Signaling Pathways that Regulate Autophagic Muscle Protein Degradation in C. *elegans*

by

Chris Bialas

Bachelor of Philosophy, University of Pittsburgh, 2010

Submitted to the Graduate Faculty of

the University of Pittsburgh in partial fulfillment

of the requirements for the degree of

Bachelor of Philosophy

University of Pittsburgh

2010

University of Pittsburgh

College of Arts and Sciences

This thesis was presented

by

Chris Bialas

It was defended on

April 10th 2010

And approved by:

John Boyle, PhD, Assistant Professor, Dept. Biological Sciences

Kirill Kiselyov, PhD, Assistant Professor, Dept. Biological Sciences

Lew Jacobson, PhD,Professor, Dept. Biological Sciences

Chris Link, Professor, PhD University of Colorado Boulder

Signaling Pathways that Regulate Autophagic Muscle Protein Degradation in C. *elegans*

Chris Bialas, B.S.

University of Pittsburgh, 2010

Protein degradation in *C. elegans* muscle cells is very tightly controlled by opposing signaling cascades. Excessive FGFR, low IGFR or TGF-B signaling, starvation or denervation all lead to muscle protein breakdown. Here we show that under conditions of activated FGFR, low IGFR or low TGF-B activity or mutationally hyperactived MAP kinase, soluble protein in muscle cytosol is degraded through autophagy. Degradation under these conditions is prevented by a reduction of function mutation in *unc-51* (encodes Atg1 homologue), by RNAi knockdown of BEC-1 (beclin), or ATG-7 (Atg7 homologue) or by treatment with N6,N6-dimethyladenosine, a presumed inhibitor of type III PtdIns-3-kinase. We infer that the MAP kinase cascade positively regulates autophagy when the FGFR signal is too high or the IGFR signal too low . However, we have yet to identify how a MAPK signal plays into TGF-B regulation of autophagy. Protein degradation following starvation or disruption of cholinergic signaling (ACh-deficient *cha-1* mutant) is not prevented by interference with autophagy, consistent with the known sensitivity to proteasome inhibitors. We have also implicated the signaling protein RSK as necessary for autophagic signaling either in parallel with or downstream of MAPK The metabolic reserve of protein in muscle can be mobilized by catabolism in response to failure of any one of a variety of signals of 'healthy' conditions. To achieve versatility and flexibility of response, muscle cells evidently use multiple proteolytic systems and regulate them independently by integrating more than one regulatory input to each degradation system.

Table of Contents

Figures List

Table List

I. Introduction

<u>Section 1 Vertebrate Muscles</u>

The vertebrate muscle cell is composed mostly of protein and serves as a means of motility. During times of starvation it also serves as a metabolic reserve. The muscle is composed of individual contractile units known as sarcomeres, which facilitate motility through the ability to contract and relax.   There are three types of vertebrate muscle: cardiac, smooth and skeletal. Cardiac and smooth muscles are controlled without conscious thought and facilitate essential life functions such as the beating of the heart and peristalsis, which aids in the movement of food. Skeletal muscle (from here referred to as only muscle) on the other hand, is controlled by the organism's conscious.

The muscle cell is composed of sarcomeres which are organized into myofibril which is in turn organized into muscle fiber (Figure 1). It is these fibers that give skeletal muscle its striated appearance. Sarcomeres are anchored and centered around a massive protein known as Titin and composed of the alternating proteins Actin and Myosin(Labeit and Kolmerer, 1995; Small and Squire, 1972; Watkins and Cullen, 1986). The myosin regions are known as the A-bands which are attached to a central M region. Partially overlapping the A bands and extending to Z band located at the end of the sarcomere are the polar Actin filaments. The Actin filament are arranged in an alternating head to tail fashion between the individual sarcomeres and are tethered together at a Z band.

Myosin filaments are composed of two chains of alpha helical rods with globular heads at one end that contain ATPase activity when activated by interactions with Actin(Dolcini et al., 1970).   Upon the hydrolysis of ATP into ADP and Pi a conformational change occurs in myosin which generates a sliding force which is responsible for the muscle contraction(Holmes, 1995; Huxley, 1969).

Fibrous Actin (F-Actin) is the other major building block of the sarcomere and is composed of an array of 13 globular Actin (G Actin) monomers in a helical conformation.

Mixed in with this Actin array are the Ca^{2+} binding regulatory proteins Tropomyosin and Troponin, which regulate contraction(Tobacman, 1996).

Contraction is a process that is highly regulated through neuronal imputs and the concentration of Ca^{2+} ions. When the sarcomere is relaxed, the myosin ATPase heads are facing away from the Actin filaments and towards the M band. This is known as the blocked confirmation because tropomyosin is blocked by its regulator troponin(al-Khayat et al., 1995; Cantino and Squire, 1986; Lehman et al., 1995; Lorenz et al., 1995). Upon nervous stimulation, Ca^{2+} is released from the sarcoplasmic reticulum and binds troponin which shifts tropomysin into its closed state, activating myosin's ATPase activity (Geeves and Conibear, 1995; Haselgrove and Huxley, 1973; Lehrer, 1994; Parry and Squire, 1973). Driven by ATP hydrolysis, the myosin fibers will generate a sliding force pulling on the Actin fibers, thus contracting the sarcomere(Jontes et al., 1995; Whittaker et al., 1995). Upon removal of the Ca^{2+} signal Troponin will shift back into its original state of blocking Tropomyosin which will in turn deactivate Myosin's ATPase activity resulting in muscle relaxation.

(Figure Removed due to copyright, please see origional figure at source)

Figure 1. The basic structure of a mammalian sarcomere(Squire, 1997)

Aside from functions in motility and defense, another key function of muscle is to serve as a metabolic reservoir that can be mobilized during times of starvation or stress. Under such

conditions, the massive amount of protein located in the muscle cells can be catabolized to serve as a source of energy or material for essential cellular processes until the stress condition is reversed. Protein levels are controlled via either synthesizing more protein or degrading it via either proteases (enzymes that cleave proteins), ubiquitin tagging and the protesome (discussed briefly in this section) and autophagy (discussed in section 3). In order to help facilitate this task, cells ranging from yeast to mammals employ numerous signaling pathways (Figure 2). Errors in these pathways cause either hypertrophy (a gain in muscle fiber size) or atrophy (a loss in muscle mass).

(Figure Removed due to copyright, please see origional figure at source)

Figure 2. Signaling pathways that regulate muscle mass. Atrophy activating proteins are colored in green whereas negative atrophy regulators are colored in red.(Glass, 2005)

An increase in muscle mass is facilitated by an increase in protein synthesis which is the result of positive signaling through Insulin growth factor-1(IGF-1) and phosphatidylinositiol-3 kinase (PI3K)/ Akt pathway(Bodine et al., 2001). This was demonstrated in transgenic mice with over expression of IGF-1, which resulted in a larger muscle mass (Musaro et al., 2001). The converse was also observed in burn victims who showed muscle atrophy related to decreased Akt signaling(Sugita et al., 2005).

Akt signaling promotes hypertrophy by activating protein synthesis in two ways, firstly by promoting Target of Rapamycin (TOR) signaling (Pallafacchina, Calabria et al. 2002) and secondly by relieving inhibition of translation initiation factor eIF2B via inhibition of Glycogen Synthase Kinase 3 Beta (GSK3B)(Cross et al., 1995). Akt also discourages atrophy by disrupting the ubiquitin tagging of proteins via inhibiting FOXO, a transcription factor that stimulates the production of the tagging enzymes MaFbx and MuRF-1(Sandri et al., 2004)

Another critical signaling component in muscle cell mass is TOR which acts as a signal integrator, combining information of levels of amino acids, IGF-1 signals and a variety of other growth and nutrition signals(Burnett et al., 1998; Zhang et al., 2000). As its name implies, TOR can be inhibited by rapamycin, and when inhibited, all IGF-1/Akt signaling is stopped(Pallafacchina et al., 2002). In addition to positively regulating protein synthesis, TOR also inhibits PHAS-1 a negative regulator of translation initiation factor eIF-4E by outcompeting the binding of a PHAS-1 activator, Raptor(Proud, 2004).

As mentioned earlier, muscle atrophy is mediated via several protein degradation methods. In this section we will focus only on Ubiquitin/ protesome mediated degradation signaling, whereas autophagy mediated signaling will be discussed in section 3. Ubiquitin/protesome mediated degradation is facilitated by a 2000 kDa barrel like structure known as the protesome. The protesome feeds its targets into the active site with the help of two arm like projections and releases free amino acids. However, before a protein can be targeted for this form of degradation, it must first be tagged with a series of ubiquitin tags by the ubiquitin conjugating enzymes E1 (the ubiquitin activating enzyme),E2 (a ubiquitin conjugating enzyme) and E3 (the ubiquitin ligating enzyme) (Haas et al., 1982; Hershko and Ciechanover, 1998).

This form of degradation can be activated by the up regulation of the ubiquitin tagging enzymes(Jagoe et al., 2002) In order to up regulate the ubiquitin tagging enzymes the transcription factors MAFbx and MuRF1 are required(Sandri et al., 2004). These transcription factors are activated through the signaling of several cytokines including Tumor Necrosis Factor Alpha (TNFα)(Argiles and Lopez-Soriano, 1999). Upon binding to its receptor, TNFα activates the transcription factor NF-kB which promotes the transcription of MAFbx and MuRF1 and hence results in atrophy. TNFα signaling has been shown to be associated (and is believed to play a major role in the pathology) of muscle loss due to disuse, starvation, sepsis and cancer

related cachexia. This signal is in fact so strong, that it is alone sufficient to produce atrophy in mice(Cai et al., 2004).

Section 2 C. *elegans* as a Model of Mammalian Muscle Homeostasis

The soil nematode C. *elegans* is a small, free living soil creature that feeds on bacteria. The animal provides an excellent model organism for the study of development, genetics and various disease pathologies. The organism also provides many advantages over traditional model systems due to its size, rapid life cycle, cost/ ease of cultivation and easily accessible/ well studied genome (fully sequenced in 1998). In this section we will discuss the basics of C. *elegans* as a model system for the study of muscle signal transduction and protein break down.

(Figure Removed due to copyright, please see origional figure at source)

Figure 3. A brief history of major developments in C. *elegans* research(Ankeny, 2001)

C. *elegans* was first proposed as a model system for the study of neurobiology and development by Syndey Brenner in the mid 1960's(Ankeny, 2001). The worm was chosen out of need for a simple organism that could be used to study how the genetics of an organism are related to its behavior. The worm offered a very simple system to study this because of its extremely simple nervous system that consisted of a series of neurons arranged in a crown like structure. The organism also offered a very short development time (about 3 days at 20 C) and the ability to generate large populations very quickly(the hermaphrodite can lay about 260 eggs).

Wild type animals grow up to be about 1 mm in length and follow a simple life cycle (Figure 4). Upon hatching, worms normally undergo four larval stages before becoming egg laying adults. However, under starvation, crowding, or high temperature conditions L2 larvae can follow an alternate development route known as the dauer pathway. Dauer(German-to endure) larvae are extremely robust and can survive under such high stress conditions for about 3-4 weeks due to arrested gonad development and greatly reduced metabolism. Upon removal of the stressor dauers will continue with their development to normal wild type adults.

Worms are cultivated on agar media Petri dishes with a lawn of E. *coli* bacteria that serves as a source of food. The E. *coli* bacteria can be transformed with a plasmid that produces anti sense RNA to target genes in C. *elegans* providing an easy method for RNAi knockdowns. The plates themselves can also be doped with chemicals allowing for easy inhibitor or drug screens as well as studies of drug effects. .
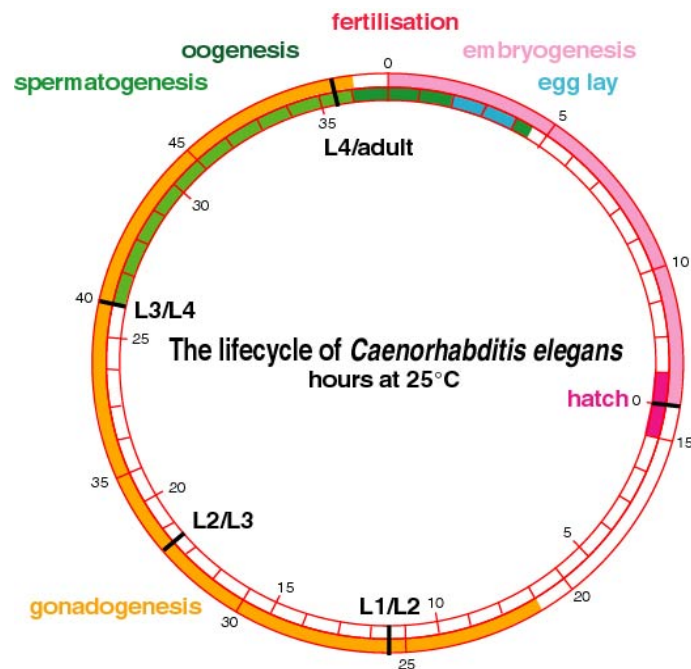

Figure 4. The lifecycle of C. elegans (Reproduced from www.nematodes.org with permission of Dr. Mark Blaxter)

Worms are an excellent model for the study of muscles and the complex signaling pathways that regulate their homeostasis. Firstly, the worm is essentially a digestive tract and gonad surrounded by muscle cells, which allows remarkable accessibility to observing muscle in live animals. Secondly, due to its sequenced and extensively studied genome, a wealth of information is rapidly accessible including data base searches for DNA, protein and RNA related information. Thirdly, there is a wide variety of tools to study the animal and its biology ranging from antibodies, to RNAi vectors, to cell specific GFP tagged strains to temperature sensitive mutations. Also, due to the animal's relative simplicity, such tools that presently do not exist can be generated rather quickly.

