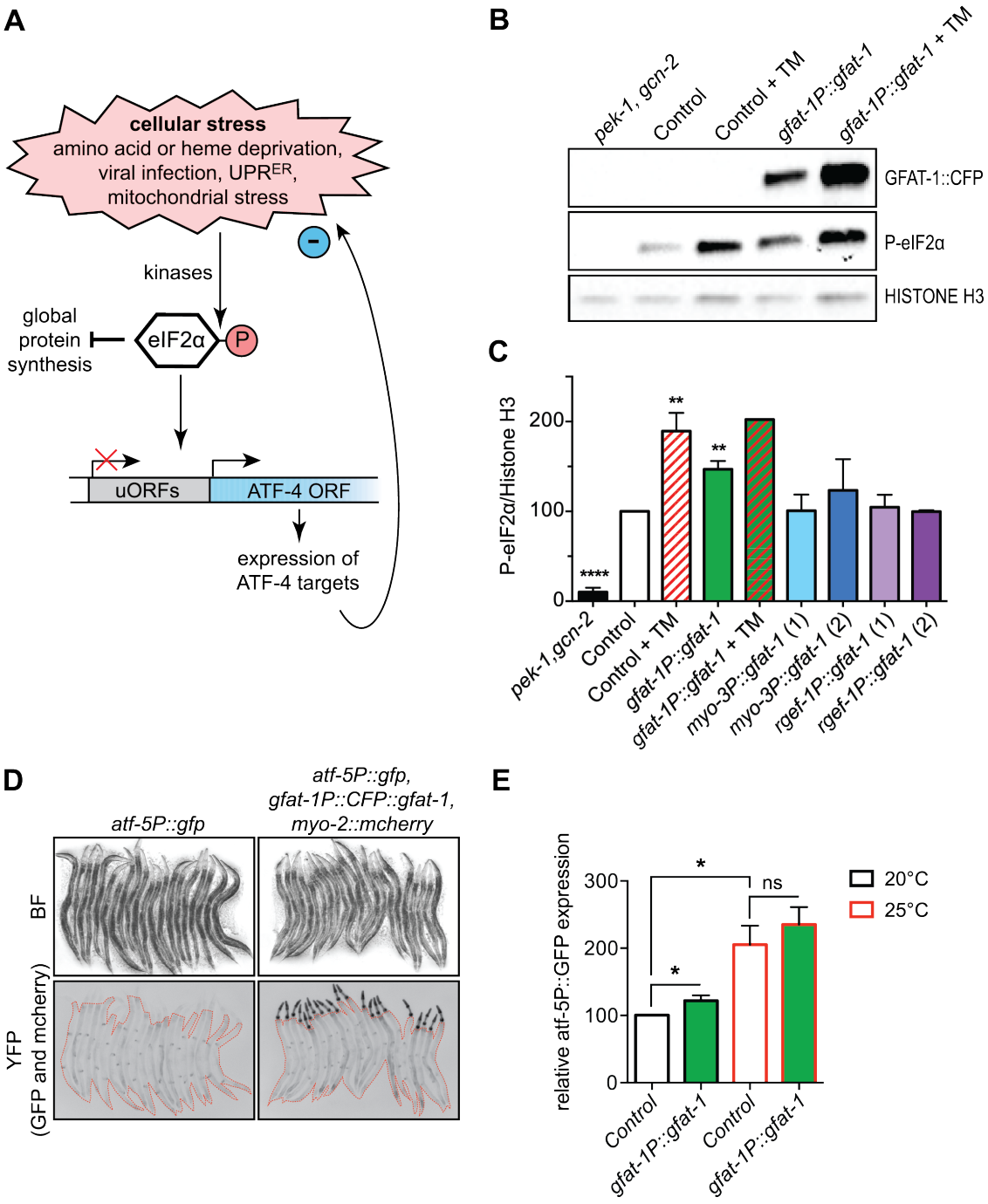


Figure 23. Mild induction of the ISR by *gfat-1* OE. A. Schematic view of the integrated stress response in mammals. Upon sensing distinct stress conditions committed kinases phosphorylate eIF2α, which causes stalling of global protein synthesis with concomitant induction of ATF4 transcription factor (ATF-5 in *C. elegans*) expression. ATF4 targets are upregulated to counteract cellular stress conditions or induce apoptosis. **B.** Representative Western blot of *gfat-1P::gfat-1* OE line at day 1 adult stage. TM, 4h Tunicamycin 10 µg/ml **C.** Quantification of Western blots (like in **B.**), N=3 (but N2+TM N=2 and *gfat-1P::gfat-1*+TM N=1), line and error bars indicate mean ± SEM, multiple t-tests, **, p<0.01, ****, p<0.0001. **D.** Representative images of *atf-5P::GFP* with and without additional *gfat-1* OE reporter strain at day 1 adult stage. Area marked by red, dashed line indicates quantified worm area, excluded is signal from pharyngeal co-injection marker *myo-2::mcherry*. **E.** Quantification of microscopy pictures of atf-5P::GFP reporter strain at day 1 adult stage (like in **D.**), worms grown at 20°C for entire experiment or shifted to 25°C at late L4 stage to cause mild heat stress, N=3, 10 worms per condition, line and error bars indicate mean ± SEM, multiple t-tests, * p<0.05.

Furthermore, we used a reporter line to assess the effect of GFAT-1 OE on *atf-5* expression downstream of P-eIF2α. We chose the *gfat-1P::gfat-1* strain for this experiment, as it shows the highest expression of transgenic GFAT-1 and P-eIF2α. Under growth conditions, we noticed a slight upregulation of GFP controlled by the *atf-5* promoter in *gfat-1* OE worms The increase in signal was mainly restricted to the hindgut of the animals (Figure 23D and 23E). To include a positive control in the assay, we shifted *C. elegans* to 25°C to provoke protein misfolding. This intervention doubled *atf-5* expression compared to unstressed controls. Under this mild heat stress, we still observed a tendency of elevated *atf-5* in the *gfat-1* OE strain (Figure 23E).

Taken together, we propose that OE of *gfat-1* might promote phosphorylation of eIF2α under standard conditions to decreased global protein synthesis and simultaneously increase ATF-5. This mild but sustained activation of the ISR might explain the improved proteostasis of *gfat-1* OE lines.

2.8 Requirement of *abcf-3* for *gfat-1* OE induced PQC improvement

After we obtained first evidence that *gfat-1* OE might act on protein translation through modulation of P-eIF2α, we, for further analysis, focused on one candidate from the RNAi suppressor screen, which had been implicated in translational regulation before. ABCF-3 (yeast GCN20) was described to work in a complex with GCN-1 to regulate phosphorylation of eIF2α (Vazquez de Aldana et al., 1995, Marton et al., 1997, Garcia-Barrio et al., 2000). This function of ABCF-3 fits in well with the observed modulation of P-eIF2α and ATF-5 expression in *gfat-1* OE mutants. Hence, we analyzed the role of ABCF-3 and its binding partner GCN-1, both of which we identified to be a physical interaction partners of GFAT-1 (Figure 19D), in *gfat-1* OE induced PQC improvement. To this end we generated lines with complete *abcf-3* or *gnc-1* deletions in the muscular *gfat-1* OE, polyQ background and studied protein aggregation and proteotoxicity.

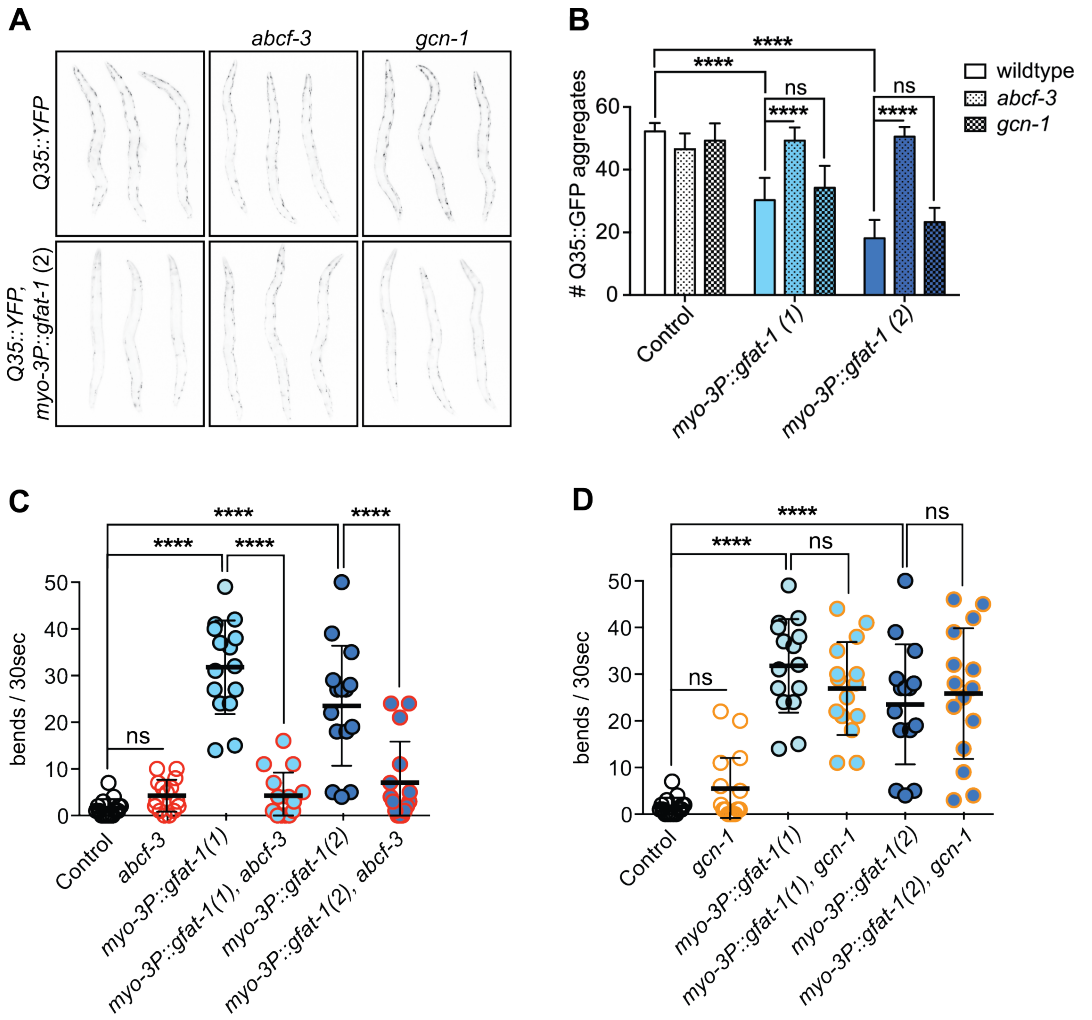


Figure 24. Requirement of *abcf-3*, but not *gcn-1* for *gfat-1* OE induced motility improvement. **A.** Representative images of polyQ aggregates in day 4 adults with and without muscular *gfat-1* OE and effects of *abcf-3* and *gcn-1* deletion. **B.** Quantification of Q35::YFP aggregates at day 4 adult stage in *unc-54P::Q35::YFP* background (as in **A.**), representative experiment of N≥3, 10 worms per condition, shown is mean +SD, statistical test 2way ANOVA, Tukey´s multiple comparisons test, ****, p<0.0001. **C.** Effect of muscular *gfat-1* OE and of *abcf-3* deletion on motility in *unc-54P::Q35::YFP* background at day 8 of adulthood as measured by swimming assay, 15 worms per condition, N≥2, line and error bars indicate mean ± SD of representative experiment, 1way ANOVA, Tukey´s multiple comparisons test, ****, p<0.0001. **D.** Effect of muscular *gfat-1* OE and of *gcn-1* deletion on motility in *unc-54P::Q35::YFP* background at day 8 of adulthood as measured by swimming assay, 15 worms per condition, N=2, line and error bars indicate mean ±SD of representative experiment, 1way ANOVA, Tukey´s multiple comparisons test, ****, p<0.0001, ns, not significant.

