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# OLD-AGE MUSCLE ATROPHY: CELLULAR MECHANISMS AND BEHAVIORAL CONSEQUENCES



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BEHAVIORAL CONSEQUENCES

AN EXPERIMENTAL STUDY IN THE RAT

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*Cover: Inkwalk of adult rat (4 months) on front, and inkwalk of aged rat (30 months) on the back.*

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To my family

## Abstract

With advancing age, humans and rodents alike lose about one third of the skeletal muscle mass. A process referred to as old-age muscle atrophy or sarcopenia. Atrophy is a major contributor to disability and morbidity among elderly adults hence the aim of this thesis is to shed light on the molecular mechanisms underlying old-age associated muscle atrophy and behavioral changes related to age in a rat model.

In **Paper I**, we characterized the growth patterns, survival and behavioral alterations linked to advancing age in the rat. The median survival age was, on average, between 29–30 months for both female and male Sprague–Dawley (SD) rats. There was a gradual decline in locomotor activity and explorative behavior associated with age, while disturbances in both coordination and balance did not become evident until later time points. In old age, weight carrying capacity, limb movement and temperature threshold were also impaired. While body weight continues to increase over the better part of the life span of rats, the behavioral changes in old age associated with a decrease in both total body weight and, in particular, muscle mass. Dietary restriction (DR) was found to increase median life span expectancy and impede the development of sarcopenia, and to retard the pace of behavioral aging.

In **Paper II**, we used two-dimensional gel electrophoresis and mass spectrometry techniques to determine changes in protein expression as well as cDNA profiling to assess transcriptional regulations in skeletal muscle of adult and aged male SD rats. Among the highly expressed proteins, thirty-five were differentially expressed in aged muscle. Proteins and mRNA transcripts involved in redox homeostasis and iron load were increased, representing novel components previously not associated with sarcopenia. Iron levels in tissue were elevated in senescence, paralleling an increase in transferrin. Proteins involved in redox homeostasis were found to display a complex pattern of changes involving increases in SOD1 and decreases in SOD2. Together these results suggest that an elevated iron load is a significant component of sarcopenia with a potential to be exploited clinically and that the mitochondria of aged striated muscle may be more vulnerable to radicals produced during cell respiration.

Muscle atrophy, in many conditions, shares a common mechanism for up-regulation of the muscle-specific ubiquitin E3-ligases Atrogin-1 and MuRF1. E3-ligases are part of the ubiquitin proteasome system (UPS) utilized for protein degradation during muscle atrophy. In **Paper III**, we show that Atrogin-1 and MuRF1 are down-regulated in old age-associated muscle atrophy. Our results suggest that this is mediated by AKT-induced inactivation of FOXO4. DR impeded sarcopenia as well as both FOXO4 inactivation and up-regulation of Atrogin-1 and MuRF1 transcripts. Our findings allow us to conclude that sarcopenia is mechanistically different from acute atrophies induced by disuse, disease, and denervation.

The 26S proteasome is responsible for most cytosolic proteolysis. Molecules that inhibit or specially tag proteasomes are helpful tools for analysis of the UPS. In **Paper IV**, we present a new class of proteasome inhibitors, considerably extended in comparison to the commonly used fluorescent substrates and peptide-based inhibitors. Modification of the most active compound, Ada-Ahx<sub>3</sub>L<sub>3</sub>VS, capable of proteasome inhibition in living cells, afforded a new set of radio- and affinity labels. N-terminal extension of peptide vinyl sulfones was found to have a profound influence on both their efficacy and selectivity as proteasome inhibitors. Results demonstrated that such extensions greatly enhanced inhibition and largely obliterated their selectivity towards individual catalytic activities.

The role of the UPS in aging-related muscle atrophy is highly controversial. In **Paper V**, we showed an accumulation of assembled proteasome particles with a corresponding increase in both proteasomal activity and protein degradation in old age muscle atrophy. This was accompanied by a wide range of UPS enzyme-regulation, including an increase in the activity of deubiquitylating enzymes. The accumulation of proteasomes was found to correlate well with muscle wasting. Both the accumulation of proteasome particles as well as the progression of muscle atrophy, were impeded when the normal pattern of aging was challenged by DR. In contrast to many conditions with UPS-associated muscle catabolism, the accumulation of proteasomes during senile muscle atrophy is not caused by transcriptional induction, but rather by decreases in their degradation. The lysosomal pathway is a candidate for degrading proteasomes. In **Paper V**, we demonstrated that impaired lysosomal function, achieved through chloroquine treatment, induced accumulation of proteasomes in adult rats. This emphasizes the existence of a functional link between the lysosomal pathway and the UPS suggesting that a decline in lysosomal function may contribute to increased proteasomal proteolysis in old-age skeletal muscle atrophy.