C. *elegans* muscles and the signaling pathways that govern them are remarkably similar to vertebrate muscle(Figure 5) in terms of structure, function and the signaling pathways that regulate muscle homeostasis (Figure 6). However, some fundamental differences do exist such that C. elegans muscles are mono nucleate as opposed to vertebrate which are multi nucleate.

(Figure Removed due to copyright, please see origional figure at source)

Figure 5. Sarcomere assembly in *C. elegans* muscle (Moerman, D. G. and Williams, B. D. (January 16, 2006), *WormBook*,)
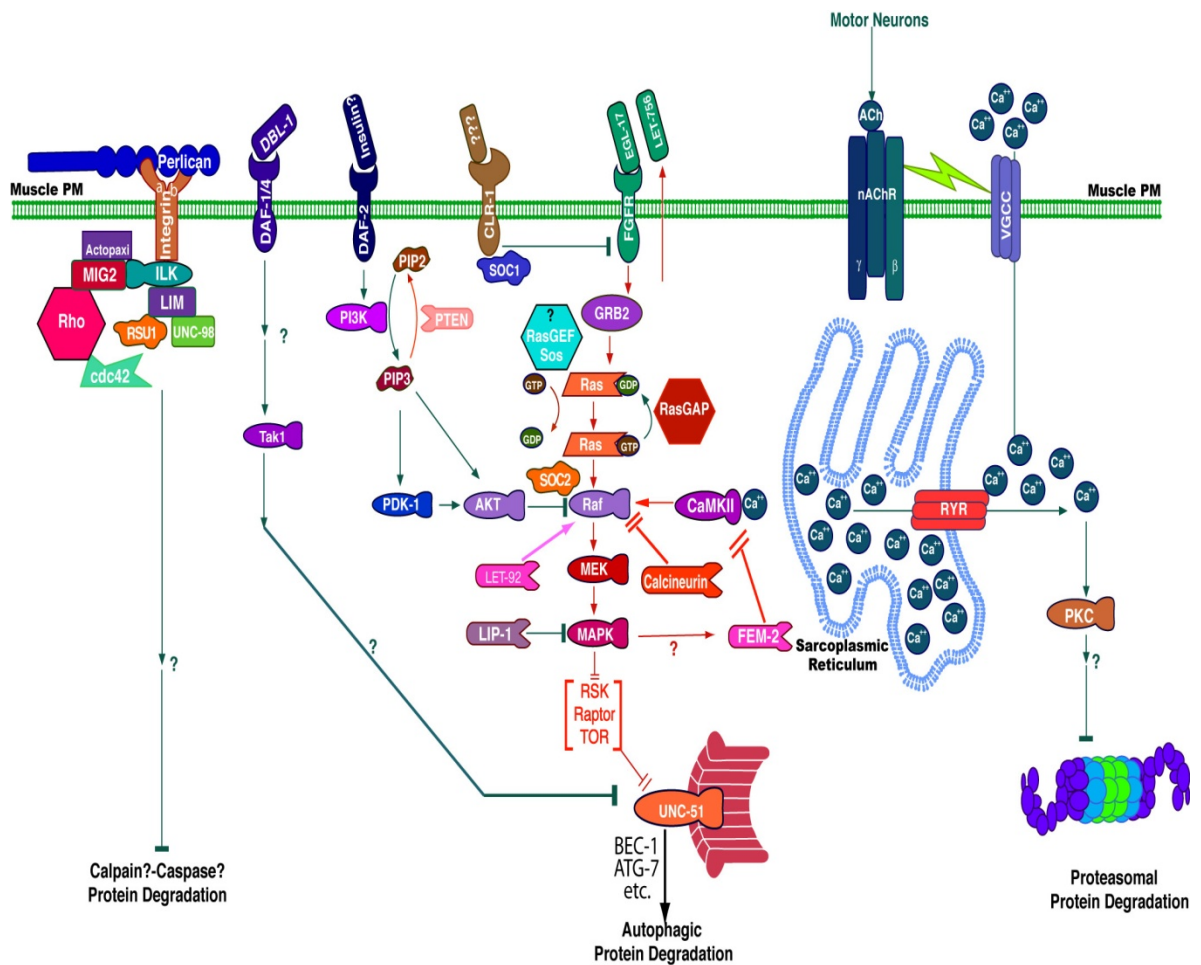
Figure 6 Proposed signaling pathways of muscles protein degradation in C. elegans muscles

While there exist many advantages to the use of C. *elegans* as a model system for muscle protein degradation, there do exist some fundamental limitations. First, as of now no one has been able to successfully culture worm cells. This presents a problem as it is difficult to study certain cell specific phenomena that could normally be done by culturing the specific cell type. For example, if one wishes to study a particular protein's expression in body wall muscles via western blot one must use the entire animal resulting in noise from other cell types. Second, certain worm genes are controlled by operons something not found in most higher eukaryotes.

<u>Section 3 Autophagy</u>

Autophagy (Greek *auto*, 'self', and *phagy*, 'to eat') is a cellular process in which portions of the cytosol are sequestered in double membrane vesicles (autophagosomes) that are then fused with a lysosome for breakdown and recycling. Autophagosomes were observed in electron micrographs as early as the 1950's, however it was not unit the late 90's that the molecular mechanisms of autophagy began to be studied(Klionsky, 2007) in yeast cells. While not yet fully understood, autophagy has proven to be evolutionarily conserved across many species and a critical component of diverse cellular process and pathologies such as homeostasis, catabolism, cell death, neuro-degeneration and cancer(He and Klionsky, 2009).

Three types of autophagy have been characterized, each with unique mechanisms and functions: micro-autophagy, macro-autophagy(autophagy) and chaperone mediated autophagy (Figure1)(Klionsky, 2005; Massey et al., 2006). In micro-autophagy an invagination of the lysosome membrane engulfs cellular material and translocates it directly into the lysosome lumen for degradation and recycling (Levine and Klionsky, 2004; Yorimitsu and Klionsky, 2005). The function of this process has not yet been elucidated in higher mammals, but, has been identified as the primary means of organelle degradation in fungi(Mizushima et al., 2008). During macro-autophagy, the target material is engulfed in a double membrane bound autophagosomes of unknown origin which then fuse with the lysosome and have their contents degraded by the lysosomal hydrolases (Levine and Klionsky, 2004; Melendez and Neufeld, 2008). Both macro and micro-autophagy can degrade large structures through both selective and bulk mechanisms. Chaperone mediated autophagy on the other hand, is used by mammalian cells to digest soluble proteins. Targets are tagged with a chaperone protein (i.e. hsc 70) which is then recognized by a receptor on the lysosome membrane and engulfed for degradation(Cuervo et al., 2005).

(Figure Removed due to copyright, please see origional figure at source)

Figure 7. A representation of the three types of autophagy(He and Klionsky, 2009)

The autophagic process is characterized by seven mechanistically distinct steps, induction, cargo recognition/selection, vesicle formation, lysosome fusion, breakdown and cargo release, and involves about 30 different genes(Table 1) (Kundu and Thompson, 2008).

Table 1. Autophagy proteins: orthologues and C. elegans reagents

Initiation				
Yeast	Human	C. elegans	RNAi?	Function
Atg1P	ULK	UNC-51	Yes	S/T kinase
Nucleation				
Atg6p	beclin	BEC-1	Yes	PI3K reg.
VPS34	PI3K class III	vps-34	Yes	PI3 kinase
Autophagosome				
Atg3P	ATG3	Y55F3AM.4	Yes	E2-like; conj Atg8 to PE
Atg4p	ATG4	Y87G2A.3	Yes	Cys protease
Atg5p	ATG5	ATG-5	No	Conj. to Atg12
Atg7p	ATG7	ATG-7	Yes	E1-like; act Atg8 and 12
Atg8p	LC-3	lgg-1	Yes	UBQ-like prot.
Atg10p	ATG10	D2085.2	Yes	Conj. Atg12 to Atg5
Atg12p	APG12-like	lgg-3	Yes	UBQ-like prot.
Atg16p	APG16-like	K06A1.5	Yes	Atg5-12 complex

In healthy, unstressed, somatic cells, basal levels of autophagy are rather low and are kept in check by the action of the S/Y kinase TOR, which integrates the various signals such as nutrient levels, hormones and energy(Chang and Neufeld, 2009; Chen et al., 2008). In yeast cells, under nutrient poor conditions, TOR becomes inactivated, which allows Atg1 to complex with Atg13 and Atg17(Kamada et al., 2000) and create a scaffold onto which other proteins will

bind and serve as a nucleation site for autophagosome formation(Figure (Cheong et al., 2008; Cheong et al., 2005; Kabeya et al., 2005; Kawamata et al., 2008; Suzuki et al., 2007).

(Figure Removed due to copyright, please see origional figure at source)

Figure8. A representation of some of the major steps of Autophagosome Maturation Adopted from:(Megalou and Tavernarakis, 2009)

The next (optional) step in autophagy is the selection of cargo. Two example of selective autophagy are the yeast Cvt (cytoplasm to vacuole targeting) pathway and the mammalian clearance of ubiquinated cytosolic structures. In the yeast Cvt pathway, the protein prApe1 contains a vacuole destination tag that is also picked up by Atg19. prApe1 and Atg19 complex with the adaptor Atg19 which in turn complex with Atg8 and the autophagosome(Scott et al., 2001; Shintani et al., 2002). The other example of selective autophagy is the clearance of ubiquinated or aggregate prone-structures which are toxic to many mammalian cells (Lewy Bodies for example can lead to the neurodegenerative disease MLB). The mammalian adaptor

SQSTM1 (p62/sequestosome) binds to such ubiquinated targets and then to the mammalian Atg8 homologue which brings targets into the autophagosomes for degradation(Pankiv et al., 2007)

Once the autophagic scaffold is made ready, nucleation of the autophagosome is initiated by the class III phosphatidylinositol 3-kinase complex composed of Vps15, Atg-14 and Atg6/Vps30(Itakura et al., 2008; Kihara et al., 2001; Liang et al., 1999; Sun et al., 2008). This complex produces PtdIns3P which recruits Atg18, Atg20, Atg21 and Atg24 to the nucleation scaffold site (Nice et al., 2002; Obara et al., 2008; Stromhaug et al., 2004). Finally, Atg12, Atg15 and Atg16 attach by self oligomerization and recruit Atg8-Phosphsphatidylethanoloamine, the proposed lipid carrier which begins the vesicle formation(Fujita et al., 2008; Hanada et al., 2007; Suzuki et al., 2001; Suzuki et al., 2007). Currently the source of the membrane is unknown, although the mitochondria, Golgi complex and E.R. have all been proposed as sources.(Juhasz and Neufeld, 2006).

Upon vesicle formation Atg-8 is cleaved back into the cytoplasm by ATG-4 and the fusion machinery consisting of Ypt7, Sec18 and the SNARE proteins Vam3, Vam7, Vti1, Ykt6 Ccz1 and Mon 1 fuse the autophagosome with a nearby lysosome(Kirisako et al., 2000; Klionsky, 2005). Degradation of the inner membrane and interior structures are facilitated by the lipase Atg-15 and Cathepsins B&D(Epple et al., 2001; Teter et al., 2001). Digested material is then spewed forth from the autophagolysosome by the permeases Avt3, Avt4 and Atg22 thus ending the autophagic degradation process(Yang et al., 2006)

Due to its ability to degrade large portions of cytosol and upset cellular homeostasis, autophagy is tightly controlled through several evolutionarly conserved regulatory pathways, most culminating in the inactivation of the TOR complex(He and Klionsky, 2009). The first such pathway is the glucose sensing Ras-PKA pathway (see figure 2).   In yeast the Protein Kinase A (PKA) complex is composed of three catalytic subunits, Tpk1, Tpk2, Tpk3 and the cAMP binding regulatory subunit Bcy1. Under nutrient rich conditions Ras1 and Ras2 produce cAMP which binds to Bcy1 and activate PKA. This in turn phosphorylates and activates TOR, thusly inhibiting autophagy(Budovskaya et al., 2004) Additionally, according to predicted interaction data, PKA may inhibit autophagy by phosphorylating Atg1 and preventing it from complexing with Atg13 and Atg17 (Budovskaya et al., 2004).

The insulin "energy sensing" pathway also plays a critical role in autophagy regulation. Upon binding to its receptor on the cellular membrane, Insulin causes the receptor to autophosphorylate and recruit/ phosphorylate insulin receptor substrate 1 and 2 (IRS1 and IRS2). The IRS proteins then form a docking scaffold for p85. P85 generates PtdIn3k which recruits phosphoinositide-dependent protein kinase 1 (PDK1). PDK1 then phosphorylates and activates PKB/Akt(Arico et al., 2001). PKB/Akt signaling normally causes Tumor Suppressor Genes 1 and 2 (TSC1 and TSC2) to complex and through Rheb activate TOR inhibiting autophagy(Manning et al., 2002). However, PKB/Akt activation is immediately negatively regulated by 3'-phosphoinositide phosphatase (PTEN) which shuts down the PKB/Akt signaling and ultimately inhibits TOR, removing the inhibition on autophagy.