In the absence of *gfat-1* OE neither *abcf-3* nor *gcn-1* deletion aggravated the accumulation of polyQ aggregates in the nematodes´ body wall muscle (Figure 24A and 24B). As observed before, overexpression of *gfat-1* in muscle was beneficial and decreased the load of protein aggregates. The deletion of *abcf-3* in *gfat-1* OE

background led to a complete reversal of the *gfat-1* induced benefits and leveled the number of polyQ aggregates in the *gfat-1* OE strain and the control strain. Deletion of *gcn-1* on the other hand did not counteract the PQC benefits induced by *gfat-1* OE and the amount of polyQ aggregates remained low (Figure 24A und 24B). To confirm these results in an independent assay, we performed a quantitative motility assay in aged worms. The swimming capacity of polyQ worms was not altered by *abcf-3* deletion. However, the improvement in motility seen in muscular *gfat-1* OE was completely dependent on *abcf-3* and reverted to the state of paralysis typical of the polyQ controls upon *abcf-3* deletion (Figure 24C). Although GCN-1 and ABCF-3 were shown to work in a complex to facilitate eIF2α phosphorylation, GCN-1 does not seem to be essential for the improved PQC caused by *gfat-1* OE. In the polyQ as well as the polyQ, *gfat-1* OE worms the deletion of *gcn-1* had no effect on motility (Figure 24D).

To conclude, we generated evidence supporting the hypothesis of a novel mode of action for the HP enzyme GFAT-1: Together with its binding partner ABCF-3, GFAT-1 protein modulates the ISR independent of unfavorable environmental conditions and thereby counters proteotoxicity. The enzymatic activity of GFAT-1 is only partially required for its newly identified function.

3. DISCUSSION

3.1. Implication of GFAT-1 regulation in the ER stress response

The production of the HP end product UDP-GlcNAc and its isomer UDP-GalNAc is essential for cell survival. Given the interlinking of the HP with several other metabolic pathways and the high energy expense necessary to produce these aminosugars, it is conceivable that the HP is tightly regulated. This regulation focuses on GFAT-1 as it is the rate-limiting enzyme in UDP-GlcNAc biosynthesis. Increasing the abundance of *gfat-1* transcript and consequently GFAT-1 protein could be one mechanism to boost HP activity. And while this method is widely used to enhance the capacity of innumerable biological processes and can be especially useful to induce rather persistent changes, it is a comparatively slow adaption. While gene transcription, mRNA translation and protein folding will at least require several minutes, the activation of proteins by PTMs like phosphorylation is only a matter of seconds (Anfinsen, 1973, Milo and Phillips, 2015, Blazek et al., 2015). In any case, it was described that, in tissue culture and in a mouse model, *gfat-1* transcription is target of distinct signaling cascades and is elevated under specific conditions, such as disruption of the ER homeostasis or nutrient shortage (Wang et al., 2014, Chaveroux et al., 2016, Moloughney et al., 2016).

When we challenged *C. elegans* with stressful environmental conditions, we expected to recapitulate these results. Interestingly, we observed a surprising stability of *gfat-1* transcript levels between diverse treatments. Neither nutrient deprivation, nor glucose supplementation or ER stress altered the amount of *gfat-1* mRNA (Figure 8A and 8B). This lack of transcriptional induction of *gfat-1* could have diverse reasons. Although *gfat-1* structure and function are highly conserved between species, it is possible, that in *C. elegans* unlike in tissue culture and mice, *gfat-1* transcription is not increased in response to stress. Alternatively, *gfat-1* mRNA might be regulated differentially in distinct tissues or might be elevated only in select cells. In this case, it is very likely that changes would not be picked up by qRT-PCR on extracts of whole worms as the increased expression in certain cells could be diluted too strongly by unaffected cells or be canceled out by other cells, which reduce *gfat-1* expression. Also, it is conceivable, that merely raising the amount of GFAT-1 might not boost HP activity. We know that introducing the *gfat-1 gof*

mutations identified in *C. elegans* into murine cells, does not increase the amount of GFAT-1 protein, but still confers TM resistance (Moritz Horn and Martin Denzel, personal communication). This argues, that elevation of GFAT-1 level is not necessary to activate the HP and promote UDP-GlcNAc production. Furthermore, this observation indicates that wildtype GFAT-1 is constantly feedback inhibited because it fails to amass UDP-GlcNAc even under TM challenge although its physical protein amount would be sufficient for it.

In contrast to our qRT-PCR results, we found an induction of GFAT-1 protein under various stress conditions (Figure 8D). This difference could either originate from shortcomings in detecting according changes on mRNA level by qRT-PCR or reflect the real picture. Triggered by stress, GFAT-1 protein might be stabilized by PTMs, its translation enhanced or proteolytic mechanisms inhibited, leading to its protein accumulation while transcript levels would remain unaltered. Using a translational reporter line allowed us to study tissue distribution and expression pattern beyond changes in GFAT-1 protein amount. Interestingly, high temperature, oxidative stress and elevated glucose all triggered the formation of foci with high GFAT-1 expression in seam cells and pharynx (Figure 8C). It is not clear, whether these structures fulfill any functions or whether they are a byproduct of failing homeostasis mechanisms under conditions that were, judging by the small body size of the worms, severely challenging. At any rate, boosting HP flux by *gof* mutations in *gfat-1* did not influence *C. elegans´* sensitivity to heat and oxidative stress.

The observed changes following exposure to the ER stress inducing drugs TM and thapsigargin seem physiologically more relevant: The distribution of GFAT-1 remains smooth as in the controls kept under standard growth conditions but there was a remarkable induction of GFAT-1 in the intestine. As this tissue is usually one of the first ones to come in contact with environmental toxins and the expression of many detoxification genes is restricted to the intestine, it suggests a specific role for GFAT-1 in countering the effect of the administered drugs (McGhee, 2007). Indeed, we know that TM resistance can be induced by simple overexpression of *gfat-1* and no circumvention of feedback inhibition is required (Figure 13E). The compound TM is naturally produced by soil bacteria, mainly by *Streptomyces* strains, and the soil nematode *C. elegans*, which feeds on bacteria is not unlikely to come in contact with the toxin (Takatsuki et al., 1971). Hence, GFAT-1 induction in the intestine could be a physiological TM detoxification mechanism. Thapsigargin on the other hand is of plant origin and therefore less likely to pose a challenge in the natural environment of *C. elegans* (Thastrup et al., 1989). In this case, GFAT-1 induction is probably only a consequence of the disturbed ER homeostasis and does not constitute a remedy as

gfat-1 gof mutations failed to boost thapsigargin resistance (Martin Denzel, personal communication). We concluded that although GFAT-1 protein level and tissue distribution are responsive to diverse challenges and culture conditions, it is unlikely that GFAT-1 fulfills an important function in directly counteracting unfavorable environmental conditions in general beyond its clear requirement for detoxification of TM (Figure 10). However, an increase in GFAT-1 expression, albeit not per se protective, might still be relevant in assisting to maintain physiological processes like protein glycosylation and folding under stressful conditions. This is supported by the observation that UPR^{ER} activation in a single tissue measurably elevated the amount of UDP-GlcNAc in whole worm lysates (Figure 9C). Especially under stress, it is essential to reasonably allocate available resources and stimulation of the cost-intensive HP would be strongly contraindicated, if it was not relieving the situation in any way.

3.2. Unaltered Transcriptome and Proteome by *gfat-1 gof* mutations

The identification of concrete downstream effects of *gfat-1* gof mutations at the basis of improved PQC mechanisms proved to be very challenging. Analyzing transcriptome and proteome of the mutants did not reveal evident regulation of specific pathways and processes (Figure 11). Considering that genetic mutations or interventions that cause longevity typically show differential expression of tens to hundreds of genes, this finding was surprising (Murphy et al., 2003, Halaschek-Wiener et al., 2005, Ruzanov et al., 2007, Depuydt et al., 2014). As UDP-GlcNAc is essential for protein glycosylation, which can directly affect protein stability, significant changes in the abundance of at least glycoproteins in *gfat-1 gof* lines compared to wildtype controls would have been plausible (Vegarud and Christnsen, 1975, Wang et al., 1996, Kozarsky et al., 1988, Garner et al., 2001). We do, however, not know whether the modulation of accessible UDP-GlcNAc within a *C. elegans* cell alters the pool of glycosylated proteins or the glycosylation state of proteins. Thus, it is possible that UDP-GlcNAc is not the limiting resource for glycosylation reactions and increasing its amount does not entail changes in the glycoproteome. Alternatively, if UDP-GlcNAc availability does affect protein glycosylation, it might well influence protein properties other than stability, like other PTMs and phosphorylation state or association with interaction partners. These possible consequences of UDP-GlcNAc accumulation would have not been picked

up by proteome analysis. Unfortunately, our approaches to gain a comprehensive picture of the *C. elegans gfat-1 gof* glycoproteome did not succeed. To date, the global analysis of the exceptionally diverse classes of glycoproteins remains challenging (Xiao et al., 2018, You et al., 2018). In the *C. elegans* system, we were able to identify only a very limited number of glycoproteins (data not shown), which did not advance our understanding of molecular changes evoked by *gfat-1 gof* mutations.

3.3. Cell autonomous PQC improvement by muscle-specific GFAT-1 OE

As the attempt to generate candidates for downstream effectors of *gfat-1 gof* by transcriptomic, proteomic and glycoproteomic analyses was not successful we set out to dissect consequences of HP activation in a tissue-specific manner. The *C. elegans* strains we generated overexpressed *gfat-1* either under its endogenous promoter, a body wall muscle-specific, a pan neuronal or an intestine-specific promoter.

The OE of *gfat-1* in the worm gut was of interest, because of the observed local induction of GFAT-1 in this tissue after experiencing ER stress and its potential role in detoxification. Furthermore, one can hypothesize that the intestine as major metabolic tissue might be especially sensitive to boosting the HP. This, in fact, seemed to be true, despite not in regards to the benefits caused by *gfat-1 gof*. Even after repeated attempts no stable transgenic line with intestinal overexpression of *gfat-1* could be generated. The slow development and small body size as signs of poor health as well as the low plasmid transmission rate and very restricted and patchy transgene expression observed in the obtained worms argued for toxicity of elevated GFAT-1 in the worm intestine. Possibly, the transgenic elevation of GFAT-1 leads to detrimental metabolic imbalances or, as part of the ER stress response, it might signal the presence of environmental stress or unfolded proteins. This, in turn, could trigger a maladaptive stress response, which could be causal for the transgene´s deleterious effects (Roth et al., 2014).