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## List of Publications

This thesis is based on the following papers, which are referred to by their Roman numerals:

- I. **Mikael Altun**, Esbjörn Bergman, Erik Edström, Hans Johnson and Brun Ulfhake. (2007) Behavioral Impairments of the Aging Rat. *Physiology & Behavior*, doi: 10.1016/j.physbeh.2007.06.017.
- II. **Mikael Altun**, Erik Edström, Eric Spooner, Amilcar Flores-Moralez, Esbjörn Bergman, Petra Tollet-Egnell, Gunnar Norstedt, Benedikt M. Kessler and Brun Ulfhake. (2007) Iron Load and Redox Stress in Skeletal Muscle of Aged Rats. *Muscle & Nerve*, 36, 223-233.
- III. Erik Edström, **Mikael Altun**, Martin Hägglund, and Brun Ulfhake. (2006) Atrogin-1/MAFbx and MuRF1 Are Downregulated in Aging-Related Loss of Skeletal Muscle. *J Gerontol A Biol Sci Med Sci*, 61, 663-674.
- IV. Benedikt M. Kessler, Domenico Tortorella, **Mikael Altun**, Alexei F. Kisselev, Edda Fiebiger, Brian G. Hekking, Hidde L. Ploegh, and Herman S. Overkleeft. (2001) Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping specificities of the catalytic beta subunits. *Chemistry & Biology*, 8, 913-929.
- V. **Mikael Altun**, Brun Ulfhake and Benedikt M. Kessler. (2007) Increased proteolysis due to proteasome accumulation in old-age muscle atrophy. Manuscript submitted.

## List of Abbreviations

19S	Proteasome regulatory complex
20S	Proteasome catalytic core
26S	Proteasome complex
2DE	Two-dimensional electrophoreses
AKT	Protein kinase B
ANOVA	Analysis of variance
Atrogin-1	Atrophy gene 1 (a.k.a MAFbx)
CMA	Chaperone mediated autophagy
DR	Dietary restriction
DUB	Deubiquitylating enzyme
ECF	Enhanced chemifluorescence
ECL	Enhanced chemiluminescence
EE	Enriched/complex environment
FOXO	Forkhead box O
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene expression omnibus
GH	Growth hormone
GHR	Growth hormone receptor
GSSG	Glutathione disulfide
HRP	Horseradish peroxidase
hUMPI/POMP	Human Proteasome maturation factor 1
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IL	Interleukin
INF	Interferon
IRS	Insulin receptor substrate
MBR	Muscle to whole body weight ratio
MN	Motoneuron
MU	Motor unit
MuRF1	Muscle ring-finger protein 1
NCBI	National Center for Biotechnology Information non-redundant
OD	Optical density
PAC	Proteasome assembling chaperone
ROS	Reactive oxygen species
Shc	Src homology 2 domain containing
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
Ub	Ubiquitin
UPP	Ubiquitin-proteasome pathway
UPS	Ubiquitin-proteasome system
USP	Ubiquitin-specific processing protease

## 1 Introduction

### 1.1 General Introduction – Aging

With advancing age, an organism faces an increasing probability of death. Furthermore, aging is commonly associated with the progressive decline of functional capacities and increased morbidity. Similar to expected life-span, the pace and extent of emerging aging-related functional impairments tend to be species specific (cf. the pacific salmon and the hydra (Martinez, 1998)) and often, even vary among individuals within a species. Since aging is not uniform, we talk about *patterns of aging* that can be more or less successful.

### 1.2 Theories on aging

Expected life-span is species specific and commonly closely linked to the species' pattern of breeding and bearing offspring. Expected life-span may have evolved as a species-specific survival strategy, however, this does not explain why we *age* with a progressive decline in functional capacity or why expected life-span show alterations in a historical perspective. Over the years a range of theories aimed at explaining these aspects of aging have been launched (e.g. Wilmoth et al., 2000). At one end, is the theory of *programmed aging* (Lamberts et al., 1997; Weisman, 1891) claiming that aging is only the final chapter in the genetically governed developmental process of an organism. At the other end, there are theories, such as the *disposable soma theory* (Kirkwood, 2005; Kirkwood and Austad, 2000), that claim that there has been no natural selection for growing old and aging only occurs in rare niches where a species (i.e. humans and their domesticated animals) is highly favored or able to control the environment (i.e. harsh competition for limited resources in *wild life*) that normally determine its expected life-span. According to the latter theory, only under such conditions described above is aging prevalent among species members (Medawar, 1952; *idem*). It is outside the scope of this thesis to discuss the pros and cons of these theories (reviewed in e.g. Holliday, 2004; Kirkwood, 2002), however, it is relevant to discuss what mechanism(s) underpin *aging*. Below, a brief account of the current concepts and mechanism(s) in the processes of aging follows, with focus in mammals.

### 1.3 Mechanisms of aging

#### 1.3.1 Genetic make-up and epigenetic modifications

The genome holds the blue print for cell and organism survival machineries. Over the past decade a number of genes that influence life span have been identified mainly through systematic mutagenesis of simpler organisms including *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and *Drosophila melanogaster* (e.g. Hekimi, 2006; Hekimi and Guarente, 2003; Kenyon, 2005; Liang et al., 2003; Mackay, 2002; Pletcher et al., 2005). Many of the identified genes regulate cell metabolism (growth), cell repair and stress responses and are conserved through evolution as is the case of the sirtuin class of enzymes (Guarente and Picard, 2005; Johnson et al., 1999). One may consider that these genes are conserved based on their function in cell maintenance and adaptation to environmental cues and pressures (*idem*). We may expect the list of identified genes that modulate life-span to expand in the upcoming years.