Ras is yet another critical signaling protein involved in autophagy regulation. By integrating signals from hormones, and amino acids Ras signals through its effectors Raf-1 and MAPK to activate autophagy in a way that appears to be independent of TOR inhibition (Furuta et al., 2004; Pattingre et al., 2003).

(Figure Removed due to copyright, please see origional figure at source)

Figure 9. A schematic of autophagic regulation due to hormones, amino acids, energy and nutrient levels. (a) The mammalian pathway (b) The yeast pathway (He and Klionsky, 2009)

<u>Section 4 The role of Autophagy in Disease</u>

As mentioned previously, autophagy has implications in many cellular processes such cell survival and death. Under conditions of cellular stress(i.e. oxidative, starvation), knockdowns of various Atg genes accelerates cellular death, suggesting cytoprotective effects of autophagy.(Levine and Yuan, 2005; Maiuri et al., 2007)   On the other hand, rampant autophagy(via *Beclin-1* upregulation) in mammals has also been shown to cause cell death, however it is unclear if overactive autophagy leads to apoptosis or if the cause of death is the destruction of large portions of the cytosol.(Pattingre et al., 2005; Scott et al., 2007)

(Figure Removed due to copyright, please see origional figure at source)

Figure 10. Mixed functions of Autophagy in select human disease(Mizushima et al., 2008)

Malfunction in autophagy leads to a plethora of disease and disorders (see Figure 10), including cancer(Mathew et al., 2007). For example, 40-70 percent of breast, prostate or ovarian tumors are linked to a monoallelicly deleted *Beclin 1* gene which quite strongly suggests a link between autophagy and cancer (Qu et al., 2003). Autophagy appears to play a preventative/protective role in cancer as *atg4c* has shown anti-tumor effects in animal models, yet the mechanism remains largely unknown(Marino et al., 2007). One hypothesis suggests that

under stress conditions(such as those caused by a tumor), autophagy prevents cell growth and proliferation.

Neurodegenaration is a common symptom of aging and recent studies have linked autophagic function to both the prevention and progression of neurogeneration. One such study has shown that mice with neural tissue specific *atg5* and *beclin 1* knockouts develop inclusion bodies in their neurons as well as neurological disorders including motor defects and abnormal reflexes suggesting autophagy is necessary to maintain healthy neural tissue(Levine and Kroemer, 2008; Mathew et al., 2007). Up regulation of autophagy (via TOR inhibitors) in polyglutamine diseased mice and fruit fly models have shown that neurodegeneration is slowed under autophagic conditions, showing promise for autophagy based treatments of neurological disorders.(Rubinsztein et al., 2007) Despite its promise in the combating of disease, up regulation of autophagy has been shown as a component of Alzheimer's disease pathology(Yu et al., 2005). During the disease progression, patient neurons have shown unusually high amounts of autophagic structures. Also, Aβ, the toxic protein product can be produced within these structures and actually cleaved to its toxic form by proteases sequestered within autophagic structures. Therefore, autophagy may actually contribute to Alzheimer's disease progression, complicating its use as a potential therapeutic agent.

II Research Objectives

This work will focus on demonstrating, that autophagy and no other method of protein degradation is responsible for LacZ::myosin cytosolic reporter degradation in low IGF, active FGFR or MAPK situations. We will utilize RNAi, mutational studies and chemical inhibitors such as Dimethyladenosine (Kovacs et al., 1998). We show autophagy either a lack of IGF signaling high FGFR or high MAPK signaling do in fact result in autophagy. Western blot analysis as well as LacZ staining indicate that when autophagy is triggered (through low IGFR or hyperactive FGFR), RNAi knockdowns or mutations in several autophagy genes (*bec-1, atg-7, unc-51*) preserve higher levels of reporter protein than untreated, wild-type, or null-RNAi controls, suggesting that protein degradation under these conditions requires autophagy. When *bec-1 or atg-7* RNAi was used against *cha-1* mutants (mutants that degrade muscle protein through the proteasome pathway), the RNAi offered no protection against degradation. These data support the notion that IGF and FGFR signaling are responsible for regulating autophagy-based degradation.

Western blotting against MAPK-P under degradation conditions induced by high FGFR signaling or low IGFR signaling indicate that in both instances MAPK-P is activate (Szewczyk and Jacobson, 2003; Szewczyk et al., 2007). This suggests that both the IGFR and FGFR pathways flow through MAPK.

When similar staining and RNAi experiments were performed with mutants that possess low TGF-β signaling, we experienced similar protein preservation effects suggesting that autophagy is necessary for degradation in that situation. Western blot analysis for MAPK-P showed that MAPK is not activated when low TGF-β signaling induces autophagy. We believe that this form of degradation signals in parallel with MAPK or feeds in downstream.

Finally, we attempt to elucidate what signaling proteins are downstream of MAPK. Through sequence information of the UNC-51, RSK and Raptor proteins, possible sites (RXRXXS/T) for phosphorylation by RSK have been found. This combined with data that show that inhibitin of RSK prevents autophagy as well as reported protein interactions in the literature (Carriere, et. al. 2008) I propose the following model:

IGFR/FGFR→→→MAPK→RSK —||Raptor→TOR—||UNC-51→→→autophagy

[where → is stimulatory and —|| is inhibitory]

III Materials and Methods

Media

NGM agar:

This was the primary medium on which the nematodes and their food source bacteria were cultivated. It was prepared by mixing 2.5 g Bacto-Peptone, 3g NaCl and 17 g Bacto-agar in 975 ml of ddH20. This mixture was then autoclaved for 35 minutes and 1 ml of 0.5% cholesterol in 95% ethanol was added. Upon cooling for 1 hour, 2 ml of sterile 0.5 Molar $CaCl_2$, 1ml of sterile 1 M $MgSo_4$ and 25 ml of sterile 1 M phosphate buffer pH 6 (800 mM Kh_2PO_4 and 200 mM K_2HPO_4 autoclaved for 35 minutes) were added and mixed. For general growth the mixture was distributed into 100 mm x 15mm Petri plates at a volume of about 30 ml per plate. For drug/inhibitor studies the mixture was poured into 60mm x 15 mm Petri plate to a volume of exactly 5ml. Once dry, the plastes were seeded with 500 ml of cultivated OP 50 E. *coli* bacteria

RNAi lite agar:

This was used for all RNAi experiments. Plates were prepared by mixing1.5 g/L NaCl, 4 g/L tryptone, 20g/L agar. This mixture was then autoclaved for 35 minutes and 1 ml of 0.5% cholesterol in 95% ethanol was added. Upon cooling for 1 hour the following were added: 25 mM KPO_4 buffer pH 6, 1 mM $CaCl_2$, 25 mg/L carbenicillin, 0.5 mM isopropyl-ß-D-thiogalactopyranoside, 0.2% w/v lactose. The mixture was distributed into 100 mm x 15mm Petri plates at a volume of about 30 ml per plate and stored at 16 C until seeding with RNAi generating bacteria.

Starvation Plates:

NGM agar plates were made following the above protocol with the exception of the addition of 10mg of Kanamycin and 200 mg of Strepomycin antibiotics per liter of media. These plates were not seeded with E. *coli*

<u>BU transfer buffer</u>:

BU buffer was used to transfer animals between plates and for age synchronization. BU was prepared by mixing 701 mg Na2HPO4, 400 mg NaCl and 300 ml of KH2PO4 in 100 ml of ddH20 and autoclaving for 35 minutes.

<u>BU+Kan+SM starvation transfer buffer</u>:

100 ml of sterile BU was mixed with 100 ul of 10mg/ml of Kanamycin and 100 ul of 200 mg/ml of Strepomycin antibiotics . Unused buffer was stored at 4C.

Bacterial Strains:

E. coli bacteria were cultured using standard methods in TB (1 g Tryptone, 500 mg NaCl and 150ul of 1 M NaOH in 100 ml ddH20. Autoclaved 35 minutes) and stored at 4C until their use as a food source for the worms. The strain OP50 uracil auxotroph) was obtained from the *Caenorhabditis* Genetics Center (CGC) and used as the primary food source for all experiments.

Growth and maintenance of nematodes

Table 2. C. *elegans* Strains:

Strain	Genetic Markers
PD 55	*ccIs55(unc-54::lacZ)V*
PJ1070	*ccIs55(unc-54::lacZ);* clr-1(e1745) II
PJ1228	*ccIs55(unc-54::lacZ)V ;daf-4(m592ts)III*
PJ1115	*ccIs55 (unc-54::lacZ)V;* gaIs37[Ef1a::Dmek hs::mpk-1] IV
PJ1123	daf-2(m41) III; ccIs55(unc-54::lacZ) V
PJ1700	daf-2(m41) III; unc-51(e369) ccIs55 (unc-54::lacZ) V Note: The m41 daf-2 allele is in fact a true temperature sensitive mutation in the receptor itself(Gems et al., 1998)
PJ1263	gaIs37(EF1a::Dmek hs::mpk-1) IV; unc-51(e369) unc-54::lacZ(ccIs55)
PJ1013	cha-1(p1182ts) IV; unc-54::lacZ(ccIs55) V

All strains were grown and maintained on NGM agar plates at 16C (PD 55 was maintained at 20C).

Gravity Age Synchronization:

Worms growing on NGM plates were age synchronized by washing the animals from the plates with BU and collecting them in a test tube. After settling under gravity for 3 minutes, the top 0.5 ml of the mixture containing L1 larvae was withdrawn and placed on an NGM plate seeded with bacteria.

Experimental Techniques:

RNA interference:

Feeder strains of *E. coli* HT115(DE3) (Timmons et al., 2001) containing plasmids to produce gene-specific RNAi (Fraser et al., 2000) were obtained from Cambridge GeneService (UK). Feeder strains were grown as lawns on NGM Lite plates. Several L4 larvae were picked to each plate and the subsequent generation of young adult animals were placed on NGM plates for 4 hours or for drug experiments onto NGM plates doped with the drug of interest for 72H. Animals were then examined via Histochemical Staining or Western Blot analysis. For each experiment, a negative control was included, consisting of *E. coli* HT115(DE3) carrying a plasmid vector with no RNAi insert (null RNAi).

Histochemical Staining and Microscopy:

Animals were fixed and stained for ß-galactosidase activity with 5-Br-4-Cl-3-indolyl-ß-D-galactopyranoside (X-gal) according to Zdinak et al. (Zdinak et al., 1997b) as modified from Fire (Fire, 1992). About 20 worms were picked from plates onto 20 ul of BU on a frosted glass slide. Slides were then dried in a 26esiccators under vacuum for about 30 minutes. Upon drying, the slides were fixed in -20C acetone for 3.5 minutes. Afterwards, the slides were dried on an aluminum block for about 7 minutes at room temperature or until all Acetone was visibly evaporated. Staining was performed by adding 20 ul of X gal in 0.4 ug/ml oxidation buffer and sealing the slides in a large Petri dish with a moist paper towel. Staining times were governed by visual examination of stained controls (wild type or mutants at permissive temperature) included with every experiment; the usual times were 2-3 hr at room temperature. Stained animals were

photographed digitally under bright-field illumination. About 5-10 images containing anywhere from 1 to 5 animals were photographed. All experiments were repeated at least twice to assure reproducibility. Finally, all images were post-processed with Adobe Photoshop to construct composite images and to equalize illumination quality and color among images acquired over several years on two different microscopes. Color and brightness adjustments were applied equally to images from a given experiment, so as not to alter the relative stain intensities among genotypes or treatments.

Rsk inhibitor treatment:

BI-D1870 was tested in vitro (Bain et al., 2007) against 71 protein kinases. RSK1 and RSK2 were inhibited >98% at 0.2 microM inhibitor. Of the other kinases only PLK1 was inhibited strongly (95%); the next most sensitive was Aurora B at 22% residual activity. This high specificity and effectiveness led us to select this inhibitor.

Temperature sensitive mutants animals were grown according to standard methods and gravity age synchronized L4 larvae were placed on NGM plates with 0.1 uM of Rsk inhibitor BI-D1870 (B-Bridge International)(Bain et al., 2007) or 5 ul of DMSO control diffused for 24 hours before use. Worms were then shifted to either the permissive 16 C or non-permissive 25C for 72 hours. Afterwards, animals were harvested for histochemical staining and or western blot analysis.

N6,N6-Dimethyl Adenosine treatment:

      This compound was identified first by Kovacs *et al.(Kovacs et al., 1998)* as an inhibitor of autophagy in hepatic cells. It is believed to block a class III PtdIns-3-kinase. We have not followed up this observation by rigorously examining what this drug targets in C. *elegans*. We are therefore unsure of its exact target.

      Temperature sensitive mutants animals were grown according to standard methods and gravity age synchronized L4 larvae were placed on NGM plates with a final concentration of 0.5 mM of Dimethyl Adenosine (Toronto Research) (Kovacs et al., 1998)or 5 ml of DMSO control diffused for 24 hours before use. Worms were then shifted to either the permissive 16 C or non-permissive 25C for 72 hours. Afterwards, animals were harvested for histochemical staining and or western blot analysis.