We, beyond that, chose neurons as tissue for GFAT-1 OE for two main reasons. First, neurodegeneration is arguably the severest consequence of age-associated protein aggregation. Here, the broad PQC benefit of *gfat-1 gof* mutations could be of particular physiological relevance. Second, we were very interested in the question whether the activation of the HP boosts proteostasis directly and cell

autonomously or whether it worked cell non-autonomously by cross tissue signaling. Neurons are classically responsible to integrate stimuli and signals and based on this information induce responses in distal tissues or even raise systemic responses. Furthermore, it was already reported that neuron-specific activation of the IRE-1 branch of the UPR^{ER} by OE of XBP-1s can systemically improve ER stress resistance and prolongs life (Taylor and Dillin, 2013). GFAT-1 is a described UPR^{ER} target and *gfat-1 gof* mutations have comparable effects as neuronal XBP-1s OE. Hence, we wondered whether GFAT-1 would also play a special role in nervous tissue and whether a similar mechanism was responsible for the phenotype of the distinct transgenic lines. Interestingly, neuronal GFAT-1 OE could not confer systemic ER stress resistance and the worms remained sensitive to TM (Figure 13E), while at the same time it did promote autophagy if not significantly, due to the high variability of the assay (Figure 14C). It is difficult to sensibly monitor autophagy in *C. elegans* and from our experiments we cannot deduce if the autophagic flux was elevated exclusively in a cell autonomous manner or if it was additionally increased in distal tissues. However, because the globally assessed autophagic flux is more than 150% of controls, we believe neuronal GFAT-1 OE to act cell non-autonomously in promoting autophagy. It is quite surprising that the induction of autophagy lacked any positive effect on protein aggregation models in the worm gut, in muscle and even in neurons itself (Figure 15, 16B, 17B and 17C).

Finally, we were particularly interested in the effects of GFAT-1 OE in muscle tissue because recessive mutations in human GFAT-1 (GFPT1) cause congenital myasthenic syndrome, also called limb-girdle myasthenia with tubular aggregates. Major clinical manifestations of this disease include early onset limb muscle weakness, disruptions of neuromuscular junction transmission and abnormal muscle morphology (Senderek et al., 2011, Guergueltcheva et al. 2012, Selcen et al., 2013). Decreased GFAT-1 expression levels rather than a decline in enzymatic function seemed to be responsible for the severe muscular and neuro-muscular phenotype (Senderek et al., 2011, Willems et al., 2016). In one severely affected patient, which exhibited myopathy already in utero, an intronic mutation could be identified that disrupted the muscle-specific GFAT-1 isoform (Selcen et al., 2013).

These findings indicate an increased importance for GFAT-1 in nervous and especially muscle tissue and raised the questions: Is the OE of GFAT-1 protein in muscle particularly beneficial for *C. elegans* and does it have exclusively cell autonomous benefits or does it work across tissues to improve PQC? Like with neuronal GFAT-1 OE, we observed an increased autophagic flux in *C. elegans* with muscle-specific GFAT-1 OE. Again, the extent of induction is relatively large,

especially in the transgenic line with high GFAT-1 expression levels. Here, the autophagic flux is at about 200% relative to wildtype controls (Figure 13C, 14B and 14C). We would argue, therefore, that muscular GFAT-1 OE systemically boosts autophagy and that the size of the effect depends on GFAT-1 protein levels. What is more, we could measure a marginally elevated resistance to low doses of TM at high muscular GFAT-1 expression levels in one set of experiments (Figure 19D and 13E).

Another piece of evidence in support of cell non-autonomous consequences of muscular GFAT-1 OE was gained by studying intestinal and neuronal polyQ models. We were surprised to learn that GFAT-1 OE in muscle apparently disturbed the intestinal proteostasis network seeing that it potently accelerated polyQ aggregate formation in the gut (Figure 15A and 15B). Furthermore, muscle-specific GFAT-1 OE decreased the lifespan of worms expressing polyQ repeats under a neuronal promoter. All of these observations indicate a cross-tissue effect of muscular GFAT-1 OE, although not necessarily improving PQC as we had expected.

The strongest phenotype of muscular GFAT-1 OE was, however, cell autonomous and beneficial: in muscle-specific protein aggregation models, transgenic GFAT-1 expression strongly decreased the load of polyQ aggregates and sustained their solubility and, most importantly, maintained the worms´ motility into old age (Figure 16 and 17). This finding could be of physiological significance as there are numerous diseases of muscle tissue with concurring protein aggregation even if the most famous protein folding diseases like AD, PD and HD are all signified by accumulation of aggregated proteins in the central nervous system. Protein aggregate myopathies (PAMs), also including congenital myasthenic syndrome, reportedly share advanced age as the main risk factor but are, beyond that, a highly diverse group of hereditary and sporadic diseases. They show a large heterogeneity regarding onset of symptoms, clinical manifestations and species of aggregating proteins. Many characteristics of these diseases like their underlying causes are often still poorly understood, hence there is, in many cases, no effective therapy available (Sharma and Goebel, 2005, Goebel and Müller, 2006, Naddaf et al., 2018). Only recently, it was described that the age dependent accumulation of aggregated proteins in muscle is not only an accompanying symptom of disturbed processes leading to PAMs but is itself a contributor to declining muscle function and loss of muscle mass (Ayyadevara et al., 2016). It could be really interesting to model some of these diseases in *C. elegans* and assess whether muscular GFAT-1 OE can alleviate symptoms.

3.4 Dual Role of GFAT-1

It was the major focus of my work to elucidate the downstream effects of GFAT-1 OE and HP activation leading to improved proteostasis. As transcriptomic and proteomic analyses could not generate a set of candidates, we approached the question from a different angle and set out to identify physical interaction partners of GFAT-1. We speculated that GFAT-1 might fulfill another role within cells beyond producing UDP-GlcNAc as part of the HP and that it might do so by binding to other proteins. By forming connections with other proteins GFAT-1 might increase their stability, trigger conformational changes, which might be activating or deactivating, it could block or foster interactions with additional factors or it might influence subcellular distribution or concentration of its binding partners. The rationale behind the hypothesis of a dual role of GFAT-1 as enzyme in the HP and as protein in a complex was mainly based on two of our findings: First, some of the observed effects present in GFAT-1 OE lines were not recapitulated in *gfat-1 gof* lines. While *C. elegans* with GFAT-1 OE under its endogenous promoter for example showed mildly improved motility in the polyQ strain, *gfat-1 gof* mutants were completely paralyzed like controls (Figure 17C and data not shown). In my opinion, this can only be explained if a possibility to regulate GFAT-1 enzymatic activity is crucial for this phenotype or if GFAT-1 protein is improving PQC by an additional mechanism distinct from UDP-GlcNAc production. Second, muscular OE even of enzymatic dead GFAT-1K720R in polyQ background could moderately improve the worms´ motility in a swimming assay (Figure 19 E). This experiment clearly argued for a function of GFAT-1 protein in PQC independent of its enzymatic activity.

We presumed that the identification of GFAT-1 binding partners might help us to better understand this intriguing finding. Using co-IP of GFAT-1 and consecutive MS analysis we were able to describe 119 GFAT-1 interacting proteins with a FDR of 0.01 or lower (Figure 20D). Unfortunately, the group of physical GFAT-1 interactors we identified did not strongly cluster into specific biological processes and we could not gain conclusive evidence to which pathways might be regulated through interaction with GFAT-1. Thus, we considered all of these proteins potential downstream effectors of GFAT-1 OE. We further tested their role in muscle PQC besides many other genes implicated in proteostasis, longevity regulation and glycosylation reactions, in a selective RNAi suppressor screen. Indeed, knock down of several binding partners of GFAT-1 could revert the improved motility of muscle GFAT-1 OE to controls while simultaneously they did not aggravate the polyQ phenotype itself.

3.5 Cross-talk of GFAT-1 and the ISR

It was reported before that the HP can sense the metabolic status of cells and, as way of downstream signaling and adapting, alter the O-GlcNAcylation pattern of proteins accordingly (Lubas et al., 1997, Yang et al., 2002, Hanover et al., 2012, Ruan et al., 2013, Berrabah et al., 2014). One process, which is controlled by O-GlcNAcylation and thereby the HP and OGT, is protein synthesis. Jang and coworkers described that eIF2α has three O-GlcNAcylation sites. At high occupancy of these sites eIF2α phosphorylation can be impeded and thereby O-GlcNAc modification of eIF2α can support normal protein translation even under ER stress conditions (Jang et al., 2015). Another study determined the dynamic O-GlcNAc modification of translation initiation factor eIF4GI as an underlying mechanism for stress granule dissolution and selective translation of stress-related mRNAs upon heat shock (Zhang et al., 2018). The work of both research groups illustrates the regulatory role of UDP-GlcNAc availability and O-GlcNAcylation status in selective translation.

Here, we demonstrate for the first time an additional role for GFAT-1 protein levels in proteostasis. We propose cross-talk and control between the ISR and GFAT-1 to be responsible for the GFAT-1 OE induced PQC benefits, which were, at least partially, independent of steady-state UDP-GlcNAc availability (Figure 25).

With this work we could identify previously unknown physical interaction partners of GFAT-1 (Figure 20). Interestingly, the group of GFAT-1 binding partners was enriched for factors involved in protein synthesis, namely tRNA synthetases, ribosomal proteins and translation initiation and elongation factors. The list furthermore contained, two known modulators of GCN-2 activity (Vazquez de Aldana et al., 1995, Marton et al., 1997). The deletion of either ABCF-3 or GCN-1 did not aggravate polyQ toxicity, but ABCF-3 was absolutely essential for the improved motility and decreased aggregation caused by GFAT-1 OE in polyQ background (Figure 24). Our study showed that GFAT-1 OE mildly induced the phosphorylation of eIF2α and increase ATF-5 expression and this could plausibly be achieved through interaction with ABCF-3 (Figure 23 and 25). Previous studies demonstrated the physical binding of GCN-1 to GCN-2 to be absolutely critical for activation of GCN-2 by uncharged tRNA (Marton et al., 1993, Kubota et al., 2000). It was, however, also suggested that GCN-1 merely fulfills a scaffolding function causing the required proximity between GCN-2 and, for example, uncharged tRNA molecules at the translating ribosome (Castilho et al., 2014). Observations that support this notion showed that the requirement for GCN-1 in GCN-2 activation can be circumvented by

OE of tRNAs, generating a surplus of the activating agent, or by *gof* mutations in GCN-2, which abolish tRNA binding necessity (Garcia-Barrio et al., 2000, Qiu et al., 2002). As GCN-1 is not essential for GFAT-1 OE induced PQC benefits, we propose that the complex formed by GFAT-1 and ABCF-3 can by itself promote GCN-2 activation and eIF2α phosphorylation. This could be achieved by several mechanisms: First, it is possible that GFAT-1 can assume GCN-1 function in forming a hub for essential factors in GCN-2 activation. By binding to ribosomes, tRNA synthetases and the GCN-2 modulator ABCF-3 it could generate an environment that facilitates the induction of the ISR (Figure 20). Alternatively, GFAT-1 could act through stabilizing ABCF-3 protein. It was proposed that ABCF-3 is quickly degraded if it does not form a complex with GCN-1 (Vazquez de Aldana et al., 1995). GFAT-1 could, through its binding to ABCF-3, have a comparable effect and GFAT-1 OE might lead to stabilization of higher amounts of ABCF-3, supporting GCN-2 activation. Another possibility is a change in ABCF-3 ability to activate GCN-2 by increased GFAT-1 binding under GFAT-1 OE conditions. Of course, the ABCF-3/GFAT-1 complex could potentially also hinder eIF2α dephosphorylation by a distinct and unknown mechanism independent of GCN-2, ribosome and uncharged tRNAs, for example by promoting GADD34 function (Novoa et al., 2001).