Based on the comparison of genome data, it appears that long-lived species have put more resources in cellular repair and maintenance machineries than short-lived ones (e.g. Kirkwood, 2002; Mackay, 2002; Sanz et al., 2006). Furthermore, variations in mutations of these and other genes may, in part, explain inter-individual variation of life-span within species (*idem*).

During life and perhaps most evident during cell-differentiation in the very early stages of development, the DNA (in cells) is subject to modifications including DNA methylation and chromatin organization (Allegrucci et al., 2005; Bannister and Kouzarides, 2005; Lu et al., 2006; Ooi and Henikoff, 2007; Qiu, 2006; see also <http://dcb.nci.nih.gov/workshoprpt.cfm>) Until recently, these modifications that affect the accessibility of DNA sequences for transcription, were considered exclusive to non-germ-line cells and therefore would not be transmitted to offspring. However, recent reports now indicate that epigenetic modification may be transmittable (Allegrucci et al., 2005; Ooi and Henikoff, 2007).

There are data indicating that the extent and pattern of DNA methylation may change during aging (Bandyopadhyay and Medrano, 2003; Martin, 2005) and major alterations of the epigenetics may result in genome instability (see also below). Therefore it will be of great importance to reveal if epigenetic modifications contribute to the phenotype alterations encountered in cellular aging.

### 1.3.2 Genome stability

In the living organism, DNA (nuclear as well as mitochondrial) is vulnerable to modifications and mutations by exogenous and endogenous sources, collectively these are referred to as DNA damage (e.g. Matsuoka et al., 2007). The main chemical alteration to DNA is through oxidative modifications (Fishel et al., 2007; Lombard et al., 2005). To keep DNA free from modifications and base pair mutations, a large number of genes that encode for DNA repair enzymes are employed (*idem*). However, the watch-keeping and maintenance of DNA integrity is not without flaws (*idem*; Jazwinski, 2000; Lans and Hoeijmakers, 2006) and DNA lesions may accumulate and result in genome instability (Andressoo et al., 2006; Lans and Hoeijmakers, 2006; Mostoslavsky et al., 2006; van de Ven et al., 2007). A common notion is that the probability of genome instability increases with advancing age; a process that may progress into cancer transformation or accentuated/overt aging. Replicating cells with damaged DNA which is unable to be repaired, may be removed by apoptosis to prevent DNA error(s) from causing harm to the organism. The situation is slightly different in terminally differentiated cells such as myocytes or neurons. In these cells, DNA repair mainly occurs through transcriptional coupled repair; since the cells do not undergo further cell division, they are not proof-read and repaired by the global machinery (Andressoo and Hoeijmakers, 2005; Fishel et al., 2007). There are new data indicating that neurons may develop genome-instability and then re-enter cell-cycling for apoptosis to occur.

### 1.3.3 Replicative senescence

Cells that divide are also under the control of the telomeres and associated enzymes; a mechanism by which the number of cell divisions can be restricted (e.g. Blackburn, 2000). The telomeres are repetitive stretches of noncoding DNA and are thought to serve as protective buffers for gene-carrying regions of the DNA (reviewed in Ahmed and Tollefsbol, 2001). If

the telomeres become too shortened, the cells can not continue to divide and therefore enters into a state of replicative senescence that is associated with the emergence of aging stigmata. We are just beginning to understand the significance of this regulatory machinery and its possible role in the aging of organisms (Ben-Porath and Weinberg, 2005; Blasco, 2007; Chai et al., 2005; Cristofalo et al., 2004; Monaghan and Haussmann, 2006; Sarin et al., 2005). Although available evidence suggests this may be true for cellular senescence in replicating cells, it has not been proven for determining the lifespan of an organism. Transgenic animals deficient of telomerase show no decrease in lifespan until the sixth generation (Rudolph et al., 1999), making it safe to assume that the individual organism will die long before replicative senescence will take its toll on cell division. Furthermore, the major hallmarks of aging in complex organisms, such as mammals, stem from changes in postmitotic cells (myocytes and neurons) which with few exceptions do not replicate and where no telomere shortening can be detected (Renault et al., 2002).

#### *1.3.4 Cell metabolism and the amassing of non-degradable waste*

Errors of metabolism, exogenous chemicals, somatic mutations and cell stress are some of the factors that inflict damage to proteins and lipids (carbonylation, oxidative modifications, protein unfolding/erroneous folding) making them not easily degradable or recyclable (Bokov et al., 2004; Halliwell, 2006). Such damaged molecules accumulate over time and appear as intracellular deposits, or inclusion bodies, that are commonly visible in aging postmitotic cells since they are not diluted by cell divisions (Cuervo et al., 2005; Terman and Brunk, 2005; Terman et al., 2007). Age-pigment, or lipofuscin, is perhaps the most abundant species of nondegradable waste material. Its accumulation has been associated with metabolic rate and redox stress (Kurz et al., 2007; Terman et al., 2007; see below). In this sense, aging bear resemblance to several degenerative diseases and is often considered a co-factor in the natural progression of late-onset neurodegenerative diseases (e.g. Bokov et al., 2004; Korolainen et al., 2006).