Starvation Experiments:

 Upon reaching early adulthood, age synchronized animals were washed with BU+Sm+Kan buffer, spun down in a centrifuge and rewashed two additional times. The worms were then plated onto unseeded starvation plates with no visible bacterial contamination for up to 72 hours.

Western Blot

Reagents:

4x SDS buffer:

The buffer was prepared to 0.16 M Tris, 20% glycerol, 8% 2-Mercaptoethanol, 8% SDS and 0.01% Bromphenyl Blue.

TBS buffer:

The buffer was prepared to 10 mM Tris-HCl, and 150mM NaCl. The solution was then adjusted to pH to 8 with HCL

TBS-T buffer:

The buffer was prepared to 10 mM Tris-HCl, 150mM NaCl and 0.2% Tween-20. The solution was adjusted to pH to 8 with HCL

Running Buffer:

The buffer was prepared to 25mM Tris, 192 mM glycine, and 0.1% SDS. After mixing the buffer was adjusted to pH to 8.6 with NaOH.

Electroblot Buffer:

The buffer was prepared to 20% methanol, 25mM Tris, and 192 mM Glycine.

Blotto:

5% non-Fat Dry Milk in TBS-T (for LacZ)

5% BSA in TBS-T (for MAPK or RSK)

AP buffer:

This buffer was prepared to 100mM Tris-HCl, 100mM NaCl and 5mM $MgCl_2$. After mixing the solution was adjusted to pH 9.5 with HCl.

Western Blot Procedure:

Samples were collected by picking 30 worms into 20 ul of ddH20, and were stored at -20C until use. Upon use, 7ul of 4X SDS sample bufferwas added to each tube and they were lightly boiled for 2 minutes. Sample were then agitated on a vortex genie shaker for 5 minutes and centrifuged for 1 minute at 13,000 RPM. Samples were then loaded on a 4-20%(for B gal, 12% for phospho antibodies) SDS polyacrylamide gel (Lonza PageR gold precast gels) and resolved at a voltage of 120V for about 2 hours in a Mighty Small II gel apparatus (Hoeffer Scientific Products).

Once resolved samples were transferred to a PMDF membrane (Millipore Immobilon-P) soaked in Methanol, ddh20 and electroblot buffer for 2, 3, and 5 minutes respectively, using a genie blot apparatus(Idea Scientific Company). Transfer was performed over one and a half hours at 24 V in electroblot buffer.

<u>Blotting for B-galactosidase</u>:

The membrane was then placed in Blotto for 1 hour at room temperature with light agitation.   The membrane was washed 4 times for 5 minutes with TBS buffer at room temperature with light agitation. Afterwards, the membrane was incubated with 12 ul of mouse monoclonal anti Beta galactosidase (DSHB no. JIE7) antibody in 20 mL of Blotto overnight at 4C with light agitation.

The membrane was then washed 4 times for 5 minutes with TBS buffer at room temperature with light agitation. It was then placed in 20 ul of alkaline phosphatase conjugated donkey anti-mouse (Jackson) antibody in 20 ml of blotto and incubated for 1 hour at room temperature with light agitation. The membrane was washed 4 times for 5 minutes with TBS buffer at room temperature with light agitation

Finally, the antibody was detected with 20 ml of AP buffer and 66 ul of 5-Bromo-4-Chloro-Indolyl Phosphate (50mg/ml in 100% N,N`-dimethylformamide) and 132 ul of Nitro Blue Tetrazolium (50 mg/ml in 70% N,N`-dimethylformamide). Typical detection time was 5 minutes.

<u>Blotting for phospho-MAPK</u>:

The membrane was placed in Blotto for 1 hour at room temperature with light agitation. The membrane was washed 4 times for 5 minutes with TBS buffer at room temperature with light agitation. Afterwards the membrane was incubated in 20 ul of rabbit monoclonal anti phospho-MAPK (Cell Signaling no. 9102) antibody in 20 mL of Blotto overnight at 4C with light agitation.

The membrane was then washed 4 times for 5 minutes with TBS buffer at room temperature with light agitation. It was then placed in 5 ul of Horse Radish Peroxidase conjugated donkey anti-rabbit (Jackson) antibody in 20 ml of blotto and incubated for 1 hour at room temperature with light agitation. The membrane was then washed 4 times for 5 minutes with TBS buffer at room temperature with light agitation

Finally, the antibody was detected with 20 ml of TMB peroxidase solution (KPL Labs). Typical detection time was 30 minutes.

IV Results

Autophagy is required for protein degradation under conditions of low IGFR, High FGFR or high MAPK signaling

Various signaling pathways have been shown to be involved in regulating the degradation of protein in C. *elegans* muscle. These include the FGF pathway(Szewczyk and Jacobson, 2003), the IGF (Thompson and Palmer, 1998) as well as the TGF- β pathway (Deloriea et. al, submitted for publication). The goal of this work was to elucidate which proteolytic mechanism is responsible for the actual degradation of cystolic muscle protein under the control of these hormonal factors.

To do so we used transgenic C. *elegans* strains that contained β-Galactosidase fusion protein driven by a muscle specific myosin promoter. Transgenic animals also contained one of several temperature sensitive "trigger genotypes" that upon activation, resulted in muscle protein degradation. As a result, at permissive temperatures (16C) animals were essentially wild type and contained reporter protein in their muscle cell cytosol. The levels of muscle reporter protein were easily observable through histochemical staining. However, when up shifted to non permissive temperature (25C), the mutant genotype became activated and the animals degraded the reporter protein.

We activated the mutation in each trigger genotype and treated the animals with a method hypothesized to disrupt autophagic signaling, (and hence autophagic protein degradation). We then examined the levels of muscle reporter protein through histochemical staining. A summary of such experiments are shown in Figure 1.

Transgenic animals with hyperactive FGF receptors at non-permissive temperatures were first shown to degrade muscle reporter protein. When the same strain was pre-treated with RNAi against bec-1 or atg-7 (both signaling proteins believed to be involved in autophagy (Melendez et al., 2003) the animals showed significantly higher levels of β -galactosidase reporter. In a separate experiment, animals were treated with autophagy inhibitor N6, N6-dimethyl adenosine (diMeAdo) that is believed to disrupt class III PtdIns-3-Kinase signaling that has been shown to

prevent autophagy in hepatocytes (Kovacs et al., 1998). These also showed higher levels of the reporter protein. Animals with a mutation that results in a reduction of function in UNC-51 (homologue of a yeast autophagic signaling protein ATG-1 also showed preservation of β -galactosidase when shifted to non-permissive temperature. These results suggest that autophagy is required for degradation of muscle protein following activation of the FGF receptor.

Similar experiments were performed in transgenic worms that contained the β -Galactosidase construct as well as a loss of function temperature sensitive mutation in the DAF-2 IGF receptor. Upon up shifting to 25C, these animals degraded the muscle reporter protein. Animals pre treated with bec-1 or atg-7 RNAi and then shifted to non-permissive temperature resulted in preservation of the reporter protein. Treatment with the autophagy inhibitor followed up by a temperature up shift also resulted in muscle reporter protein preservation. Finally, double mutants that contained the IFGF mutation as well as an UNC-51 rof mutation also showed preservation of muscle cell protein at the non-permissive condition. These results implicate autophagy is required for IGF mediated protein degradation.

It has been shown that MAPK plays a role in controlling protein degradation downstream of both the IGF and FGF signals (see introduction) (Szewczyk et al., 2007). Therefore we repeated the same series of experiments in a system in which MAPK is constitutively expressed and activated via phosphorylation. To achieve a condition of constitutively active MAPK we used a strain that contains a transgene with a heat shock promoter that drives a constitutively active Drosophila MEK (the signaling protein that phosphorylates and activates MAPK) as well as an E-IFIα promoter that drives the expression of worm MAPK.

To activate the transgene, animals were first heat shocked for 30 minutes at 37C before being raised to the non permissive 25C. These animals were shown to degrade muscle reporter protein in a similar fashion to the previous two mutants at non permissive temperature. When these worms were pre-treated with RNAi against bec-1 or atg-7 followed by a heat shock and temperature up shift, they preserved cytosolic reporter protein. A second construct containing the MAPK transgene as well as a *unc-51* rof was also heat shocked and temperature up shifted. The *unc-51* mutation was shown to preserve muscle reporter protein in a similar fashion as it did in IFG or FGF signaling mutants. Autophagy is therefore, required for muscle protein degradation whenever MAPK is activated.
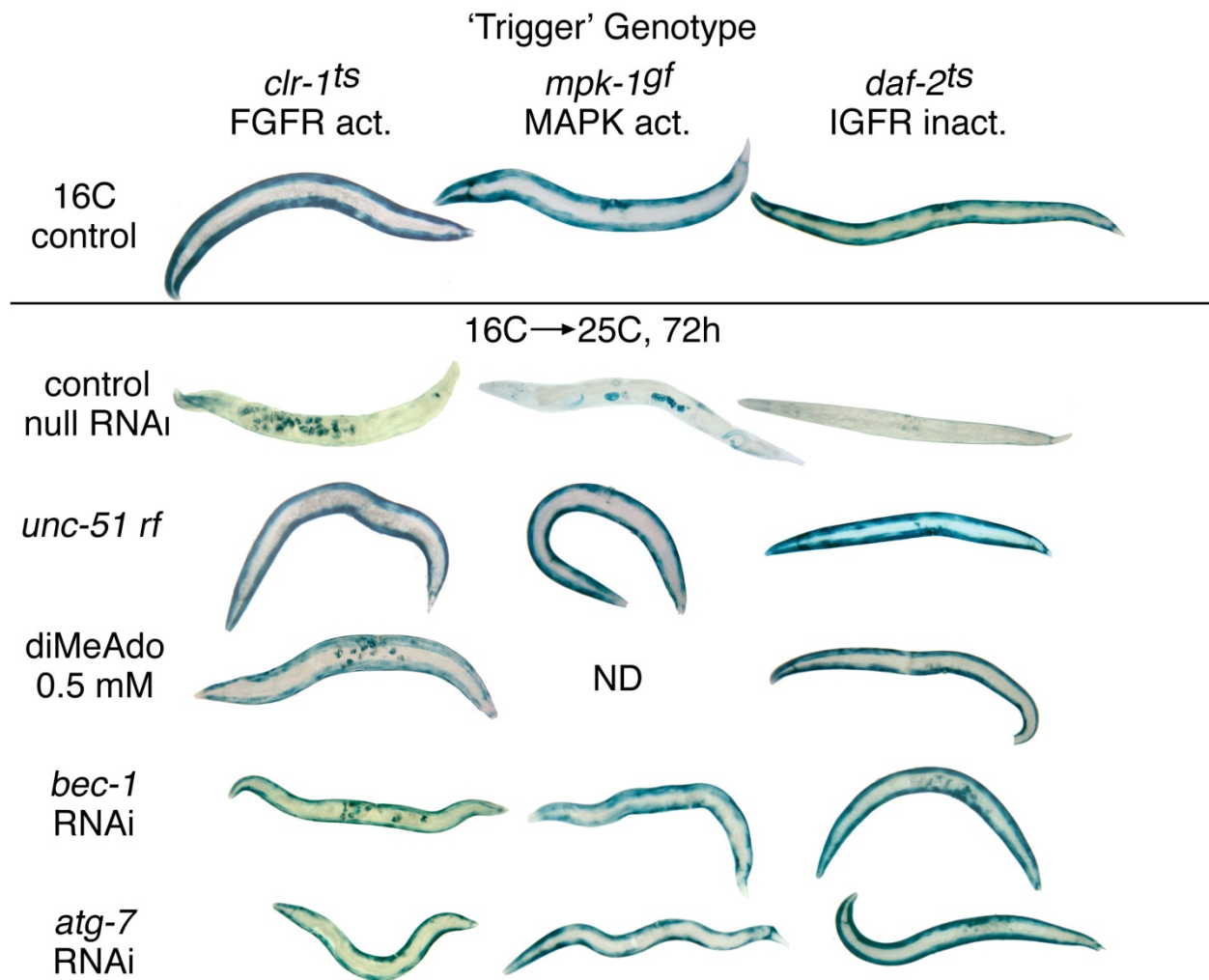
'Trigger' Genotype

	clr-1^{ts} FGFR act.	*mpk-1^{gf}* MAPK act.	*daf-2^{ts}* IGFR inact.

16C control

16C→25C, 72h

control null RNAi

unc-51 rf

diMeAdo 0.5 mM — ND

bec-1 RNAi

atg-7 RNAi

Figure 11. Histochemical stains for β-galactosidase of "Trigger Genotype" animals treated with an inhibitor of a protein that is believed to be involved in autophagic signaling. Control worms at permissive temperature containing one of the three trigger genotypes were shown to stain well under permissive temperatures of 16C, essentially resembling WT animals. IGF lf t.s. and FGF gf t.s. animals were up shifted to non-permissive temperatures of 25C to induce the protein degrading mutation (mpk-1^{gf} animals were first heat shocked for 30 minutes at 37C before up shift). L4-Animals pre-treated with RNAi, were placed on plates with bacteria that produce the anti-sense RNA and their adult progeny were stained. Animals treated with 0.5mM diMeAdo in DMSO were placed on plates containing the diffused drug and then up shifted to non permissive temperature. Double mutants that contained

UNC-51 rof as well as the trigger genotype were simply raised to non permissive temperature before staining (MPK-1^{gf} were first heat shocked for 30 minutes at 37C).