The mild induction of the ISR resulting from GFAT-1 OE could explain the observed boost in PQC and ameliorated proteotoxicity. A slight decrease in protein synthesis can improve protein folding in the ER by a lower load of erroneously produced proteins and better accessibility to chaperones and the proteolytic pathway, which can counteract protein aggregation (Syntichaki et al., 2007, Hansen et al., 2007, Pan et al., 2007, Hipkiss, 2007, Tsaytler et al., 2011, Das et al., 2015, Schneider and Bertolotti, 2015). Additionally, activation of the ISR promotes the expression of ATF4 (ATF-5) and few other transcription factors and induces specific transcriptional programs that tune proteostasis mechanisms like autophagy (B'chir et al., 2013, Pakos-Zebrucka et al., 2016). Furthermore, ISR downstream changes in metabolism and amino acid uptake could be beneficial under conditions of protein aggregation (Harding et al., 2003, Seo et al., 2009, Wang et al., 2010a, Quirós et al., 2017). Besides entrapping components of the PQC machinery, non-degradable protein aggregates might also restrict critical resources like the pool of cycling amino acids (Cummings et al., 1998, Chai et al., 1999). A shift in metabolism and elevation of amino acid biosynthesis might help to overcome this restriction of resources trough protein aggregation. Beyond that, it was already reported that ATF4 fulfills a special role in modulating toxicity of protein aggregation diseases and tissue maintenance. While ATF4 expression slowed down accumulation of prion and polyQ

aggregates, it, at the same time, contributed to age related muscle atrophy (Hetz et al., 2007, Sayers et al., 2013, Hernández et al., 2017). It is established that the extent and duration of eIF2α phosphorylation, ISR activation and ATF4 induction are crucial for the downstream programs and the mild induction by GFAT-1 OE seems to have decidedly positive effects on PQC (Pakos-Zebrucka et al., 2016).

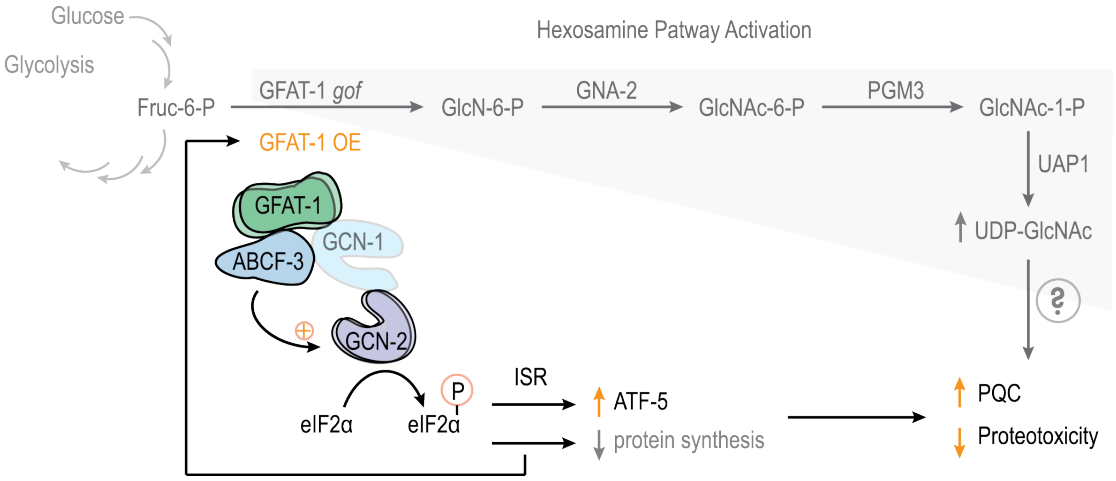


Figure 25. Model for the dual role of GFAT-1 in UDP-GlcNAc production and ISR modulation. GFAT-1 *gof* mutations potently increase UDP-GlcNAc production to activate PQC and decrease proteotoxicity via an unidentified mechanism. By contrast, OE of GFAT-1 does not elevate steady-state UDP-GlcNAc levels but still boosts PQC and reduces proteotoxicity by modulation of the ISR. GFAT-1 activates GCN-2 through its binding partners ABCF-3 and GCN-1. The resulting increase in P-eIF2α leads to a mild induction of ATF-5, responsible for the improved proteostasis. Activation of the ISR in turn induces GFAT-1 expression leading to cross-talk between the HP and the ISR via a positive feedback loop (Chaveroux et al., 2016). PGM3, Phosphoacetylglucosamine mutase; UAP1, UDP-N-acetylglucosamine pyrophosphorylase

Chaveroux and colleagues recently identified GFAT-1 as target of activated GCN-2 and thereby suggested for the first time a connection between GFAT-1 and the ISR (Chaveroux et al., 2016). We supported this finding by data establishing that GFAT-1 OE, in turn, can tune the ISR. We propose that physical interaction of GFAT-1 and its binding partner ABCF-3 is essential to connect the HP to the ISR and cause the GFAT-1 OE induced PQC improvement and decrease in proteotoxicity. Future work on the interconnection and cross-regulation of both pathways will be highly interesting and could be relevant in the context of protein aggregation disease

interventions or in the maintenance of healthy and functional skeletal muscle tissue during the aging process.

4. Future perspectives

My results presented in this thesis open up a number of important questions, which could initiate interesting follow-up studies. Some possible future directions are described in this final section.

4.1 Analyzing an implication of GFAT-1 in ER stress resistance

Our study showed that *gfat-1 gof* mutations do not confer a general stress resistance. A special role for GFAT-1 under stress conditions is, however, still likely as its expression is significantly upregulated upon stress. This effect was particularly pronounced under ER stress and we propose to further study the role for GFAT-1 in this context. We should also consider that GFAT-1 OE and *gfat-1 gof* might have distinct effects under ER stress conditions and therefore compare and contrast the different transgenic lines.

First, one should assess the resistance of GFAT-1 OE and *gfat-1 gof C. elegans* strains in diverse ER stress conditions, such as treatment with thapsigargin, dithiothreitol or RNAi against factors of the ERAD machinery. With these experiments we can elucidate whether GFAT-1 protein levels are critical in a challenging environment or whether its enzymatic activity and the amount of UDP-GlcNAc produced also play a major role.

Second, it could be quite informative to study the role of gfat-1 in modulating stress signaling pathways. To this end, one would analyze the induction of diverse stress-responsive pathways in GFAT-1 OE and *gfat-1 gof* strains in comparison to controls and *gfat-1* RNAi treated worms. Tunicamycin, to which GFAT-1 OE and *gfat-1 gof* strains are highly resistant, as well as the previously mentioned agents should be used to trigger ER stress. The induction of the different branches of the UPR and several compartment specific chaperones as well as accumulation of ERAD substrate within the ER lumen could be either measured by qRT-PCR or by using available reporter lines. Addressing the effectiveness of autophagic and proteasomal protein degradation would be much more challenging but the suggested experiments could generate first evidence of GFAT-1 being involved in coordination or tuning of distinct stress pathways.

4.2 Investigating the role of GFAT-1 in normal muscle aging

Muscular GFAT-1 OE was highly beneficial in a muscle-specific polyQ proteotoxicity model. Not only was the load of aggregates significantly decreased but also the worms´ motility could be maintained into high age when polyQ controls were completely paralyzed. But aggregating proteins are only one of the many causes for loss of muscle mass, strength and function. Especially sarcopenia is a very prevalent conditions and poses a public health problem as it is one of the major causes of disability in the elderly and a contributor to their morbidity (Baumgartner et al., 1998, Janssen et al., 2002, Lauretani et al., 2003, Candow and Chilibeck, 2005, Pérez-Zepeda et al., 2017). We were wondering whether the upregulation of GFAT-1 was generally improving muscle tissue maintenance beyond its role in counteracting proteotoxicity. If GFAT-1 OE would be effective against the age-associated loss of muscle integrity it could open up new possibilities of sarcopenia treatment and help preserve the quality of life in older people. Hence, it could be highly relevant to compare muscle function in *C. elegans* controls and muscle-specific GFAT-1 OE strains at different age. The rather recent development of several new tools and techniques allows to reliably monitor many parameters of muscle function, like force production, speed, range of motion and an activity index in nematodes besides roughly assessing motility and muscle fiber architecture (Restif et al., 2014, Beron et al., 2015, Rahman et al., 2018). We propose that these techniques could be adapted to develop a comprehensive picture of the role of GFAT-1 in muscle aging. If, indeed, GFAT-1 OE can delay the age-associated loss of muscle function, it would be interesting to test whether the same downstream effectors are required as in the GFAT-1 OE induced polyQ resistance.

4.3 Understanding the link between GFAT-1 and the ISR

Our data indicate that tuning of the ISR by physical interaction of GFAT-1 with ABCF-3 is required for the GFAT-1 OE induced reduction of proteotoxicity. Therefore, our study supports a previous report on a connection between the HP and activation of GCN-2 (Chaveroux et al., 2016). However, the crosstalk between the metabolic HP and the ISR remains poorly understood.

First, a pharmacological approach is possible to specifically address the need for increased P-eIF2α levels in transmitting GFAT-1 OE into boosting PQC. ISRIB is

a commercially available compound that was shown to decrease the phosphorylation state of eIF2α by activating its GEF eIF2B (Sidrauski et al., 2015b). Thereby, ISRIB prohibits the induction of the ISR with its downstream effects like the expression of ATF4 (ATF-5) and the set of its target genes (Sidrauski et al., 2013, Sidrauski et al., 2015a). It would be straightforward to test whether muscle-specific GFAT-1 OE can still ameliorate polyQ toxicity when the ISR is blocked by ISRIB supplementation. This experiment can confirm or refute our current hypothesis that GFAT-1 OE modulates the ISR to exert its positive effects on PQC.

Second, it is important to understand how the GFAT-1/ABCF-3 interaction modulates the ISR. To answer this question, one should determine the dependence of the PQC benefits by GFAT-1 OE on further factors of the ISR like GCN-2, PEK-1 and ATF-5 by RNAi knock down or genetic deletions. This could generate first evidence of the target of the GFAT-1/ABCF-3 complex.

Third, the generation of a *C. elegans* line with endogenously tagged ABCF-3 protein by CRISPR/Cas technology would allow us to detect changes in protein abundance or subcellular localization caused by GFAT-1 OE, which could be responsible for tweaking ISR signaling.