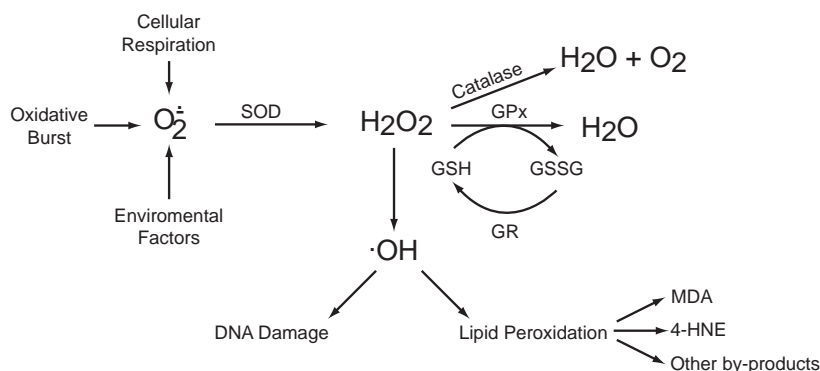
#### *1.3.5 Cell respiration and the free radical theory of aging*

According to the “free radical theory of aging,” (Harman, 1981; Harman, 1994) cellular damage caused by free radicals will accumulate during an organism’s life time. Free radicals, as reactive oxygen species, are formed in the body as a result of metabolism and are toxic if not scavenged or tightly controlled. The mitochondria, ancient bacterial symbionts with their own DNA (mtDNA), are the “power plant” of cells and the site of cellular respiration. Mitochondria convert dietary calories to energy through oxidative phosphorylation releasing radical oxygen species (ROS) as a by-product. While mitochondria produce most of the cellular ROS, radicals are also generated by several other enzymatic reactions, for example, immune reactions (immune cells use targeted release of ROS to defend against invading microorganisms) and in cellular signaling pathways (Beckman and Ames, 1998).

Post mitotic cells with a high metabolic rate, such as neurons, may be particularly vulnerable to aging associated oxidative injury, due to their high metabolic activity. Therefore, if the generation of high levels of ROS (Halliwell, 1992; Keller et al., 1998) in these cells exceeds the scavenging capacity, cells and tissues fall under oxidative stress (Harman, 1981; Harman, 1994).

Through reactions with DNA, RNA, lipids and proteins, free radicals are potentially toxic to cells and can subsequently damage vital cell functions (Bokov et al., 2004; Sanz et al., 2006;

Wallace, 2005). A number of genes in our genome encode for enzymes dedicated to scavenge free radicals. Data indicates that long-lived species appear to have higher expression of free radical scavengers than short-lived species. The defense system for ROS include: (i) superoxide dismutase (SOD) and catalase, that collectively remove superoxide ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ) from the cytoplasm, (ii) glutathione peroxidase glutathione that acts to reduce  $H_2O_2$  to  $H_2O$ , and (iii) vitamins E and C, which function by terminating lipid chain reactions involving peroxy radicals (reviewed by Squier, 2001). Glutathione (GSH) is regarded as a major protectant against ROS (Cooper and Kristal, 1997). GSH is directly involved in redox cycling reactions, in which the reduced form is converted to its oxidized counterpart, glutathione disulfide (GSSG). Many studies have found an age-related shift towards the oxidized form of GSH associated with an accumulation of oxidative damage to DNA in aged individuals (Barja and Herrero, 2000; de la Asuncion et al., 1996; Soong et al., 1992; and references above). The level of oxidative damage appears to correlate with life span as well as age-related impairments, including; decline in cognitive function and motor skills and a loss of muscle mass (Forster et al., 1996; and references above). Oxidative stress is probably a major contributor to DNA damage and posttranslational modifications of proteins during aging and could be instrumental in both cytoskeletal and cell membrane (lipid peroxidation) changes seen in aging (summarized in Fig. 1). However, whether aging is associated with changes in the capacity of ROS scavenging has not been fully resolved (Ashok and Ali, 1999; Benzi and Moretti, 1995; see also Ramirez-Leon et al., 1999).



**Figure 1.** Schematic illustration of cellular and environmental generation of free radicals in a cell, free radical defense and the molecular modifications/damage caused by free radicals if not scavenged.