In order to corroborate the histochemical staining data that suggests autophagy is required for muscle protein degradation in the IGFR trigger genotype, samples of 30 worms at either permissive or non permissive temperatures were lysed, homogenized and resolved on an SDS-Page gel. The samples were then blotted for the β -Galactosidase reporter protein (LacZ) (Figure 2.) A similar blotting experiment was performed for daf-2, unc-51 double mutants. The animals were up shifted to 25 C for 72 hours before being harvested, lysed, resolved on a SDS-PAGE gel and blotted against LacZ. Animals that contained the *unc*-51 rof mutation showed significantly higher levels of LacZ than animals without the mutation (Figure 2B).

Both of these results confirm that autophagy is required for degradation of LacZ protein in both trigger genotypes.
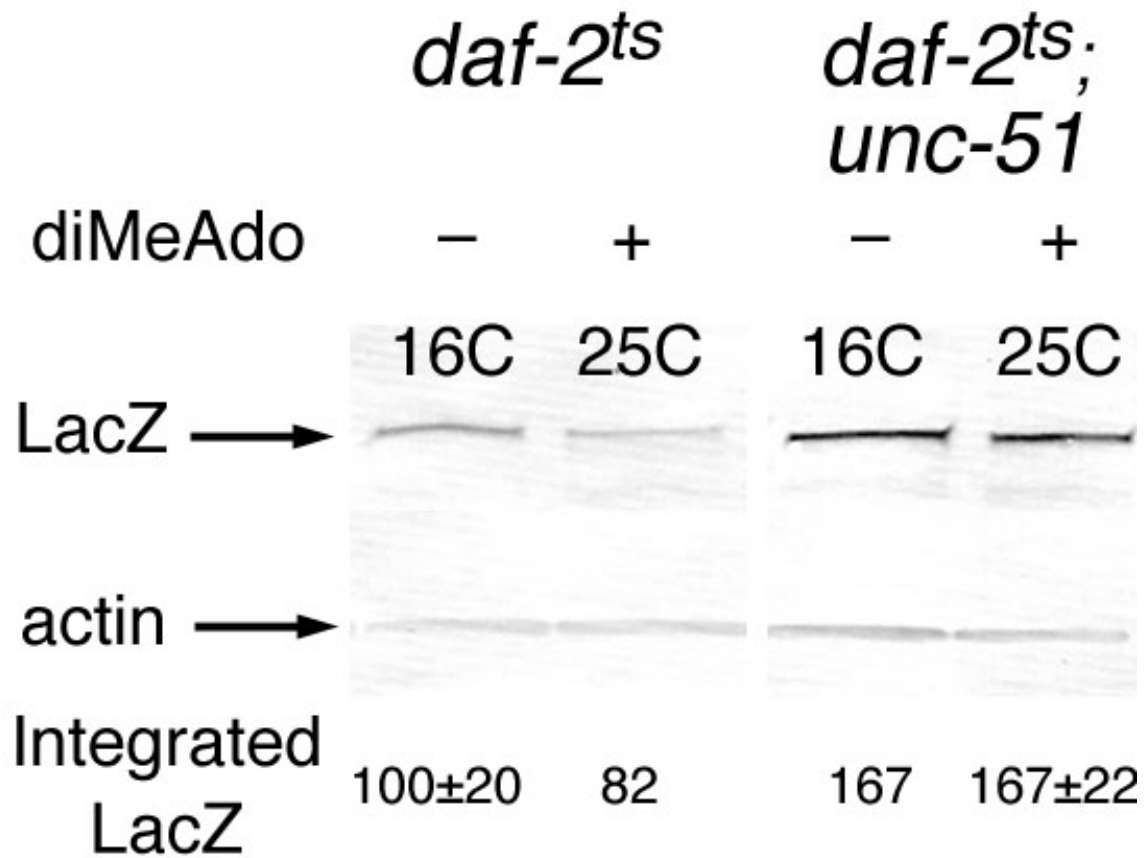
Figure 12. Western Blot analysis of β -galactosidase reporter protein levels in two signaling mutants.(B) daf-2 lf t.s. mutants lose IGFR function at 25C which results in LacZ degradation. Daf-2^{ts} mutants as well as double mutations daf-2^{ts} and *unc*-51 mutants were up shifted to 25C for 72 hours. Samples of 30 worms were then harvested in 20ul ddH20 and blotted for LacZ. Daf-2^{ts} mutants at non permissive temperatures showed a 17% reduction in LacZ levels compared to permissive controls. The double mutants showed significantly higher levels of LacZ at both 16C and 25C(67 percent higher than controls), implicating the need for autophagy in IGFR mediated protein degradation. The blot was reprobed for Actin, which serves as a loading control.

Autophagy is not required for the degradation of muscle protein under denervation conditions

The next series of experiments tested to see if autophagy was the mechanism of muscle protein degradation in denervated muscles. Under normal conditions, C. *elegans* muscle cells receive signals from motor neurons via the neurotransmitter acetylcholine. Acetylcholine facilitates the opening of a potassium channel, allowing potassium to enter the cell. Upon the change in membrane polarization due to K^+ flux, a voltage gated Ca^{2+} channel opens, which allows Ca^{2+} to enter the cell. The release of Ca^{2+} in the muscle cell is used in signaling in many processes including regulation of muscle protein degradation. To observe the levels of LacZ reporter protein as a consequence of denervation, we distrupted nerve signaling using a temperature sensitive reduction of function mutation in a neuronal choline acetyltransferase. At the non permissive 25C, the enzyme suffers a conformational change that greatly reduces its ability to synthesize acetylcholine, resulting in a lack of neuronal signals into muscle cells(Figure 3) . Upon the activation of this mutation for 48 hours, animals experienced muscle protein degradation.

Neither treatment with 0.5 mM diMeAdo for 48 hours nor pretreatment for 72 hours with RNAi against bec-1 or atg-7 yielded any preservation of LacZ. This shows that autophagy is not necessary for protein degradation in the muscles as the result of denervation. This is consistent with prior evidence showing that under these conditions, protein degradation is mediated by the ubiquitin-proteasome system (Szewczyk et al., 2000).
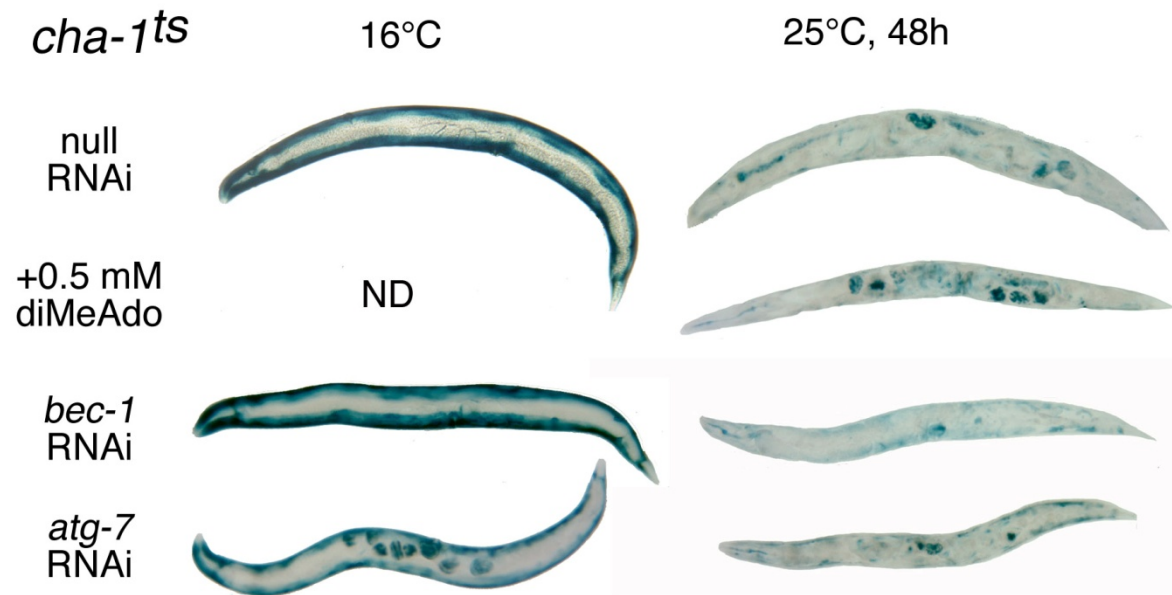
Figure 13. Histochemical stains β -Galactosidase in cha-1 rof t.s. mutants (simulate denervation) after 48 hours of up shift to non-permissive temperature show muscle protein degradation.   Control worms treated with a null RNAi vector show normal muscle protein staining at permissive temperature whereas the same worms upshifted to 25C do nto show much staining. Worms treated with 0.5 mM diMeAdo in DMSO show little or no preservation of reporter protein at non permissive temperature. Animals pretreated with RNAi against bec-1 or atg-7 for 72 hours before a 48 hour up shift to non-permissive temperature also show no preservation of LacZ. This shows that autophagy is not required for protein degradation in denervated muscles.

Starvation induced degradation does not require autophagy

Another cause of muscle protein degradation in C. *elegans* is starvation(Zdinak et al., 1997a). In response to a lack of food the animal catabolises lipids and muscle protein to use as energy until a food source is found. To investigate if autophagy is a possible mechanism of breakdown of muscle protein under starvation conditions, a transgenic line of worms that contained the LacZ reporter protein was used as well as one that also contained the *unc*-51 rof mutation. Animals were extensively washed with Streptomycin and Kanamycin buffer and placed on bacteria free plates for 48 hours. Afterwards, the animals were histochemicaly stained for 3 hours. Animals with and without the *unc-51* rof mutation both showed equally high levels for reporter protein breakdown. Animals treated with diMeAdo also showed comparable levels of protein breakdown in their muscles. It can therefore be concluded that autophagy is not required for starvation induced protein breakdown. It should be noted that the staining present in the head of the animals treated with diMeAdo is the result of the slow and highly controlled catabolism of protein. This is because the head contains the essential neurons, which are required for survival and hence the absolute last protein to be degraded(Szewczyk et al., 2000). A comprehensive discussion is presented in the discussion section. Also embryos are independent of the parent worm and therefore may stain under starvation conditions.
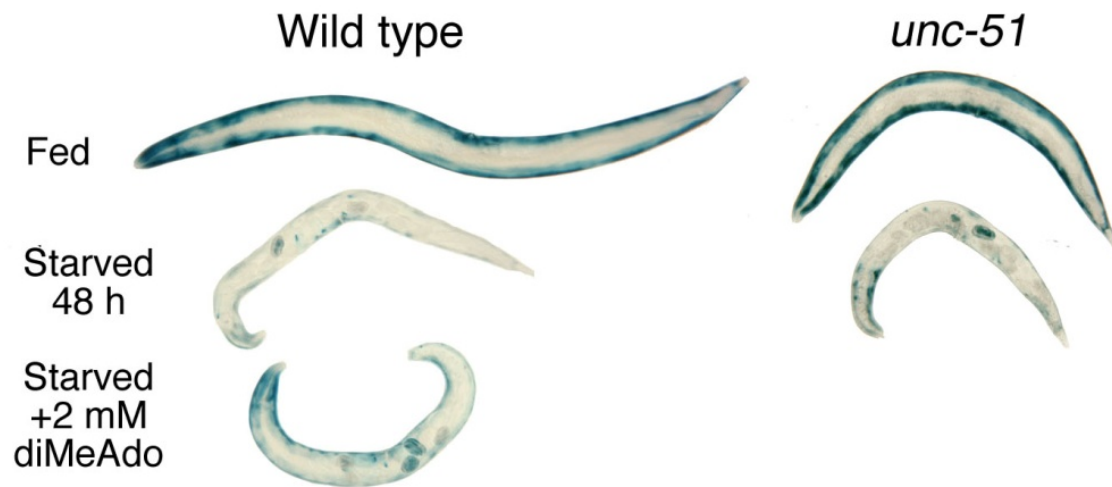
Figure 14. Worms containing the LacZ construct, starved for 48 hours and then stained for β -galactosidase reporter protein for 3 hours. Animals with and without the *unc-51* rof t.s. mutation showed little or no preservation of reporter protein. Treatment with 0.5 mM N6, N6- dimethyl adenosine in DMSO also yielded little protection against protein breakdown.

Rsk signaling is required for autophagy

After establishing that autophagy is in fact the proteolytic mechanism downstream of IGFR, FGFR and MAPK signaling we proceeded to what signaling molecules are involved downstream of MAPK. Following recent studies of autophagic signaling downstream of MAPK(Carriere et al., 2008a; Carriere et al., 2008b), we wanted to test if RSK is involved in autophagy in C. elegans.