Finally, by performing polysome profiling, we could begin to understand whether GFAT-1 OE alters overall protein synthesis and specifically identify the subset of genes with differential active expression between GFAT-1 OE mutants and controls under standard conditions and under stress. It would even allow us to probe whether GFAT-1 is binding to translating ribosomes and whether this association is influenced by ABCF-3 (del Prete et al., 2007, Chassé et al., 2017, Pringle et al., 2018).

4.4 Further evaluation of candidates from motility screen

By performing a selective RNAi suppressor screen for the GFAT-1 OE induced motility benefits in polyQ background we generated a list of 15 potential downstream effectors of GFAT-1 OE. While the deletion of the GCN-2 modulator ABCF-3 was shown to completely revert polyQ aggregation and progressive paralysis to the levels observed in controls, the other candidates were, up to now, not studied in more detail. Interestingly, the arginyl tRNA-synthetase *rars-1* and the ribosomal protein lateral stalk subunit P1 *rla-2* are included in the candidate list as well. This particular ribosomal subunit is known to interact with translation initiation

and elongation factors to promote translation initiation (Ito et al., 2014, Murakami et al., 2018, Tanzawa et al., 2018). Furthermore, the ribosomal p stalk is in close proximity of the ribosomal tRNA acceptor site (A site), which is the proposed site of action of the GCN-1/ABCF-3/GNC-2 complex (Sattlegger and Hinnebusch, 2000, Visweswaraiah et al., 2012, Graifer and Karpova, 2015). It could be exciting to study the role of GFAT-1, which we identified as binding partner of ABCF-3, RLA-2 and RARS-1, in this complex of translating ribosome, tRNA-synthetases, GCN-2 and modulators and translation initiation and elongation factor. I can imagine that its presence in the complex could modify its composition and the affinity of different molecules. Again, this could be addressed by polysome profiling as suggested previously. First, however, it is important to study the dependence of the GFAT-1 downstream effects on these factors in more detail.

Of the remaining candidates, the proteasomal subunit *rpn-1*, the mitochondrial chaperone *hsp-60* and the lysosomal proton ATPase subunit *vha-1* and vacuolar protein sorting factor *vps-33.1* are the only ones that provide a direct link to proteostasis and could therefore be considered the most promising. Beyond that, it is compelling to analyze the relevance of the GlcNAc-transferring tumor suppressor EXT gene *rib-1*, involved in heparin sulfate proteoglycan synthesis, in GFAT-1 mediated PQC changes. It is possible that GFAT-1 OE might impact on ECM composition trough RIB-1 and might hereby influence cell-cell interactions, cellular transport and multiple signaling pathways (Sarrazin et al., 2011). The study of these processes might, however, be challenging in *C. elegans.*

5. MATERIALS & METHODS

5.1 Genetics and *C. elegans* handling

5.1.1 Maintenance and culture of *C. elegans* strains

As standard all *C. elegans* strains were grown at 20°C on nematode growth medium (NGM: 2.5% bacto-agar, 0.225% bacto-peptone, 0.3% NaCl (all w/v), 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KPO_4, 5 µg/ml cholesterol) seeded with the OP50 strain of *E. coli* bacteria unless mentioned otherwise (Brenner, 1974). The strains used in experiments were outcrossed at least 4 times to the N2 Bristol control strain, which simultaneously served as wild type reference strain.
Generally, worms were maintained by transferring L4 larvae to fresh culture plates twice a week.
In case of bacterial contaminations on culture plates, worms were collected in M9 buffer (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl (all w/v), 1 mM $MgSO_4$) and spun down at 2000 x g for 2 min. Bleach solution (0.5 M KOH, 20% (v/v) NaClO) was added to the worm pellet. After the worms were completely dissolved and only eggs remained, usually after 8 to 10 min, the reaction was stopped by adding M9 buffer. Then the eggs were spun at 2500 x g for 2 min down, the liquid removed and after at least two washing steps in M9 transferred to fresh culture plates.

5.1.2 Synchronization of worm population

For all experiments with exception of the GFAT-1 IP worms were synchronized by short egg-lays. For that purpose gravid adults were transferred to culture plates and completely removed again after a period of 4 hours at the most. The eggs were allowed to develop and the animals collected for experiments as indicated, usually at late L4 larval stage or day 1 adult stage.

5.1.3 List of *C. elegans* strains

N2 Bristol (wildtype),
AA4135 *dhIs941[gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR; myo-2::mCherry]*
AGD927 *uthIs270[rab-3p::xbp-1s (constitutively active); myo-2p::tdTomato] {Taylor:2013kw}*
AA3105 *gfat-1 gof(dh468) II*
AA3106 *gfat-1 gof(dh784) II*
AA3104 *gfat-1 gof(dh785) II*
CB1370 *daf-2(e1370) III*
KU25 *pmk-1(km25) IV*
AA3589 *pmk-1(km25) IV; gfat-1(dh468) II*
PRJ112 *mutEx70[pmk-1P::pmk-1::GFP; rol-6(su1006)]*
AA3882 *gfat-1 gof(dh468) II; mutEx70[pmk-1P::pmk-1::GFP; rol-6(su1006)]*
AA3741 *gfat-1 gof(dh784) II; mutEx70[pmk-1P::pmk-1::GFP; rol-6(su1006)]*
AA3861 *gfat-1 gof(dh785) II; mutEx70[pmk-1P::pmk-1::GFP; rol-6(su1006)]*,
AA4136 *dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (1)
AA4137 *dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (2)
AA3735 *dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (1)
AA3736 *dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (2)
AA4339 *dhEx1095[(ges-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]*
AA4406 *dhIs1095[(ges-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]*
DA2123 *adIs2122[(lgg-1::GFP; rol-6(su1006)]*
AA3131 *gfat-1 gof (dh785) II; adIs2122[(lgg-1::GFP; rol-6(su1006)]*
AA3951 *adIs2122[(lgg-1::GFP; rol-6(su1006)]; dhEx1041[pDC28; myo-2::mCherry (line E8)]*
AA3953 *adIs2122[(lgg-1::GFP; rol-6(su1006)]; dhEx1041[pDC28; myo-2::mCherry (line F8)]*
AA3942 *adIs2122[(lgg-1::GFP; rol-6(su1006)]; dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (1)
AA3943 *adIs2122[(lgg-1::GFP; rol-6(su1006)]; dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (2)

OG412 *drIs20 [vha-6p::Q44::YFP+rol-6(su1006)+pBluescript II]*

AA4290 *drIs20[vha-6p::Q44::YFP+rol-6(su1006) +pBluescript II]; dhIs941[(gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR); myo-2::mCherry]*

AA4288 *drIs20[vha-6p::Q44::YFP+rol-6(su1006)+pBluescript II]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (1)

AA4289 *drIs20[vha-6p::Q44::YFP+rol-6(su1006)+pBluescript II]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (2)

AA4324 *drIs20[vha-6p::Q44::YFP+rol-6(su1006)+pBluescript II]; dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry] (1)*

AA4325 *drIs20[vha-6p::Q44::YFP+rol-6(su1006)+pBluescript II]; dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry] (2)*

AM322 *N2; rmEx135(F25B3.3p::Q86::YFP)*

AA4473 *rmEx135(F25B3.3p::Q86::YFP); dhIs941[(gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR); myo-2::mCherry]*

AA4471 *rmEx135(F25B3.3p::Q86::YFP); dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (1)

AA4478 *rmEx135(F25B3.3p::Q86::YFP); dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (2)

AA4472 *rmEx135(F25B3.3p::Q86::YFP); dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (1)

AM141 *rmIs133[unc-54P::Q40::YFP]*

AA4192 *rmIs133[unc-54P::Q40::YFP]; dhIs941[(gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR); myo-2::mCherry]*

AA4190 *rmIs133[unc-54P::Q40::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (1)

AA4191 *rmIs133[unc-54P::Q40::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (2)

AA4223 *rmIs133[P(unc-54) Q40::YFP]; dhIs1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (1)

AM140 *rmIs132[unc-54P::Q35::YFP]*

AA4234 *rmIs132[unc-54P::Q35::YFP]; dhIs941[(gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR); myo-2::mCherry]*

AA4230 *rmIs132[unc-54P::Q35::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (1)

AA4233 *rmIs132[unc-54P::Q35::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (2)

AA4296 *rmIs132[unc-54P::Q35::YFP]; dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (1)
AA4297 *rmIs132[unc-54P::Q35::YFP]; dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (2)
AA3173 *N2; dhEx940[(gfat-1P::FLAG-HA::CFP::gfat-1 3'UTR); myo-2::mCherry]*
AA4200 *gcn-2(ok871) II; pek-1(ok275) X*
AA4423 *ldIs[atf-5P::GFP::unc-54 3'UTR]* (kindly provided by Dr. Keith Blackwell)
AA4474 *ldIs[atf-5P::GFP::unc-54 3'UTR]; dhIs941[(gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR); myo-2::mCherry]*
AA4479 *abcf-3(ok2237) III; rmIs132[unc-54P::Q35::YFP]*
AA4554 *abcf-3(ok2237) III; rmIs132[unc-54P::Q35::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (1)
AA4493 *abcf-3(ok2237) III; rmIs132[unc-54P::Q35::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (2)
AA4561 *gcn-1(n4827) III; rmIs132[unc-54P::Q35::YFP]*
AA4560 *gcn-1(n4827) III; rmIs132[unc-54P::Q35::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (1)
AA4559 *gcn-1(n4827) III; rmIs132[unc-54P::Q35::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (2)

All underlined strains were generated in this work by molecular cloning and microinjections, crossing or UV integration.

5.1.4 Genotyping by PCR and TagMan SNP analysis

In case genotyping was not possible by phenotypic assessment, Taq polymerase chain reaction (PCR) and agarose gel electrophoresis or SNP analysis by TaqMan were conducted. As template served the lysate obtained from incubating a single worm in 10 µl (for PCR) or 5 µl (for TaqMan SNP mapping) single worm lysis buffer (10mM Tris pH 8.3, 50mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% Triton X-100 (all v/v), 1mg/ml proteinase K (NEB)) for 60 min at 65°C and subsequently 15 min at 95°C.

PCR reaction composition	
H2O	9.49 µl
Taq Buffer	1.50 µl
50mM MgCl	0.45 µl
10mM dNTPs	0.15 µl
DMSO	0.35 µl
Taq Polymerase	0.06 µl
Primer FW	0.75 µl
Primer RV	0.75 µl
single worm lysate	1.50 µl

PCR program		
95°C	5 min	
95°C	45 sec	30 x
≈ 60°C	30 sec	
72°C	~ 60 sec/kb	
72°C	10 min	

Genotype	PCR primer	T_{anneal}	t_{elong}	PCR product
pmk-1 (km25)	FW GTTTCCACAGACAACAATGGATC RV CGTATCCAGTCATTTCAGAATCAG	65°C	45 sec	WT: 622bp mutant: -
abcf-3 (ok2237)	FW GTGGACGAGCTAACAGCTTC RV CCACGGAAGCTTGAAGATG RV2 CAACGGTAGTTGGAGTATCTC	58°C	35 sec	WT: 354bp +561bp mutant: 354bp

For TaqMan SNP mapping of *gcn-1(n4827)* specific primers were designed by and purchased from Applied Biosystems. The reaction was run according to manufacturer´s instructions on a ViiA 7 Real-Time PCR system with corresponding software. Each sample was run in technical duplicates and wildtype and mutant animals were included as controls in every reaction.