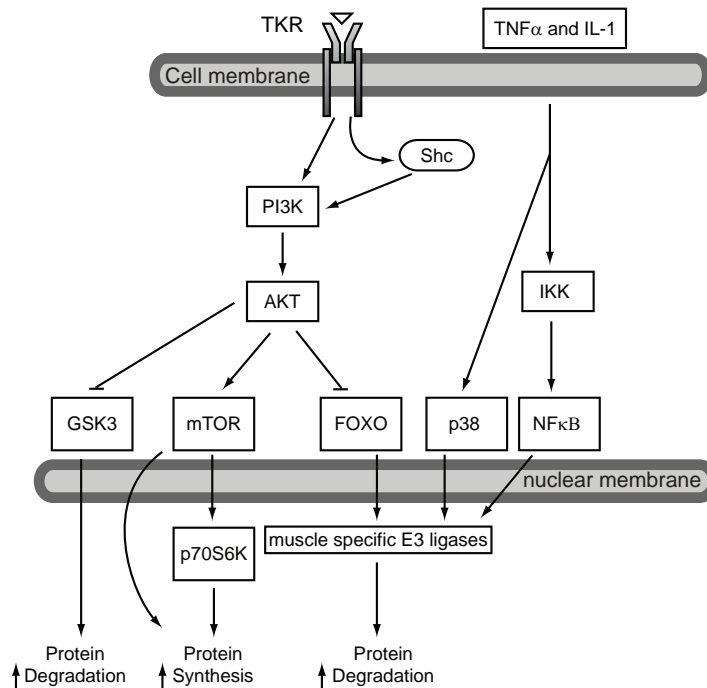
### 1.3.6 Iron and redox stress

Iron is an important and indispensable metal-ion involved in cell respiration (mitochondrial complex IV) and other enzymatic processes, but must be tightly controlled due to its reactivity. It is known from studies on aged liver, kidney, and brain, that iron in these tissues accumulates (Cook and Yu, 1998; Focht et al., 1997; Schipper, 2000) and that ferritin, the major mobile iron-binding protein carrier, increases with age (Piec et al., 2005; Rikans et al., 1997). Free  $Fe^{2+}$  is a key element in fenton chemistry and produces hydroxyl radicals ( $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$ ) through the Haber-Weiss reaction (recently reviewed by Atamna, 2004; Hayes et al., 2005). Moreover, iron load in hepatocytes has been associated with mitochondrial dysfunction (Schipper, 2000) and lipofuscin forms due to iron-catalyzed intralysosomal peroxidation (review in Brunk and Terman, 2002a). The oxidative stress induced by iron-ions

can damage DNA, lipids and proteins. Furthermore, iron-ion associated oxidative stress have been linked to such diverse conditions as cardiovascular disease, the induction of different cancers, and neurodegenerative diseases including Alzheimer's and Parkinson's (reviewed in Valko et al., 2007).

#### 1.4 Skeletal muscle atrophy

In the constant remodeling of human body tissues, the homeostasis of skeletal muscle is of great importance. It has been determined that as much as 280 grams of proteins are turned over per day in males weighing 70 kg (Mitch and Goldberg, 1996). Skeletal muscles are a reservoir for amino acids that are targeted when systemic nutrients, normally supplied by the liver, are low. A net efflux of amino acids stemming from skeletal muscles, recruited by the degradation of myofibrils, will result in muscle atrophy. This occurs not only in starvation, but also with disuse (unloading), denervation and a number of systemic diseases (cancer, Cushing's disease, kidney failure etc.) (reviewed in Attaix et al., 2005a; Glass, 2003; Glass, 2005; Lecker et al., 2006). The protein breakdown machineries mainly responsible for myofibrillar degradation are the endocytic calpains and the ubiquitin-proteasome system (UPS, (reviewed in Attaix et al., 2001; Attaix et al., 2005b; Glass, 2003; Jagoe and Goldberg, 2001)). The role of the lysosomal cathepsins in muscle atrophy is still unresolved. The lysosomal system is rap-



**Figure 2.** Schematic illustration of tyrosine kinase receptor activation and downstream signaling via the AKT pathways. Activated AKT will decrease protein degradation through deactivating FOXO and GSK3 and increase protein synthesis by activating mTOR-p70S6K signaling. Muscle specific E3 ligases promoting protein degradation by the 26S proteasome, is not only regulated by the FOXO:s, but also by NF $\kappa$ B and p38 signaling through TNF- $\alpha$  and IL-1.

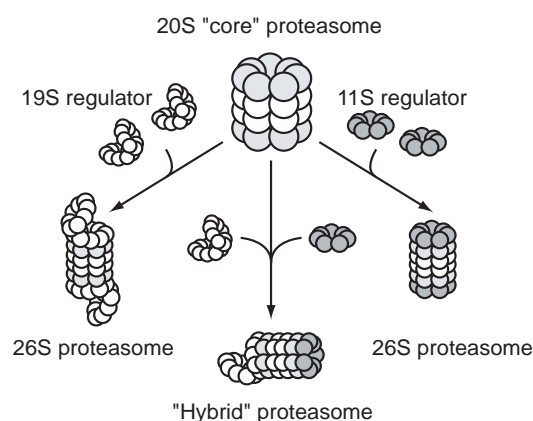
idly induced in conditions of cell starvation but does not appear to be critical for myofibrillar protein breakdown (Kettelhut et al., 1994). Instead, in a wide range of conditions (see above), muscle atrophy share a common triggering mechanism (Fig. 2) for the induction of muscle-specific E3-ligases (ligases that flag myofibrillar proteins for degradation in the proteasome (see below)) (reviewed in Glass, 2005; Lecker et al., 2004; Lecker et al., 1999; Mitch and Goldberg, 1996). The signaling pathway involved in its induction as well as the proinflammatory signals such as TNF $\alpha$  (tumor necrosis factor  $\alpha$ ) and IL-1 (interleukin-1) that in turn activates NF $\kappa$ B or p38 (summarized in Fig. 2), have been rigorously dissected experimentally (reviewed in Glass, 2005). There are solid evidence that the transcription of the E3-ligases, Atrogin-1/MuRF1 and MURF-1, are induced by the FOXO (forkhead box O) family of transcription factors, p38 and NF $\kappa$ B and are key elements involved in skeletal muscle atrophy (Bodine et al., 2001; Glass, 2005).