We utilized the same FGFR and MAPK gf animals with a LacZ reporter protein as well as a potent inhibitor of RSK, BI-D1870 (Bain et al., 2007). Animals were treated with either a 0.2 uM concentration of the drug in DMSO or with DMSO as a control and then either up shifted to non permissive 25C or left at 16C. In both FGFR and MAPK animals, controls treated with BI-D 1870 at 16C stained similarly to untreated animals. At 25 C animals treated with the drug showed substantial preservation of muscle LacZ as opposed to untreated animals which showed very little reporter protein. While we have yet to conclude where or how Rsk signaling plays a role in autophagy in C. *elegans*, we do know that it positively regulates the process either in parallel or downstream of MAPK. Considering that RSK encodes by *rsk-1* has been directly shown to be a substrate of MAPK the downstream model is favored(Arur et al., 2009).
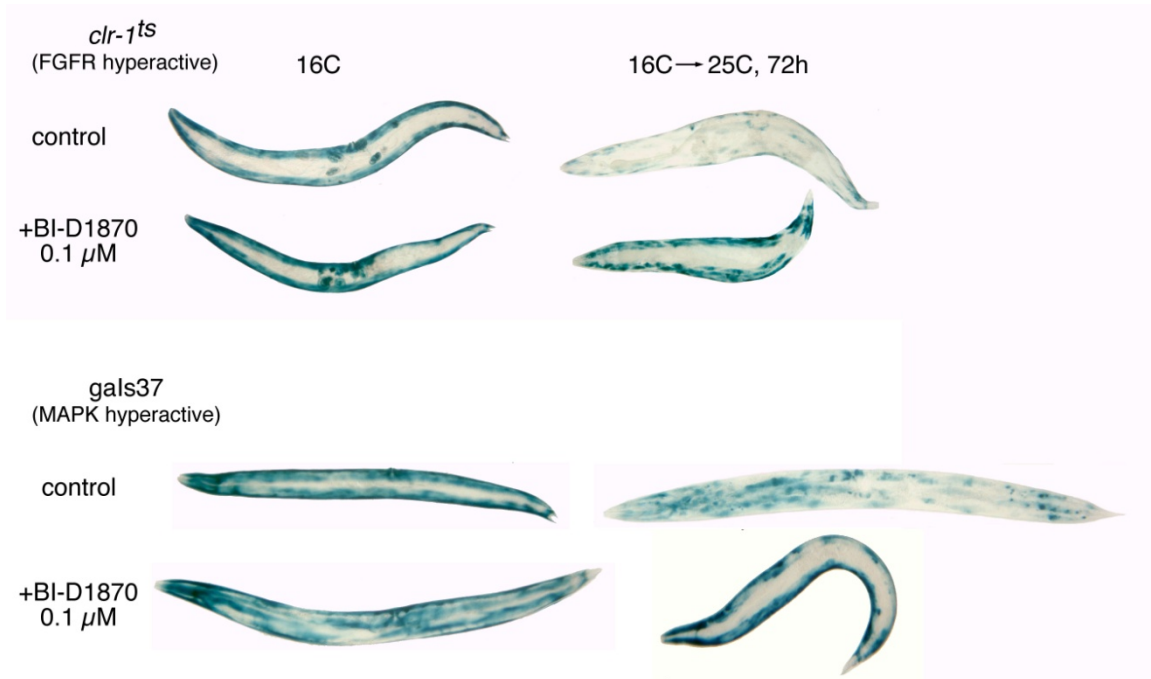
Figure 15. Histochemical stains of animals containing the FGFR or MAPK gf trigger genotypes were treated with 0.1 uM BI-D1870 in DMSO, an inhibitor of the autophagic signaling protein RSK at permissive and non permissive temperatures. Animals containing the FGFR gf t.s. mutation stained normally with or without inhibitor treatment at permissive temperature. At non permissive temperature animals treated with the inhibitor showed much higher levels of reporter protein then DMSO treated controls.   Hyperactive MAPK animals treated with the inhibitor stained similarly to untreated controls at the permissive 16C. However, at 25C animals treated with the inhibitor showed higher levels of LACZ when compared to DMSO treated controls.

Autophagy is required for degradation of reporter protein as a result of the disruption of TGF-β signaling

TGF-β signaling is required for many cellular processes such as growth and development(Massague, 1998). During development the animal undergoes programmed cell death as well as cellular remodeling in order to produce functional supercellular structures and organs. A portion if this complex process requires the breakdown and recycling of cellular proteins which is controlled by TGF-β signaling. We decided to elucidate which proteolytic mechanism is utilized in this process.

We employed *daf-4* lf t.s. mutants that also contain a LacZ reporter transgene identical to the one used in all other experiments. These animals, which are essentially wild type at 16C but, when shifted to a non permissive temperature of 25C lose TGF-β signaling. When these animals lose TGF-β signaling they degrade the muscle reporter protein(Figure 6) Treatment with 0.5mM diMeAdo and upshift to 25C for 72 hours results in higher levels of LacZ in muscles as opposed to DMSO treated controls upshifted for the same amount of time.

Double *daf-4 lf t.s./unc-51* mutants showed similar results to other *unc-51* mutants in other experiments and lessened the severity of reporter protein breakdown after 72 hours of temperature upshift to 25C when compared to *daf-4 lf t.s.* animals.

Pre treatment with RNAi against bec-1 or atg-7 for 72 hours followed by a temperature shift to non permissive for another 72 hours resulted in less LacZ degradation then null treated controls. These data indicate that autophagy is necessary for protein breakdown in muscle cells following a lack of TGF-β signaling.
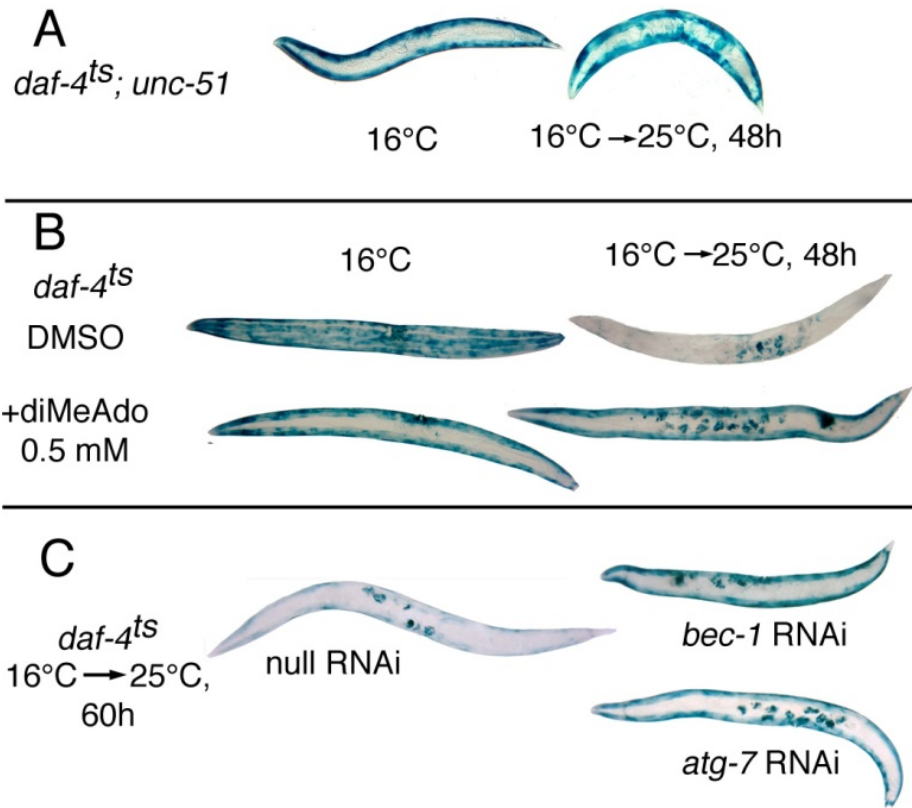
Figure 16. Histochemical stains for β -galactosidase after inhibition of believed autophagy signaling proteins in daf-4 lf t.s. mutants. (A) When daf-4 lf t.s and *unc-51* double mutants were upshifted to non permissive temperature of 25C for 72 hours degradation little LacZ occured. (B) daf-4 lf t.s mutants degrade LacZ after beigns shifted to non permissive temperatures for 72 hours. Treatment of the se animals with 0.5mM N6,-N6, dimethyl adenosine results in preservation of the reporter protein after 72 hours. (C) 72 hours of pre treatment with RNAi against bec-1 or Atg results in inhibition of degradation of muscle reporter protein after 72 hours of temperature shift to 25C.

TGF-β autophagy signaling does not activate MAPK

Because low TGF-β signaling results in autophagy just like low IGFR or high FGFR, we asked whether TGF-β signaling also requires phosphorylated MAPK in order to transduce the signal. We treated *daf-4* lf t.s. signaling mutants with 0.5diMeAdo and upshifted some to a non permissive temperature for 72 hours.

We collected samples of 30 worms in 20 ul of ddH20, lysed homogenized and resolved the samples on an SDS-PAGE gel. We then blotted against the active (phosphorylated) form of MAPK. To our surprise, the levels of MAPK-P actually decreased at 25 C regardless of treatment with diMeAdo (Figure 7). To ensure that the cause of the weaker signal was not a technical flaw (i.e. inconsistent numbers of worms in each lane), we stripped and reprobed the blot for total MAPK. Total MAPK levels were fairly consistent in all of the samples, indicating that all lanes were loaded correctly and evenly. This shows that MAPK-P is not necessary for autophagic degradation as a result of loss of TGF- β signaling.
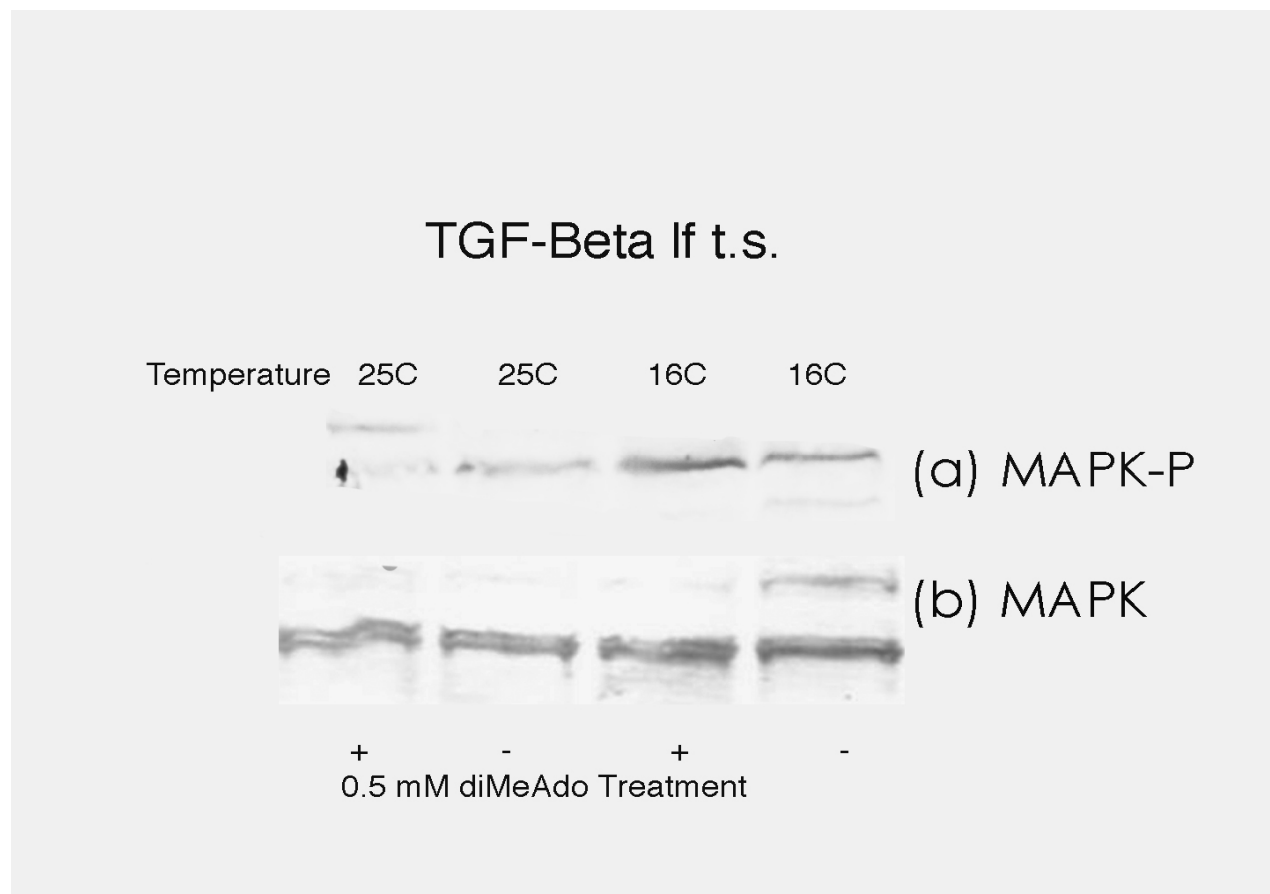
Figure 17. Western Blot analysis of MAPK and Phospho-MAPK levels in TGF- β signaling mutants. (A).   After treatment with 0.5 mM autophagy inhibitor diMeAdo in DMSO and shifted to 25C for 72 hours, worms were collected in 20ul of ddh20 and resolved on a 4-20% gel before being blotted for MAPK-P. Worms at 25C showed lower levels of MAPK-P versus 16C controls non-permissive temperatures. MAK-P levels were little affected by diMeAdo (B) A reprobe of the same blot for total MAPK revealed consistent levels of the protein under all conditions.