TaqMan reaction composition	
TaqMan Universal Master Mix II (2x), with UNG	5 µl
Primers Master Mix (40x)	0.25 µl
H_20	2 µl
single worm lysate	2.75 µl

5.1.5 Gene knockdown by RNAi

For RNAi-mediated knockdown of a specific gene worms were cultured from egg or L4 stage onward on *E. coli* HT115 (DE3) bacteria expressing dsRNA of the target gene under the control of an IPTG-inducible promoter (Timmons and Fire, 1998,

Kamath et al., 2001). NGM plates for RNAi experiments contained a final concentration of 100 µg/µl ampicillin and 1mM IPTG to select for vector carrying HT115 bacteria and induce dsRNA expression. All RNAi clones used in this study were obtained from either the Ahringer or Vidal library (Kamath and Ahringer, 2003, Rual et al., 2004). All clones, despite excluded candidates from the RNAi suppressor screen, were confirmed by plasmid purification (MiniPrep, Qiagen) and sequencing with the L4440 seq RV primer.

5.1.6 UV integration of extrachromosomal arrays

L4 larvae and young adults expressing a plasmid as extrachromosomal array were UV irradiated (0.03 Joule/cm2 on Viber Lourmat BLX-254 crosslinker) to achieve stable integration of the transgene in the genome. The at least 100 irradiated P0 worms were kept in the dark for at least 24h to prevent further light induced DNA damage. At least 4 F1 animals of every surviving P0 worm were singled out and the F2 and F3 generation were scanned for stable integrands, which were then outcrossed to controls at least 4 times.

5.1.7 Worm imaging

General handling of *C. elegans* was done on a Leica M80 stereomicroscope.
Worm stacks were arranged on unseeded NGM plates on ice and images were taken on a Leica M165FC fluorescence microscope using a Leica DFC 3000G camera and Leica Application Suite. The worm area was determined and the mean pixel intensity/worm area calculated using Photoshop (Microsoft office).
Images of single worms at higher magnification were obtained on a Carl Zeiss Axio Imager Z1 connected to a Zeiss Axiocam 506 mono camera using AxioVision software. Pictures were processed and fluorescence intensity was determined using Adobe Photoshop CS5.

5.1.8 PolyQ aggregate quantification

Quantification of polyQ aggregates in *C. elegans* intestine and muscle was done from images of aligned worms taken on a Leica M165FC fluorescence microscope (see 5.1.7) at magnification of about 8 fold. The worms were synchronized to a 4 hours time window and were grown until analysis at late L4 larval stage in *unc-54P::Q40* background, at day 4 adult stage in *unc-54P::Q35* background and in day 8 adult stage in *vha-6P::Q44* background. Images of 10 worms were taken for each biological replicate, the colors were inverted in Photoshop for easier aggregate distinction and aggregates were counted by eye. To avoid bias worms were selected and arranged for analysis on a Leica M80 light microscope and aggregate quantification was performed blinded.

5.1.9 Motility assay

To assess motility in a swimming assay worms were synchronized to a 4 hours time window and were cultured at standard conditions to day 5, day 8, day 10 or day 12 of adulthood as indicated in individual experiments. Worms that were able to move at least slightly after touch stimulus on culture plates were transferred to M9 buffer on unseeded NGM plates and were allowed to adjust to new conditions for about 30 sec. Afterwards full body bends within a 30 sec interval were recorded by counting. Except for the RNAi suppressor screen 15 worms per genotype and condition were analyzed for each biological replicate. To avoid bias all experiments were carried out blinded.

5.1.10 Selective RNAi screen for *gfat-1* mediators

Eggs of the muscular *gfat-1* OE strain AA4137 were collected in M9 after bleaching and roughly 50 eggs were transferred to NGM plates containing 40 µM 5-Fluoro-2′-deoxyuridine (FUDR, Sigma), seeded with HT115 bacteria expressing select RNAi clones. If animals showed a strong developmental delay or appeared sick, RNAi knockdown of the specific genes was only started at late L4 larval stage. At day 4 of adulthood worms were transferred to fresh culture plates and at day 12 motility was assessed on plates by comparing to *luciferase*, *gfat-1* and *gna-2* RNAi as well as the

polyQ strain without *gfat-1* OE as controls. Therefore worms were picked to a marked small circle on culture plate and their ability to leave the circle within 1 minute was scored. Worms that performed similar to *gfat-1* and *gna-2* RNAi or the polyQ strain on *luciferase* RNAi were evaluated in a motility assay as described in 5.1.9 with only 3 worms per test condition and 5 worms per control condition. RNAi clones causing motility within the range of error of *gfat-1* and *gna-2* RNAi controls or the polyQ strain on *luciferase* RNAi were defined as primary hits. The effect of these candidate RNAi clones was next measured in the polyQ background without *gfat-1* OE (AM140) as counter screen. The worms were grown to day 5 of adulthood and a motility assay performed as described above. If the motility on specific RNAi clones was below the range of error of *luciferase*, *gfat-1* and *gna-2* RNAi as controls the candidates were not considered secondary hits and excluded from further analysis. The remaining candidates were confirmed in another round of screening at day 10 and counter screening at day 5 and without FUDR in culture plates. The same criteria as previously described were applied to exclude candidates and determine true hits.

5.1.11 Stress assays

To analyze the effect of stress on *gfat-1* mRNA expression wildtype N2 Bristol worms were cultured to late L4 larval stage under standard condition. Subsequently they were exposed to stress conditions for 8h, harvested, RNA was extracted and mRNA levels compared. To check the effect of stress on GFAT-1 protein expression *gfat-1P::CFP::gfat-1* worms were, likewise, grown to late L4 larval stage under standard condition and images were taken after 14 h exposure to stress conditions.
C. elegans was shifted to 30°C to induce heat stress or transferred to culture plates containing 10 µg/ml Tunicamycin. Worms kept on standard NGM/OP50 plates served as reference.
Worms were exposed to 100 mM Paraquat, 5 µM Thapsigargin or 2% Glucose in liquid medium (100 mM NaCl, 0.1% K_2HPO_4, 0.6% KH_2PO_4 (all w/v), 5 µg/ml cholesterol) containing OP50 bacteria, or exposed to starvation in M9 buffer. As control for these conditions worms were grown in liquid medium containing OP50 bacteria. All nematodes kept in liquid medium were slowly shaken at 20°C.
Tunicamycin resistance of transgenic *C. elegans* strains was tested on NGM plates containing 0, 4, 6, 8 or 10 µg/ml Tunicamycin as described previously (Denzel et al.,

2014). At least 75 eggs per strain and concentration were transferred to Tunicamycin plates. All animals that developed to L4 larval stage or adulthood after 4 days were scored as resistant. Paraquat resistance was tested by transferring 60 late L4 *C. elegans* larvae to NGM plates containing 50mM Paraquat. The survival was scored after 24 h.

5.1.12 Lifespan analysis

Worms were synchronized by egglay and adult lifespan analysis was performed with 180 animals per genotype at 20°C on *E. coli* OP50. The young adult stage was defined as day 0 and survival was scored every day or every second day.
Worms with internal hatching or bursting vulva as well as worms that crawled off the agar were censored.

5.2 Molecular biology

5.2.1 Molecular cloning

Plasmids for tissue-specific overexpression of *gfat-1* were constructed using a GeneArt® Seamless PLUS Cloning and Assembly Kit (Invitrogen) according to manufacturer´s instructions. DNA fragments were generated by PCR with Phusion® High-Fidelity DNA Polymerase (NEB) with proofreading capacity. All promoter fragments for tissue-specific *gfat-1* expression were amplified from genomic DNA of wildtype *C. elegans*. The *gfat-1* ORF and 3UTR of 3,354 bp as well as the pDC6 vector were amplified from the *gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1-3´UTR* plasmid (Denzel et al., 2014). Promoter, ORF and vector fragments were fused in a single reaction using the following manufacturer´s instructions.
Assembled plasmids were transformed by 45 sec heat shock at 42°C into chemically competent *E. coli* DH5α (self made) and positive clones selected by Ampicillin resistance.

Plasmids were purified using Mini- or Midikits (Qiagen) following manufacturer's instructions and the correct assembly of fragments was confirmed for each plasmid by sequencing (eurofins Genomics).

Target		Sequence
myo-3P	FW	CAGGTCGACTCTAGATATGGTGGCCGATTTTGAGT
	RV	TACCGGATCCTCTAGATTAGATGGATCTAGTGGTCG
rgef-1P	FW	TCTAGAATCCCGTTTGGGACAAGAA
	RV	TACCGGATCCTCTAGACGTCGTCGTCGTCGATGCCG
ges-1P	FW	GCAGGTCGACTCTAGAAAGCTTAATGAAGTTTATTTC
	RV	TACCGGATCCTCTAGACTGAATTCAAAGATAAGATA
gfat-1 ORF	FW	ATGTGCGGAATTTTCGCCTA
	RV	TTACTCGACGGTAACTGACT
vector 1 (tags)	FW	CTAGAGGATCCGGTACCGGTA
	RV	GAAAATTCCGCACATACCGATCCCACCTCCGCCTTTG
vector 2 (backbone)	FW	GTTACCGTCGAGTAAAGCGTCCCGTCTTCTGCCCA
	RV	TCTAGAGTCGACCTGCAGGC

5.2.2 RNA extraction and quantitative RT-PCR

Populations of about 500 L4 larvae or day1 adult *C. elegans* were harvested in M9 and after 2 washing steps in cold M9 the worm pellet taken up in 700 µl QIAzol reagent (Qiagen) and snap frozen in liquid nitrogen. The samples were subjected to 6 freeze/thaw cycles (liquid nitrogen/37°C water bath) and homogenization with 1.0 mm Zirconia/Silica beads (FisherScientific) in a TissueLyser LT (Qiagen) for 15 min at full speed to crack the worms´ cuticle and release RNA. 120 µl Chloroform were added to 600 µl QIAzol solution, the components were mixed by vortexing and incubated for 2 min at RT. After 15 min centrifugation at 12000 x g and 4°C the aqueous phase was collected for total RNA extraction using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The RNA quantitiy and quality were determined on a NanoDrop 2000c (peqLab) and cDNA was prepared using the iScript cDNA Synthesis Kit (BioRad). Power SYBR Green Master Mix (Applied Biosystems) was used to quantify cDNA on a ViiA 7 Real-Time PCR system (Applied Biosystems). For each sample four technical replicates were pipetted on a 384-well plate by use of the JANUS automated workstation (PerkinElmer). The expression of target RNA was calculated from comparative CT values, normalized to *ama-1* as internal control, and compared to wildtype or untreated controls using the corresponding ViiA 7 software. All unpublished primers were validated by determination of their standard curves and melting properties.