One of the main characteristics of old-age in humans is the loss of muscle mass, known as sarcopenia. In contrast to the wide range of conditions described above, part of this thesis describes how sarcopenia is not associated with an induction of the muscle-specific E3-ligases Atrogin-1 and MuRF1 (**Paper III** and **V**) but may, through a different mechanism be due, at least in part, to increased proteasomal proteolysis (see **Paper V** and General Discussion).

## 1.5 Ubiquitin Proteasome System

### 1.5.1 The Proteasome

The major component of the ubiquitin proteasome system (UPS) is the 26S proteasome which is a large multi-catalytic protease complex composed of a 700kDa inner 20S core associated with two 900kDa 19S (PA700) caps (Fig. 3; Matthews et al., 1989; McGuire et al., 1988; Tanaka et al., 1988). The outer rings, which interact with the 19S regulatory caps, consist of seven highly homologous  $\alpha$ -subunits ( $\alpha$ 1- $\alpha$ 7). The two inner rings are assembled from seven distinct  $\beta$ -subunits ( $\beta$ 1- $\beta$ 7), where three of these are catalytically active ( $\beta$ 1,  $\beta$ 2,  $\beta$ 5) and mediate the caspase-like, trypsin-like, and chymotrypsin-like activity, respectively (Fenteany et al., 1995; Groll et al., 1997; Kisselev et al., 1999; Lowe et al., 1995).



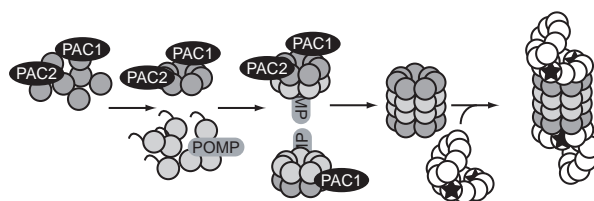
**Figure 3.** Schematic illustration of the different proteasome species present in cells.

Upon treatment with  $\text{INF}\gamma$  or  $\text{TNF}\alpha$ , the catalytic  $\beta$  subunits can be replaced by the  $\beta 1i$ ,  $\beta 2i$ ,  $\beta 5i$  subunits (Brown et al., 1991; Gaczynska et al., 1993; Griffin et al., 1998; Groettrup et al., 1997; Schmidt and Kloetzel, 1997) and is then referred to as the immuno-proteasome, a unique proteasome that has different cleaving sites and a faster enzymatic cleaving rate. To make it even more complicated, it has been observed that  $\text{INF}\gamma$  not only induces the formation of “pure” immuno-proteasomes, but also 20S complexes formed by both immuno- and constitutive-subunits. For example, the  $\beta 5i$  subunit is found to be present in the 20S without the other immuno-proteasome subunits (Fenteany et al., 1995). These observations raise the question about the general role of the immuno-proteasome and its “hybrid” species. Several studies addressing the generation of antigenic epitopes from immuno-proteasomes suggest that this system has evolved to optimize antigenic peptide production with major histocompatibility complex class I specificity, therefore enhancing presentation by major histocompatibility complex molecules and subsequent immune responses (Sijts et al., 2000).

The regulatory components of the 26S proteasome are the 19S, 11S and the newly discovered PA200. The 19S regulatory complex, responsible for the recognition of ubiquitylated-proteins, deubiquitination, unfolding, and processing substrates into the 20S proteolytic core, can be divided into a base- and lid- subcomplex. The base is composed of six ATPases belonging to the triple “AAA” family (Rpt1-6, all of which are ATP dependent with a variety of cellular activities) and two non-ATPase subunits, which bind to the 20S core. The ATPase subunits are those believed to be involved in the unfolding of substrates and channeling them into the protease. The residual eight subunits of the 19S form the lid, however, their function is not yet fully understood (Braun et al., 1999; Glickman et al., 1999; Strickland et al., 2000). The 19S regulatory complex can be replaced by another complex, the PA28 or 11S regulatory complex, which replaces one or both of the 19S regulatory caps after treatment with  $\text{INF}\gamma$  (Dubiel et al., 1992; Ma et al., 1992). This complex is composed of two distinct  $\alpha$ - and  $\beta$ -subunits that form a hexa or heptameric ring (Knowlton et al., 1997). The 11S complex also exhibits ATPase activities, similar to the 19S, but to date, there is little known about the exact function of the 11S regulatory complex (Preckel et al., 1999). A proposed function of the 11S, is an accelerated release of proteasome breakdown products which is more optimal for the generation of antigenic peptides (Whitby et al., 2000). The recently discovered PA200 has an asymmetric dome-like structure with major and minor lobes that has been suggested to binds to the 20S as a monomer (Ortega et al., 2005). The least is known about PA200, it has been described to be located in the nucleus and linked to DNA repair (Ustrell et al., 2002).