V Discussion

In this work I have made four major points. First, autophagy is the mechanism of cytosolic muscle protein degradation following activation of FGFR signaling, MAPK signaling or deactivation of IGFR signaling or TGF-β signaling. This is the first time that this has every been shown for muscle in any organism. Second, I have demonstrated that autophagy is not the method of degradation of protein under starvation or denervation conditions. Third, I have implicated RSK as a positive regulator of autophagy. Finally, I have established that low TGF-β does not trigger degradation by activating MAPK, as was observed for low IFGR or high FGFR signaling.

The need for multiple proteolytic systems

Our work has demonstrated that there are at least two distinct proteolytic systems in C. elegans: the ubiquitin mediated proteasome pathway and autophagy, both of which are controlled through very different signaling cascades and are active under different conditions.   Proteasome mediated degradation is the result of denervation or starvation whereas autophagy is the result of low IGFR, high FGFR or low TGF-β signaling.

These data raise questions as to why organisms would need multiple systems to degrade protein and what selective advantages this offers for the organism. I believe that by having multiple pathways with several signaling "inputs", organisms are better suited to "fine-tune" levels of protein in response to multiple stimuli simultaneously as well as have different tools evolved for different degradation needs.

The post-embryonic development of the animal provides an example of when having two separate degradation pathways would be useful. By separating out a degradation inducing signaling that is the result of starvation from signaling that is the result of cell differentiation/ remodeling factors such as TGF-β or FGF ,the cell would be able to continue (fairly) normal development under nutrient poor conditions. Had the signaling systems been linked, or utilized only one signaling molecule in this scenario, the animal would probably perish as the signal to degrade protein would have become overwhelming. Also, in the situation of one signaling molecule or pathway, the system would be much more susceptible to saturation if multiple sources simultaneously generated the signaling molecule.

Another advantage of having such a multi-system setup would be the advantage of having the right tool for the right job, depending on the cell's protein degrading needs.   Using an analogy of common household tools, one would not use a pair of garden shears to trim one's nails, nor would one use a pair of nail clippers to trim the hedges, as either tool is best suited for one purpose and not the other. A similar case can be made for autophagy vs. proteosomal breakdown. Autophagy can be considered the "garden shears" of muscle protein breakdown, it is a high power, bulk method of breaking down cytosolic contents. (macro)Autophagy does not differentiate or require tagging (except in the case of chaperone mediated autophagy), vesicles form, contents are sequestered, degraded and recycled.

This type of breakdown is very useful for development or cellular differentiation. During organogenesis for example, undifferentiated cells must undergo enormous remodeling of the cytosol, secretory cells must have many Golgi bodies, muscle cells need their filaments and neurons require many membrane channels. All of these structures must be generated from undifferentiated cells and what better/ more efficient way to break down unnecessary structures in order to acquire the raw materials for new structures than through the bulk breakdown of unnecessary organelles? The proteasome would simply be unable to cope with such high demand. First, it would be very difficult to ubiquitinate many membrane embedded proteins or structures composed of carbohydrates and proteins due to inaccessibility of the tagging residues. Second, attempting to selectively perform this tagging process of huge sections of the cytosol would be difficult from a kinetics point of view as well as consume large amounts of energy as there would be a need for ubiquitin tags and the tagging machinery itself. It would be the equivalent of trying trim a bush with a pair of nail clippers. Third, provided that the proteins were somehow all tagged for breakdown in the first place, how would such structures fit into the narrow active site of the proteasome?   All of these important considerations become non-issues when bulk autophagy, simply engulfs an entire section of the cytoplasm without any tagging required.

Using the converse of this logic, it is clear that autophagy is not a very good tool for degradation of protein under starvation or denervation conditions. Even under starvation conditions, the cell will still continue to use and produce energy to carry out housekeeping processes. Therefore, it must acquire a source of chemical energy such as that stored in the carbon and peptide bonds of non essential proteins. This process must be carried out in a careful and controlled manner for several reasons. First, the order of degradation is important, non essential proteins must go first whereas housekeeping or essential proteins must be preserved. Second, essential-protein rich organelles must avoid degradation until all other sources have been exhausted. Third, the condition of starvation could be reversed at any moment (as is often the case in nature) and the breakdown of the cell should proceed as selectively as possible in order to avoid causing unnecessary or even permanent damage during starvation.

As in the case of starvation, muscle denervation- related protein degradation must proceed in an ordered manner. Denervation of muscle can be the result of injury, disuse or starvation. Depending on the type and severity the of injury the muscle cell may only be slightly damaged and salvageable with only specific-minor repair required. In such a situation, it would be prudent to not remodel the entire cell, but only break down damaged components for rebuilding. In the case of disuse, it would also be in the organism's best interest to slowly breakdown the disused muscles as they may be required at any given moment. Starvation induced denervation presents a similar situation; while the muscle must be broken down for energy, the nutrient tide could quickly turn and it would be preferential to break down as little of the cell as possible.

It is in these situation that autophagy would not be an efficient way to breakdown protein. Autophagy is simply too non specific and may actually do more harm than good. Returning to the tool analogy, garden shears, would be a sloppy and dangerous tool for cutting one's nails.

The need for multiple signaling inputs for one pathway

At first glance, it may appear redundant to have IFG, FGF and TGF-β signaling cascades to regulate autophagy. However, as was the case with having multiple mechanisms for degrading protein, the advantage here is again the ability to "fine tune" levels of protein with regard to multiple stimuli. For example by placing the pro autophagy Ca^{2+} and FGFR signals in the same pathway as IGFR, the cell has evolved an efficient circuit that can vary the degree of degradation at any time. It is in fact, not uncommon for several of these signal molecules to be activated simultaneously competing for their effect. The FGFR pathway may be basally active at low levels most of the time but be shut out by a stronger IGFR signal. This type of regulation makes sense given that it is hypothesized that the origin of the IGF signaling ligand is the neuron. By making the anti-degradation system constitutively active, the neuron constantly informs the muscle that a homeostatic condition exists and there is no need for breakdown. However, in the absence of the neuron or its signaling the homeostatic condition is lifted and the cell will break down protein. Therefore, by combining the counterpoised FGFR and IGFR signaling, the animal is able to constantly monitor and coordinate an optimized response to a multitude of unique situations, reducing material and energy waste.

The TGF-β receptor uses an alternative signaling mechanism

From our western-blots of TGF-β signaling mutants, we saw that MAPK-P levels were actually lower under autophagic conditions then non autophagic conditions. Because autophagy is occurring in samples shifted to 25C, we conclude that the signaling must occur through a different signaling molecule (i.e. a MAPK isoform) or feed in below MAPK.

A reason for an alternate signaling molecule could be to take stress off the use of the MAPK molecule. MAPK is an extremely ubiquitous signaling molecule, shared between many signaling pathways including cellular differentiation, adhesion, growth and is even co-opted in many disease related signaling events. Could it be that because of the complexity of MAPK signaling and the low availability of the molecule to participate in TGF-β signaling, that an alternative signaling mechanism evolved?

Autophagy signaling downstream of MAPK

Recent work on the control of autophagy by (Carriere et al., 2008a; Carriere et al., 2008b)has yielded the following hypothetical model of autophagy control downstream of MAPK signaling in mammals

MAPK→RSK→RAPTOR/TOR—I autophagy

However, this model does not fit with our current results, which show that an activated MAPK promotes autophagy mediated degradation. Also, our data show that RSK signaling is necessary for autophagy. The model that we propose is:

FGFR→→→MAPK→RSK —‖Raptor→TOR—‖UNC-51→→→autophagy

These two models are very similar except in the context that the "sense" of the signaling pathway has been reversed. Our model suggests that MAPK signaling induces autophagy whereas the Carrie model suggests that MAPK inhibits it. There are several interesting possibilities to explain this discrepancy.

One such possibility would be the presence of other signaling molecules that have yet to be accounted for or multiple isoforms of RSK, Raptor or TOR that have varying affects on autophagy. For

example, there exist several forms of RSK, could it be that the pro autophagy form that we have been working is different than the anti autophagy form that Carriere et al have been working with? TOR and Raptor also posses many different isoforms of each molecule, some of which are even insensitive to Rapamycin inhibition (need citation). It is therefore possible that that various complexes of TOR/ Raptor have varying effects on protein degradation. Because these forms are not well studied yet, we are unsure of what induces them or their interaction and that we have independently activated opposing TOR/Raptor complexes that affect autophagy differently. However, it should be noted that the differences in the isoforms of ceTOR are so minor, that it is unlikely that they have opposing functions.

A more likely possibility is that the sense of the signal is reversed between worms and mammals. Although there is strong overall homology in the worm and mammalian Raptor proteins (Jia et al., 2004), the potential RSK phosphorylation sites are not conserved. Thus, it is possible that RSK phosphorylation stimulates mammalian Raptor activity but inhibits worm Raptor.

Another very likely explanation would be that because our work was done specifically in muscle cells and that of the Carriere group was not, that our model is only true for muscle cells, where as the Carriere model is true for other cell types. I believe that both models, are in fact correct for their respective model organism and/or cell type.

VI Acknowledgements

Over the years of time spent in the Jacobson Group and throughout the writing of this work many people and institutions have greatly helped me on my way. This list is very long and it is difficult for me to properly recognize every single person who helped me, it is therefore, that I sincerely regret any omission and would like to assure them that it is not because I did not value their contribution. From the bottom of my heart I would like to thank the following:

VII References/Bibliography

al-Khayat, H.A., Yagi, N., and Squire, J.M. (1995). Structural changes in actin-tropomyosin during muscle regulation: computer modelling of low-angle X-ray diffraction data. J Mol Biol *252*, 611-632.

Ankeny, R.A. (2001). The natural history of Caenorhabditis elegans research. Nat Rev Genet *2*, 474-479.

Argiles, J.M., and Lopez-Soriano, F.J. (1999). The role of cytokines in cancer cachexia. Med Res Rev *19*, 223-248.

Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P.F., Meijer, A.J., Codogno, P., and Ogier-Denis, E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem *276*, 35243-35246.

Arur, S., Ohmachi, M., Nayak, S., Hayes, M., Miranda, A., Hay, A., Golden, A., and Schedl, T. (2009). Multiple ERK substrates execute single biological processes in Caenorhabditis elegans germ-line development. Proc Natl Acad Sci U S A *106*, 4776-4781.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevernic, I., Arthur, J.S., Alessi, D.R., and Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. Biochem J *408*, 297-315.

Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J.*, et al.* (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol *3*, 1014-1019.

Budovskaya, Y.V., Stephan, J.S., Reggiori, F., Klionsky, D.J., and Herman, P.K. (2004). The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in Saccharomyces cerevisiae. J Biol Chem *279*, 20663-20671.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., and Sabatini, D.M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc Natl Acad Sci U S A *95*, 1432-1437.

Cai, D., Frantz, J.D., Tawa, N.E., Jr., Melendez, P.A., Oh, B.C., Lidov, H.G., Hasselgren, P.O., Frontera, W.R., Lee, J., Glass, D.J.*, et al.* (2004). IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. Cell *119*, 285-298.

Cantino, M., and Squire, J. (1986). Resting myosin cross-bridge configuration in frog muscle thick filaments. J Cell Biol *102*, 610-618.

Carriere, A., Cargnello, M., Julien, L.A., Gao, H., Bonneil, E., Thibault, P., and Roux, P.P. (2008a). Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. Curr Biol *18*, 1269-1277.

Carriere, A., Ray, H., Blenis, J., and Roux, P.P. (2008b). The RSK factors of activating the Ras/MAPK signaling cascade. Front Biosci *13*, 4258-4275.

Chang, Y.Y., and Neufeld, T.P. (2009). An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. Mol Biol Cell *20*, 2004-2014.

Chen, Z.H., Kim, H.P., Sciurba, F.C., Lee, S.J., Feghali-Bostwick, C., Stolz, D.B., Dhir, R., Landreneau, R.J., Schuchert, M.J., Yousem, S.A.*, et al.* (2008). Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. PLoS One *3*, e3316.

Cheong, H., Nair, U., Geng, J., and Klionsky, D.J. (2008). The Atg1 kinase complex is involved in the regulation of protein recruitment to initiate sequestering vesicle formation for nonspecific autophagy in Saccharomyces cerevisiae. Mol Biol Cell *19*, 668-681.

Cheong, H., Yorimitsu, T., Reggiori, F., Legakis, J.E., Wang, C.W., and Klionsky, D.J. (2005). Atg17 regulates the magnitude of the autophagic response. Mol Biol Cell *16*, 3438-3453.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

Cuervo, A.M., Bergamini, E., Brunk, U.T., Droge, W., Ffrench, M., and Terman, A. (2005). Autophagy and aging: the importance of maintaining "clean" cells. Autophagy *1*, 131-140.

Dolcini, C., Fano, G., and Jorno, L. (1970). [The arrangement of the molecules of actin, tropolyosin and troponin on the thin filament. Spatial relations between the two orders of filaments]. Boll Soc Ital Biol Sper *46*, 1069-1070.

Epple, U.D., Suriapranata, I., Eskelinen, E.L., and Thumm, M. (2001). Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. J Bacteriol *183*, 5942-5955.

Fire, A. (1992). Histochemical technqiues for locating *Escherichia col*i ß-galactosidase activity in transgenic organisms. Gene Anal Tech Appl *9*, 151-158.

Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature *408*, 325-330.

Fujita, N., Itoh, T., Omori, H., Fukuda, M., Noda, T., and Yoshimori, T. (2008). The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Mol Biol Cell *19*, 2092-2100.

Furuta, S., Hidaka, E., Ogata, A., Yokota, S., and Kamata, T. (2004). Ras is involved in the negative control of autophagy through the class I PI3-kinase. Oncogene *23*, 3898-3904.

Geeves, M.A., and Conibear, P.B. (1995). The role of three-state docking of myosin S1 with actin in force generation. Biophys J *68*, 194S-199S; discussion 199S-201S.

Gems, D., Sutton, A.J., Sundermeyer, M.L., Albert, P.S., King, K.V., Edgley, M.L., Larsen, P.L., and Riddle, D.L. (1998). Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. Genetics *150*, 129-155.

Glass, D.J. (2005). Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol *37*, 1974-1984.

Haas, A.L., Warms, J.V., Hershko, A., and Rose, I.A. (1982). Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation. J Biol Chem *257*, 2543-2548.

Hanada, T., Noda, N.N., Satomi, Y., Ichimura, Y., Fujioka, Y., Takao, T., Inagaki, F., and Ohsumi, Y. (2007). The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. J Biol Chem *282*, 37298-37302.

Haselgrove, J.C., and Huxley, H.E. (1973). X-ray evidence for radial cross-bridge movement and for the sliding filament model in actively contracting skeletal muscle. J Mol Biol *77*, 549-568.

He, C., and Klionsky, D.J. (2009). Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet *43*, 67-93.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu Rev Biochem *67*, 425-479.

Holmes, K.C. (1995). The actomyosin interaction and its control by tropomyosin. Biophys J *68*, 2S-5S; discussion 6S-7S.

Huxley, H.E. (1969). The mechanism of muscular contraction. Science *164*, 1356-1365.

Itakura, E., Kishi, C., Inoue, K., and Mizushima, N. (2008). Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell *19*, 5360-5372.

Jagoe, R.T., Lecker, S.H., Gomes, M., and Goldberg, A.L. (2002). Patterns of gene expression in atrophying skeletal muscles: response to food deprivation. FASEB J *16*, 1697-1712.

Jia, K., Chen, D., and Riddle, D.L. (2004). The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. Development *131*, 3897-3906.

Jontes, J.D., Wilson-Kubalek, E.M., and Milligan, R.A. (1995). A 32 degree tail swing in brush border myosin I on ADP release. Nature *378*, 751-753.

Juhasz, G., and Neufeld, T.P. (2006). Autophagy: a forty-year search for a missing membrane source. PLoS Biol *4*, e36.

Kabeya, Y., Kamada, Y., Baba, M., Takikawa, H., Sasaki, M., and Ohsumi, Y. (2005). Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. Mol Biol Cell *16*, 2544-2553.

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., and Ohsumi, Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol *150*, 1507-1513.

Kawamata, T., Kamada, Y., Kabeya, Y., Sekito, T., and Ohsumi, Y. (2008). Organization of the pre-autophagosomal structure responsible for autophagosome formation. Mol Biol Cell *19*, 2039-2050.

Kihara, A., Noda, T., Ishihara, N., and Ohsumi, Y. (2001). Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. J Cell Biol *152*, 519-530.

Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000). The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol *151*, 263-276.

Klionsky, D.J. (2005). The molecular machinery of autophagy: unanswered questions. J Cell Sci *118*, 7-18.

Klionsky, D.J. (2007). Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol *8*, 931-937.

Kovacs, A.L., Gordon, P.B., Grotterod, E.M., and Seglen, P.O. (1998). Inhibition of hepatocytic autophagy by adenosine, adenosine analogs and AMP. Biol Chem *379*, 1341-1347.

Kundu, M., and Thompson, C.B. (2008). Autophagy: basic principles and relevance to disease. Annu Rev Pathol *3*, 427-455.

Labeit, S., and Kolmerer, B. (1995). Titins: giant proteins in charge of muscle ultrastructure and elasticity. Science *270*, 293-296.

Lehman, W., Vibert, P., Uman, P., and Craig, R. (1995). Steric-blocking by tropomyosin visualized in relaxed vertebrate muscle thin filaments. J Mol Biol *251*, 191-196.

Lehrer, S.S. (1994). The regulatory switch of the muscle thin filament: Ca2+ or myosin heads? J Muscle Res Cell Motil *15*, 232-236.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell *6*, 463-477.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell *132*, 27-42.

Levine, B., and Yuan, J. (2005). Autophagy in cell death: an innocent convict? J Clin Invest *115*, 2679-2688.

Liang, X.H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature *402*, 672-676.

Lorenz, M., Poole, K.J., Popp, D., Rosenbaum, G., and Holmes, K.C. (1995). An atomic model of the unregulated thin filament obtained by X-ray fiber diffraction on oriented actin-tropomyosin gels. J Mol Biol *246*, 108-119.

Maiuri, M.C., Zalckvar, E., Kimchi, A., and Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol *8*, 741-752.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell *10*, 151-162.

Marino, G., Salvador-Montoliu, N., Fueyo, A., Knecht, E., Mizushima, N., and Lopez-Otin, C. (2007). Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J Biol Chem *282*, 18573-18583.

Massague, J. (1998). TGF-beta signal transduction. Annu Rev Biochem *67*, 753-791.

Massey, A.C., Zhang, C., and Cuervo, A.M. (2006). Chaperone-mediated autophagy in aging and disease. Curr Top Dev Biol *73*, 205-235.

Mathew, R., Karantza-Wadsworth, V., and White, E. (2007). Role of autophagy in cancer. Nat Rev Cancer *7*, 961-967.

Megalou, E.V., and Tavernarakis, N. (2009). Autophagy in Caenorhabditis elegans. Biochim Biophys Acta *1793*, 1444-1451.

Melendez, A., and Neufeld, T.P. (2008). The cell biology of autophagy in metazoans: a developing story. Development *135*, 2347-2360.

Melendez, A., Talloczy, Z., Seaman, M., Eskelinen, E.L., Hall, D.H., and Levine, B. (2003). Autophagy genes are essential for dauer development and life-span extension in C. elegans. Science *301*, 1387-1391.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069-1075.

Musaro, A., McCullagh, K., Paul, A., Houghton, L., Dobrowolny, G., Molinaro, M., Barton, E.R., Sweeney, H.L., and Rosenthal, N. (2001). Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet *27*, 195-200.

Nice, D.C., Sato, T.K., Stromhaug, P.E., Emr, S.D., and Klionsky, D.J. (2002). Cooperative binding of the cytoplasm to vacuole targeting pathway proteins, Cvt13 and Cvt20, to phosphatidylinositol 3-phosphate at the pre-autophagosomal structure is required for selective autophagy. J Biol Chem *277*, 30198-30207.

Obara, K., Sekito, T., Niimi, K., and Ohsumi, Y. (2008). The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. J Biol Chem *283*, 23972-23980.

Pallafacchina, G., Calabria, E., Serrano, A.L., Kalhovde, J.M., and Schiaffino, S. (2002). A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. Proc Natl Acad Sci U S A *99*, 9213-9218.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem *282*, 24131-24145.

Parry, D.A., and Squire, J.M. (1973). Structural role of tropomyosin in muscle regulation: analysis of the x-ray diffraction patterns from relaxed and contracting muscles. J Mol Biol *75*, 33-55.

Pattingre, S., Bauvy, C., and Codogno, P. (2003). Amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells. J Biol Chem *278*, 16667-16674.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell *122*, 927-939.

Proud, C.G. (2004). mTOR-mediated regulation of translation factors by amino acids. Biochem Biophys Res Commun *313*, 429-436.

Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E.L., Mizushima, N., Ohsumi, Y.*, et al.* (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest *112*, 1809-1820.

Rubinsztein, D.C., Gestwicki, J.E., Murphy, L.O., and Klionsky, D.J. (2007). Potential therapeutic applications of autophagy. Nat Rev Drug Discov *6*, 304-312.

Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S.H., and Goldberg, A.L. (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell *117*, 399-412.

Scott, R.C., Juhasz, G., and Neufeld, T.P. (2007). Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. Curr Biol *17*, 1-11.

Scott, S.V., Guan, J., Hutchins, M.U., Kim, J., and Klionsky, D.J. (2001). Cvt19 is a receptor for the cytoplasm-to-vacuole targeting pathway. Mol Cell *7*, 1131-1141.

Shintani, T., Huang, W.P., Stromhaug, P.E., and Klionsky, D.J. (2002). Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. Dev Cell *3*, 825-837.

Small, J.V., and Squire, J.M. (1972). Structural basis of contraction in vertebrate smooth muscle. J Mol Biol *67*, 117-149.

Squire, J.M. (1997). Architecture and function in the muscle sarcomere. Curr Opin Struct Biol *7*, 247-257.

Stromhaug, P.E., Reggiori, F., Guan, J., Wang, C.W., and Klionsky, D.J. (2004). Atg21 is a phosphoinositide binding protein required for efficient lipidation and localization of Atg8 during uptake of aminopeptidase I by selective autophagy. Mol Biol Cell *15*, 3553-3566.

Sugita, H., Kaneki, M., Sugita, M., Yasukawa, T., Yasuhara, S., and Martyn, J.A. (2005). Burn injury impairs insulin-stimulated Akt/PKB activation in skeletal muscle. Am J Physiol Endocrinol Metab *288*, E585-591.

Sun, Q., Fan, W., Chen, K., Ding, X., Chen, S., and Zhong, Q. (2008). Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. Proc Natl Acad Sci U S A *105*, 19211-19216.

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001). The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO J *20*, 5971-5981.

Suzuki, K., Kubota, Y., Sekito, T., and Ohsumi, Y. (2007). Hierarchy of Atg proteins in pre-autophagosomal structure organization. Genes Cells *12*, 209-218.

Szewczyk, N.J., Hartman, J.J., Barmada, S.J., and Jacobson, L.A. (2000). Genetic defects in acetylcholine signalling promote protein degradation in muscle cells of Caenorhabditis elegans. J Cell Sci *113 (Pt 11)*, 2003-2010.

Szewczyk, N.J., and Jacobson, L.A. (2003). Activated EGL-15 FGF receptor promotes protein degradation in muscles of Caenorhabditis elegans. EMBO J *22*, 5058-5067.

Szewczyk, N.J., Peterson, B.K., Barmada, S.J., Parkinson, L.P., and Jacobson, L.A. (2007). Opposed growth factor signals control protein degradation in muscles of Caenorhabditis elegans. EMBO J *26*, 935-943.

Teter, S.A., Eggerton, K.P., Scott, S.V., Kim, J., Fischer, A.M., and Klionsky, D.J. (2001). Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. J Biol Chem *276*, 2083-2087.

Thompson, M.G., and Palmer, R.M. (1998). Signalling pathways regulating protein turnover in skeletal muscle. Cell Signal *10*, 1-11.

Timmons, L., Court, D.L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene *263*, 103-112.

Tobacman, L.S. (1996). Thin filament-mediated regulation of cardiac contraction. Annu Rev Physiol *58*, 447-481.

Watkins, S.C., and Cullen, M.J. (1986). A quantitative comparison of satellite cell ultrastructure in Duchenne muscular dystrophy, polymyositis, and normal controls. Muscle Nerve *9*, 724-730.

Whittaker, M., Wilson-Kubalek, E.M., Smith, J.E., Faust, L., Milligan, R.A., and Sweeney, H.L. (1995). A 35-A movement of smooth muscle myosin on ADP release. Nature *378*, 748-751.

Yang, Z., Huang, J., Geng, J., Nair, U., and Klionsky, D.J. (2006). Atg22 recycles amino acids to link the degradative and recycling functions of autophagy. Mol Biol Cell *17*, 5094-5104.

Yorimitsu, T., and Klionsky, D.J. (2005). Autophagy: molecular machinery for self-eating. Cell Death Differ *12 Suppl 2*, 1542-1552.

Yu, W.H., Cuervo, A.M., Kumar, A., Peterhoff, C.M., Schmidt, S.D., Lee, J.H., Mohan, P.S., Mercken, M., Farmery, M.R., Tjernberg, L.O.*, et al.* (2005). Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. J Cell Biol *171*, 87-98.

Zdinak, L.A., Greenberg, I.B., Szewczyk, N.J., Barmada, S.J., Cardamone-Rayner, M., Hartman, J.J., and Jacobson, L.A. (1997a). Transgene-coded chimeric proteins as reporters of intracellular proteolysis: starvation-induced catabolism of a lacZ fusion protein in muscle cells of Caenorhabditis elegans. J Cell Biochem *67*, 143-153.

Zdinak, L.A., Greenberg, I.B., Szewczyk, N.J., Barmada, S.J., Cardamone Rayner, M., Hartman, J.J., and Jacobson, L.A. (1997b). Transgene-coded chimeric proteins as reporters of intracellular proteolysis: starvation-induced catabolism of a lacZ fusion protein in muscle cells of Caenorhabditis elegans. J Cell Biochem *67*, 143-153.

Zhang, H., Stallock, J.P., Ng, J.C., Reinhard, C., and Neufeld, T.P. (2000). Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes Dev *14*, 2712-2724.