Target		Sequence	published
ama-1	FW	GGATGGAATGTGGGTTGAGA	Denzel et al., 2014
	RV	CGGATTCTTGAATTTCGCGC	
cdc-42	FW	CTGCTGGACAGGAAGATTACG	
	RV	CTCGGACATTCTCGAATGAAG	
ctl-1	FW	AGGTGATGTTCCTCTACTCGG	
	RV	TTCTTGGAACCTTGAGCAGG	
ctl-2	FW	TGGTTAACAAGGACGGAAAGGC	
	RV	TCTTCACTCCTTGAGTTGGCTTG	
gfat-1	FW	GTCCAACCACGCTACTCATTTGC	Denzel et al., 2014
	RV	TGACAGGTCCAGAATGTTGTTTGG	
gna-2	FW	ACATTCACGAAGCGGGAACACG	
	RV	GTGACAACTTGTAGACGCCAATCG	
gst-4	FW	GCCAGCTTCCCATTTTACAAG	
	RV	TTGATCTACAATTGAATCAGCGTAAG	
sod-1	FW	AATCCGAGATCCGTCACGTAGG	
	RV	CGTCTTGTCCGGCATGAACAAC	
sod-2	FW	AGCTTGTTCAACCGATCACAGGA	
	RV	TGGTAAATCTGGCAGCGAGTGC	
sod-3	FW	CACGAGGCTGTTTCGAAAGG	Denzel et al., 2014
	RV	GAATTTCAGCGCTGGTTGGA	
xbp-1	FW	CCGATCCACCTCCATCAAC	
	RV	ACCGTCTGCTCCTTCCTCAATG	
xbp-1s	FW	TGCCTTTGAATCAGCAGTGG	
	RV	ACCGTCTGCTCCTTCCTCAATG	
C17H12.8	FW	TTTGAAAGAATGCTGAACAC	Ermolaeva et al., 2013
	RV	CAATCGACAGTGAAATTCTC	
K08D8.5	FW	CTTTTCATGGGATTGGATACTGCG	
	RV	GACTCTCACGTTTGCATTGTATGG	
T24B8.5	FW	CTGTAACGAAGCAGATGTTAGAAGTG	
	RV	GGGCATTGTTCAGCAATATTTTCATC	

5.3 Biochemistry

5.3.1 LC-MS/MS Analysis

Synchronized populations of about 3000 day 1 adult worms in at least 4 biological replicates per strain were used for analysis.

The worms were harvested in M9 and after 2 washing steps in cold M9 and 1 washing step in ddH_2O the pellets were snap frozen in liquid nitrogen. The samples

were subjected to 6 freeze/thaw cycles (liquid nitrogen/37°C water bath) and sonication pulses. The total protein content of the sample was measured using BCA Protein Assays (Thermo Fisher) and UDP-HexNAc extraction was achieved in Chloroform::Methanol (1:2) for 1h on a nutator. After spinning the samples 5 minutes at high speed in a tabletop centrifuge the same volume of supernatant of each sample was transferred to glass vials. The liquid was evaporated in a EZ-2 Plus Genevac (SP Scientific) with following settings: time to final stage 15 min, final stage 4 h, low BP mixture.
The determination of absolute amounts of UDP-HexNAc were carried out by Yvonne Hinze and Jeanette Hütges from the Proteomics Core facility as described previously (Denzel et al., 2014).

5.3.2 Western blot analysis

For Western blot analysis synchronized young adult or gravid day 1 adult worms were collected in M9, snap frozen in liquid nitrogen and lysed in 4x LDS sample buffer (Thermo Fisher) containing 50 mM DTT. After a boiling and a sonication step, equal volumes were subjected to reducing SDS-PAGE and transferred to nitrocellulose membranes. Following antibody staining of specific proteins, the bands were detected on a ChemiDoc MP Imaging System (BioRad) and the intensity quantified using the corresponding Image Lab software (BioRad).

5.3.3 Immunoprecipitation

A mixed population of *gfat-1P::Flag::HA::CFP::gfat-1* and *gfat-1P::Flag::HA::CFP* worms was collected and washed in M9 buffer and lysed on ice in IP Lysis buffer (10 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, cOmplete Mini Protease and phosphatase Inhibitor Cocktail (Roche)) using a dounce homogenizer.
The total protein content of the lysates was measured using BCA Protein Assays (Thermo Fisher). 50 µl Dynabeads Protein G (Thermo Fisher) were coated with 5 µg anti-FLAG M2 antibody (Sigma) following manufacturer´s instructions and incubated with 1 mg worm lysate at 4°C for 1 h. After three washing steps in IP Lysis buffer and two washing steps in IP Wash Buffer (as IP Lysis buffer but without NP-40) the precipitating proteins were either eluted with Flag peptide in 50mM Tris/HCl ph 7.4,

120 mM NaCl for analysis by Western Blot or Silver gel or directly digested from the beads by 30 min and over night incubation with 1.5 µg Trypsin Gold (Promega) in 20 mM Tris pH 8.3 for mass spectrometric analysis.
Non-selective visualization of all precipitating was achieved using a Pierce Silver Stain Kit (Thermo Fisher) according to manufacturer´s instructions.

5.3.4 Fractionation of SDS soluble and SDS insoluble polyQ peptides

Synchronized day 1 adult *C. elegans* were harvested and washed in M9 and lysed in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% SDS, 0.5% SDO, 1% NP40, cOmplete Mini Protease and phosphatase Inhibitor Cocktail (Roche)) by freeze/thaw cycles and sonication. The insoluble material was pelleted by 20 min centrifugation at 4°C and 16,000 x g. The supernatant was collected as soluble fraction and the pellet was washed in RIPA buffer and after centrifugation solubilized in urea/SDS buffer (8 M urea, 50 mM Tris pH 8, 2% SDS, 50 mM DTT). Protein fractions were then mixed with 4x LDS sample buffer (Thermo Fisher) and separated and visualized by SDS PAGE and immunoblotting.
To detect total amounts of SDS soluble and insoluble polyQ fragments worms were directly collected in urea/SDS buffer, snap frozen in liquid nitrogen and proteins separated and detected by SDS PAGE and immunoblotting.

5.3.5 SILAC labeling of *C. elegans*

Stable isotope labeling with amino acids in *C. elegans* was carried out as described previously by Larance and colleagues (Larance et al., 2011) with the following adaptions:
The agarose content of NGM-N plates was increased from 12 g/l to 13 g/l and plates were prepared without Nystatin, only heavy isotope $^{15}N_2/^{13}C_6$-lysine was used for labeling, it was abstained from using *orn-1* RNAi and total protein samples were digested with rLys-C (Promega) instead of trypsin, *E. coli* SLE1 bacteria were concentrated 10 fold instead of 50 fold, the *C. elegans* population was synchronized prior to analysis by 4h egglay instead of filtering, the proteome of the fourth *C. elegans* generation grown on SLE1 bacteria was analyzed.

5.3.6 Proteomic Analysis (by Xinping Li and Ilian Atanassov, MPI core facility)

C. elegans samples for proteomic analysis were boiled in lysis buffer (100 mM Tris, 6 M Guanidinium chloride, 10 mM Tris(2-carboxyethyl)phosphine hydrochloride, 40 mM 2-Chloroacetamide) for 10 min, lysed at high performance in 10 cycles of 30 s soncation intervals in a Bioruptor Plus sonication device (diagenode) and boiled again. The samples were spun at 20000 x g for 20 min to get rid of debris.
The samples were diluted 1:10 in 20 mM Tris pH 8.3 / 10% acetonitrile (ACN) and after the protein concentration was measured using BCA Protein Assays (Thermo Fisher) the samples were digested over night with according amounts of Trypsin Gold (Promega) or rLys-C (Promega) in case of SILAC experiments.
The peptides were cleaned on a Supelco Visiprep SPE Vacuum Manifold (Sigma) using OASIS HLB Extraction cartridges (Waters). The columns were conditioned twice with Methanol, equilibrated twice with 0.1% formic acid, loaded with the sample, washed three times with 0.1% formic acid and the peptides eluted with 60% ACN / 0.1% formic acid. Then, the samples were dried at 30°C for roughly 4 h in a Concentrator plus speedvac (Eppendorf) set for volatile aqueous substances. The dried peptides were taken up in an adequate volume of 0.1% formic acid (usually in 20 µl) and the samples were analyzed by the Max Planck Proteomic Core facility for analysis according to the following protocol:
Peptides were separated on a 25 cm, 75 µm internal diameter PicoFrit analytical column (New Objective) packed with 1.9 µm ReproSil-Pur 120 C18-AQ media (Dr. Maisch) using an EASY-nLC 1200 (Thermo Fisher Scientific). The column was maintained at 50°C. Buffer A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Peptides were separated on a segmented gradient from 2% to 5% buffer B for 10 min, from 5% to 20% buffer B for 180 min, from 20% to 25% buffer B for 20 min and from 25% to 40% buffer B for 10 min. Alternatively, peptides were separated from 2% to 5% buffer B for 10 min, from 5% to 20% buffer B for 180 min, from 5% to 20% buffer B for 90 min and from 20% to 40% buffer B for 45 min at 200 nl/min. Eluting peptides were analyzed on a QExactive Plus or QExactive HF mass spectrometer (Thermo Fisher Scientific). Peptide precursor m/z measurements were carried out at 70000 (QExactive Plus) or 120000 (QExactive HF) resolution in the 300 to 1800 m/z range. Only the top ten most intense precursors with charge state from 2 to 7 were selected for HCD fragmentation using 27% normalized collision energy. The m/z values of the peptide fragments were measured at a resolution of 17500 (QExactive Plus) or 15000 (QExactive HF) using

a minimum AGC target of 2e5 and 80 ms maximum injection time. Upon fragmentation, precursors were put on a dynamic exclusion list for 20 or 45 sec.
To identify and quantify proteins the raw data were analyzed with MaxQuant version 1.6.1.0 (Cox and Mann, 2008) using the integrated Andromeda search engine (Cox et al., 2011). Peptide fragmentation spectra were searched against the canonical sequences of the *C. elegans* reference proteome (proteome ID UP000001940, downloaded September 2018 from UniProt). Methionine oxidation and protein N-terminal acetylation were set as variable modifications; cysteine carbamidomethylation was set as fixed modification. The digestion parameters were set to “specific” and “LysC/P”. The minimum number of peptides and razor peptides for protein identification was 1; the minimum number of unique peptides was 0. Protein identification was performed at a peptide spectrum matches and protein false discovery rate of 0.01. The “second peptide” option was “on”. Successful identifications were transferred between the different raw files using the “Match between runs” option. SILAC quantification was performed using a minimum ratio count of two. The results from the protein quantification (ProteinGroups.txt) were filtered against Possible contaminants, “Reverse” and “Only” identified by site. The SILAC ratios were log2 transformed and tested for differential expression using limma (Ritchie et al., 2015).