The  $\beta$ -subunits of the proteasome are transcribed as precursors that do not display catalytic activity until the proteasome is fully assembled and the pro-peptide part is cleaved off (Kloetzel, 2001). The assembly and maturation of this large complex is promoted by several chaperone proteins, such as proteasemblin/human proteasome maturation factor UMP1 (POMP/hUmp1), proteasome assembling chaperone one (PAC1) and two (PAC2) (Burri et al., 2000; Griffin et al., 2000; Hirano et al., 2005; Witt et al., 2000). The formation of the heptameric  $\alpha$ -ring is formed by the binding of PAC1 and PAC2 to the  $\alpha$ -subunits and to the ring assembly. The pro-beta subunits are in the presence of POMP (with binds to the propeptide) arranged on the  $\alpha$ -ring to form a complex named “half proteasome.” This structure consists of one  $\alpha$ -ring, one  $\beta$ -ring and the assembly chaperones. The maturation is completed when two “half-proteins” dimerize, the pro-peptides of the  $\beta$ -subunits are removed and the assembly chaperones degraded (see Fig. 4; Burri et al., 2000; Griffin et al., 2000; Hirano et al., 2005; Witt et al.,

2000). The assembly of the 19S caps is poorly understood. The current working model is that the six ATPase subunits (Rpt1-6) of the base first assemble as a hexameric ring, after which the other two base subunits, Rpn1 and Rpn2, associate with the 20S-proteasome (Gorbea et al., 1999). The binding of the base to the 20S has been postulated to be regulated by phosphorylation. It has been demonstrated that at least Rpt6, one of the ATPases, is phosphorylated upon its association with the  $\alpha$ 2 subunit of the 20S (Satoh et al., 2001). Little is known about the rest of the assembly of the 19S proteasome, especially with regard to the lid joining the base, although it is certain that these components interact (Fu et al., 2001; Yen et al., 2003).



**Figure 4.** Schematic illustration of the assembly process of the 20S and 26S proteasome, mediated by the chaperons PAC1, PAC2 and POMP.

The distribution pattern and the various forms of proteasome differ between different cell types. Studies examining the distribution of different proteasome forms have mainly been directed towards the 26S proteasome (i.e. 20S-19S complex). The expression patterns of 20S and 26S have been investigated in several tissues. High concentrations of the 26S proteasome have been reported in both the spleen and brain, whereas skeletal muscle contained very low amounts. In liver and skeletal muscle, the 20S proteasome species has been found to be the most abundantly expressed followed by the 26S, and then by the immuno-proteasome which is normally expressed at much lower levels (Beyette et al., 1998; Brooks et al., 2000; Peters et al., 1994). The subcellular localization of the proteasome reveals that the highest concentration of is in the cytosol, followed by microsomal and nuclear localization (Brooks et al., 2000).

The proteasome is involved in many biological processes. These include cell cycle control, by the degradation of cyclins, cell differentiation, by the destruction of transcription factors or metabolic enzymes, direct removal of misfolded or improperly assembled proteins, and stress responses, through the processing or degrading transcriptional regulators (Coux et al., 1996; Deveraux et al., 1994; Voges et al., 1999). Degradation of proteins by the proteasome can be mediated in two ways; an ubiquitin independent 20S and 26S degradation, or by the tagging with lysine48 poly-ubiquitin, a signal for 26S ubiquitin dependent proteolysis.

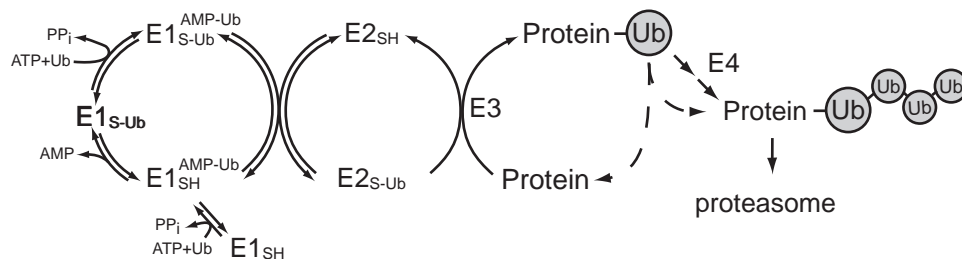
Whereas the most common and controlled way of degrading proteins appears to be via the 26S degradation of poly-ubiquitylated proteins, proteolysis by the 20S is both ubiquitin and ATP independent. Moreover, proteolysis by the 20S seems to be the route favored for the elimination of oxidatively modified proteins (Davies, 2001; Shringarpure et al., 2003). It has been shown that oxidation causes an increase in surface hydrophobicity through a partial unfolding or denaturation of the protein, and where certain hydrophobic peptides can activate the peptidase activity of eukaryotic 20S proteasomes, apparently through a pore-gating mechanism (Davies and Shringarpure, 2006; Kisselev et al., 2002).

### 1.5.2 Ubiquitin

Ubiquitin a large, 76 amino-acid protein, is expressed ubiquitously in all eukaryote cell types and its sequence is conserved from yeast to human (Jentsch et al., 1991). Ubiquitin is covalently linked to other proteins through an isopeptide bond, most commonly formed between the C-terminal glycine (Gly76) of ubiquitin and the  $\epsilon$ -NH<sub>2</sub> group of an internal lysine residue of the target protein. The current understanding is that protein modification by ubiquitin can be mediated by either the attachment of a single ubiquitin or by several single ubiquitins, referred to as mono- and multi-ubiquitination, respectively. The latter of the two can occur on the N-terminal of a protein (N-terminal ubiquitination) or in prolonged chains (poly-ubiquitination). Ubiquitin has seven lysyl residues all which can function as potential conjugation sites; Lys 6, 11, 27, 29, 33, 48 and 63 (Pickart, 2000; Weissman, 2001). Proteins destined for degradation by proteasomes are tagged by Lys48 linked poly-ubiquitylation through the ubiquitin conjugating cascade (E1, E2 and E3, and potentially elongated by E4 (Hershko and Ciechanover, 1998)). When attached to the 26S proteasome, ubiquitin is removed from the targeted protein by deubiquitylating enzymes (DUBs) (Wilkinson, 2000), that enables the recycling of the ubiquitin molecules to the cytosolic pool of “free ubiquitin.” The large number of genes encoding E3 ligases and DUBs makes it likely that these two families of proteins govern the substrate specificity of the UPS directed proteolysis (Pickart, 2001).