5.3.7 Antibodies

The following primary antibodies were used in this study: Phospho-p38 MAPK (#3597 Cell Signaling, rabbit monoclonal), GFP (JL-8 Living Colors, mouse monoclonal and #2956 Cell Signaling, rabbit monoclonal), β-Actin (ab8224 abcam, mouse monoclonal), Histone H3 (ab1791 abcam, rabbit polyclonal), α-Tubulin (T6199 Sigma, mouse monoclonal), Phospho-eIF2α (#3597 Cell Signaling, rabbit monoclonal),
The following horseradish peroxidase conjugated antibodies were used in this study: anti-mouse IgG (G-21040 Invitrogen, goat polyclonal), anti-rabbit IgG (G-21234 Invitrogen, goat polyclonal)

5.4 Statistical analysis

Results are presented as mean + SD or SEM or mean ± SD or SEM as indicated. Statistical analyses were performed using 1way ANOVA or 2way ANOVA with Tukey´s, Sidak´s or Dunnett´s multiple comparisons test or unpaired t-test. The program used was GraphPad Prism (GraphPad software). Significance levels are *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ and ****, $p<0.0001$ versus respective control.

5.5 Software

Most graphs were produced and statistical analyses performed using GraphPad PRISM 6.
GFAT-1 sequence alignments were generated using Clustal Omega and ESPript 3.0 (Sievers et al., 2011), (Robert and Gouet, 2014). A section of the molecular GFAT-1 protein structure was depicted with PyMOL using pdb code 4AMV.
Data from SILAC and GFAT-1 IP experiments was graphically displayed using Instant Clue (Nolte et al., 2018).
Bands on Western blots were quantified using Image Lab Software (Bio-Rad Laboratories GmbH).

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7. Appendix

Contributions

I performed all experiments described in this work independently, except for:

RNA sequencing on *gfat-1 gof (dh468) C. elegans* strain and controls (Figure 11A) – done by Dr. Martin Denzel (former Antebi lab member)

MS analysis of SILAC samples of *gfat-1 gof C. elegans* strain and controls (Figure 11B) and of eluate after GFAT-1 and control co-IP (Figure 20C and 20D) – done in collaboration with Dr. Ilian Atanassov and Dr. Xinping Li (Proteomic core facility, Max Planck Institute for Ageing Research)

LC-MS analysis of UDP-HexNAc content in *C. elegans* samples (Figure 13D and 18F) - done in collaboration with Yvonne Hinze and Jeanette Hütges (Metabolomic core facility, Max Planck Institute for Ageing Research)

Site directed mutagenesis to generate expression vectors for GFAT-1K720R, generation of associated *C elegans* strains and conduction of related experiments (Figure 19C, 19D and 19E) – done by Master student Vignesh Karthikaisamy under my supervision

Thanks to all these people for their important contributions.

Summplementary Material

List of all genes tested in the selective RNAi suppressor screen for motility improvement

B0218.3	*pmk-1*	hits from RNAseq
C29F7.5	*fkh-4*	
C55C3.3		
C55C3.6		
C55C3.7		
F42G8.3	*pmk-2*	
F42G8.4	*pmk-3*	
K10G6.1	*lin-31*	
T10D4.6a		
T28F2.1		
T28F2.5		
C34B2.8	*NDUFA13*	regulated in first SILAC experiment
F22D6.4	*nduf-6*	
F45H10.3	*NDUFA7*	
F53F4.10	*NDUFV2*	
F59A2.3	*cri-3*	
F59C6.5	*NDUFB10*	
W01A8.4	*nuo-6*	
Y54E10BL.5	*nduf-5*	
B0024.12	*gna-1*	known physical interactors of GFAT-1 and interactors identified by IP and MS
B0303.3		
B0336.10	*rpl-23*	
B0432.4	*misc-1*	
C02C6.1	*dyn-1*	
C04F6.1	*vit-5*	
C05E4.9	*icl-1*	
C05G5.4		
C07H6.5	*cgh-1*	
C08H9.2	*vgln-1*	
C13B9.3		
C23G10.3	*rps-3*	
C23G10.4	*rpn-2*	
C26F1.4	*rps-30*	
C27D11.1	*egl-45*	
C28D4.3	*gln-6*	
C28H8.3		
C30C11.2	*rpn-3*	
C36E8.5	*tbb-2*	
C42D8.2	*vit-2*	
C53A5.1	*ril-1*	
D2023.2	*pyc-1*	
D2085.1	*pyr-1*	
F07A11.3	*npp-5*	
F10G7.2	*tsn-1*	
F14B4.3	*rpoa-2*	
F17C11.9	*eef-1G*	
F18H3.3	*pab-2*	
F20D12.1	*csr-1*	
F21D5.1		
F22B3.4	*gfat-2*	
F26D10.10	*gln-5*	
F26D10.3	*hsp-1*	
F26E4.8	*tba-1*	
F26F4.10	*rars-1*	
F28C6.3	*cpf-1*	
F32B6.2	*mccc-1*	
F32H2.5	*fasn-1*	
F37C12.9	*rps-14*	
F39B2.10	*dnj-12*	
F40A3.6		
F41E7.5	*fipr-21*	
F42A10.1	*abcf-3*	
F43D9.4	*sip-1*	
F44E5.4		
F45E4.2	*plp-1*	
F46F11.2	*cey-2*	
F47B10.1	*suca-1*	
F49H12.5		
F53A2.7	*acaa-2*	
F54B3.3	*atad-3*	
F55A8.2	*egl-4*	
F55H2.6	*clu-1*	
F56D12.5	*vig-1*	
F56E10.4	*rps-27*	
F56H1.4	*rpt-5*	
F57F10.1	*abts-3*	
F58B3.5	*mrs-1*	
H43I07.2	*rpac-40*	
K01C8.1		
K01C8.9	*nst-1*	
K02F3.2		
K07C5.4		
K09F5.2	*vit-1*	
K11E8.1	*unc-43*	
K11H3.3		
M01E11.5	*cey-3*	

R02D3.1		known physical interactors of GFAT-1 and interactors identified by IP and MS
R03D7.1	*metr-1*	
R09A1.1	*ergo-1*	
R11A5.4	*pck-2*	
R11A8.6	*irs-1*	
T01C3.7	*fib-1*	
T02G5.9	*kars-1*	
T04A8.5		
T05F1.1	*nra-2*	
T05G5.3	*cdk-1*	
T07A9.9		
T07D3.9		
T08B2.7	*ech-1.2*	
T10B10.2	*ucr-2.2*	
T20H4.3	*pars-1*	
T21E12.4	*dhc-1*	
T21G5.3	*glh-1*	
T22D1.9	*rpn-1*	
T24C4.1	*ucr-2.3*	
T27E4.3	*hsp-16.48*	
T27E4.8	*hsp-16.1*	
T28D6.2	*tba-7*	
W01B11.3	*nol-5*	
W06H3.1	*immt-2*	
W07E11.1		
W08E3.3	*ola-1*	
W09B6.1	*pod-2*	
W09C5.6	*rpl-31*	
Y106G6H.2	*pab-1*	
Y18D10A.17	*car-1*	
Y41E3.10	*eef-1B.2*	
Y41E3.4	*qars-1*	
Y43F4B.5		
Y46G5A.31	*gsy-1*	
Y46H3A.2	*hsp-16.41*	
Y48G9A.3	*gcn-1*	
Y54E10A.9	*vbh-1*	
Y54E2A.11	*eif-3.B*	
Y55F3AM.8	*immp-2*	
Y59A8A.3	*tcc-1*	
Y62E10A.1	*rla-2*	
Y66H1A.2	*dpm-1*	
Y66H1B.2	*fln-1*	
Y71F9AL.17	*copa-1*	
Y74C10AR.1	*eif-3.I*	known physical interactors of GFAT-1 and interactors identified by IP and MS
Y87G2A.5	*glp-4*	
Y87G2A.8	*gpi-1*	
ZC434.5	*ears-1*	
ZK381.4	*pgl-1*	
ZK593.5	*dnc-1*	
ZK669.4		
ZK829.4	*gdh-1*	
ZK973.6	*anc-1*	
B0025.1	*vps-34*	autophagy
B0261.2	*let-363*	
B0336.8	*lgg-3*	
C32D5.9	*lgg-1*	
M7.5	*atg-7*	
T19E7.3	*bec-1*	
T22H9.2	*atg-9*	
ZK593.6	*lgg-2*	
B0205.3	*rpn-10*	proteasome
C47E12.5	*uba-1*	
F23F1.8	*rpt-4*	
F25B5.4	*ubq-1*	
F57B9.10	*rpn-6.1*	
T06D8.8	*rpn-9*	
T19E7.2	*skn-1*	
Y110A7A.14	*pas-3*	
Y38A8.2	*pbs-3*	
C15H9.6	*hsp-3*	ER PQC, UPR and chaperones
C41C4.4	*ire-1*	
F43E2.8	*hsp-4*	
F45E6.2	*atf-6*	
F46C3.1	*pek-1*	
F54C9.2	*stc-1*	
R74.3	*xbp-1*	
T05E11.3	*enpl-1*	
Y38A10A.5	*crt-1*	
Y53C10A.12	*hsf-1*	
ZK632.6	*cnx-1*	

C30H6.6	*haf-1*	MT PQC, UPR and chaperones
C37H5.8	*hsp-6*	
F22B7.5	*dnj-10*	
F46F11.4	*ubl-5*	
Y22D7AL.5	*hsp-60*	
ZC376.7	*atfs-1*	
ZK1193.5	*dve-1*	
C47E12.3	*edem-1*	ERAD
F19B6.2	*ufd-1*	
F45D3.5	*sel-1*	
F55A11.3	*sel-11*	
C09B8.6	*hsp-25*	chaperones
C12C8.1	*hsp-70*	
F26D10.3	*hsp-1*	
F38A5.13	*dnj-11*	
T05C12.7	*cct-1*	
T27E4.3	*hsp-16.48*	
Y46H3A.3	*hsp-16.2*	
B0303.9	*vps-33.1*	lysosomes
C05D11.2	*vps-16*	
F20B6.2	*vha-12*	
F32A6.3	*vps-41*	
F41C3.5		
M04G12.2	*cpz-2*	
R10E11.8	*vha-1*	
R13A5.1	*cup-5*	
W03C9.3	*rab-7*	

B0205.4		glycosylation
B0285.5	*hse-5*	
B0464.4	*bre-3*	
C03E10.4	*gly-20*	
C08B11.8		
C08H9.3		
F12F6.3	*rib-1*	
F13H10.4	*mogs-1*	
F14B6.6		
F21D5.1		
F26H9.8	*uggt-2*	
F48C1.1	*aman-3*	
F48E3.3	*uggt-1*	
F56H6.5	*gmd-2*	
K04G7.3	*ogt-1*	
K08F8.3	*fut-1*	
K09E4.2		
T03G11.4	*mans-3*	
T09A5.11	*ostb-1*	
T12A2.2	*stt-3*	
T15D6.2	*gly-16*	
T20B5.3	*oga-1*	
T24D1.4	*tag-179*	
T26A5.4	*algn-1*	
Y110A2AL.14	*sqv-2*	
Y60A3A.14	*algn-7*	
Y66A7A.6	*gly-8*	
C02C2.3	*cup-4*	others
F38A6.1	*pha-4*	
K08F4.7	*gst-4*	
K11D9.2	*sca-1*	
R13H8.1	*daf-16*	
F15H9.4	*sri-16*	

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt.

Die von mir vorgelegte Dissertation ist von Prof. Dr. Adam Antebi betreut worden.

Köln, März 2019
Sarah Denzel