### 1.5.3 Ubiquitin conjugating cascade

The ubiquitin conjugating cascade is a sophisticated mechanism involving three primary enzyme families; ubiquitin-activating enzyme (E1), ubiquitin-carrier protein (E2) and ubiquitin-protein ligase (E3). In order for poly-ubiquitylation to occur, some substrates may also require the action of the chain elongation factor (E4) (Koegl et al., 1999; Weissman, 2001). To date, only two E1 (Jin et al., 2007) and approximately 30 E2 enzymes have been described, while several hundred tentative E3 ligases have been identified (Ardley and Robinson, 2005; Wilkinson, 2000). The E3 ligases are subdivided into at least four different classes based on their activity and structural domains: HECT-type, RING-type, PHD-type, and U-box containing (Wilkinson, 2000). The fact that there are a large number of E3 ligases and their role in tagging ubiquitin to specific substrates delineates them as a key element for the specificity and sophistication of the cascade.



**Figure 5.** Schematic illustration of the different steps in the ubiquitin conjugating cascade tagging proteins for degradation by the proteasome.

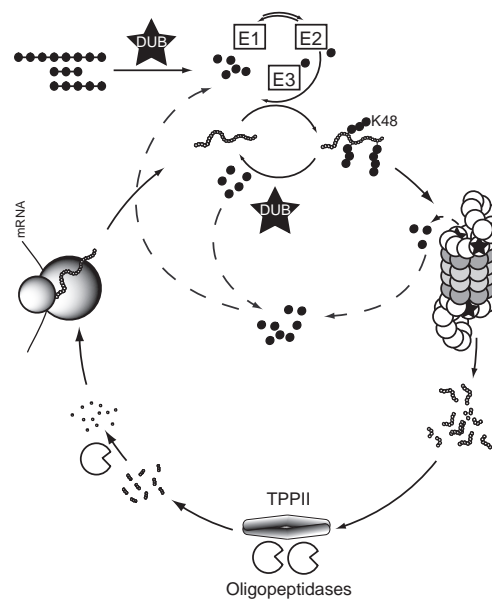
The first step of the ubiquitin conjugating cascade is an ATP-dependent process by which the ubiquitin-activating enzyme (E1) forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin. As a second step, the ubiquitin-carrier enzyme (E2) accepts ubiquitin from the E1, by transthioylation, a required step that again involves the carboxyl terminus of ubiquitin.

As the third and final step in this cascade, the ubiquitin protein ligase (E3) catalyses the transfer of ubiquitin from the E2 enzyme to the  $\epsilon$ -amino group of a lysine residue on the substrate (see Fig. 5; Hershko and Ciechanover, 1998).

#### 1.5.4 Deubiquitylating enzymes (DUBs)

The DUBs are characterized into at least five distinct subfamilies based on sequence similarities and likely mechanism of action (reviewed in Nijman et al., 2005). These are; the USP DUB family, or ubiquitin-specific processing protease group; the ubiquitin-specific cysteine protease subfamily, or ubiquitin carboxy-terminal hydrolases (UCHs); the OTU (*ovarian tumor*)-related proteases; ataxin-3 subfamily (the only member identified so far belonging to this subfamily); and finally Rpn11/POH1 subfamily, a subunit of the proteasome itself which carries features of a metalloprotease specific for protein-linked ubiquitin. Known specific function(s) of the different DUBs is being involved in the processing of ubiquitin precursors, the editing or rescuing of ubiquitin conjugates, the coupling of protein deubiquitination and degradation by the proteasome, the disassembly of ubiquitin oligomers, and membrane protein trafficking.

Since all ubiquitin proteins are transcribed as precursors, the DUBs become important controllers of free ubiquitin, by cleaving the chains and releasing them as ubiquitin monomers. The reason why ubiquitin is synthesized as a pre-form and its need to be cleaved is not known, however, it has been speculated as a quality control step.



**Figure 6.** Schematic illustration of the major components of the ubiquitin proteasome system. Proteins targeted for degradation will get tagged with ubiquitin by the ubiquitin conjugating cascade (E1, E2 and E3), deubiquitylated by deubiquitylating enzymes (DUB) and degraded by the 26S proteasome. Peptides generated by the proteasome will get further processed to free aminoacids in several steps involving several peptidases incl. tripeptidylpeptidase II (TPPII) and different oligopeptidases.

The chain of ubiquitin must be released from a substrate before it may enter the catalytic core of the proteasome. The DUB that is responsible for the main bulk of unanchored/released polyubiquitin is USP14 (Hu et al., 2005). DUBs are therefore of great importance for ubiquitin recycling and allow for its re-use. Failure to properly detach polyubiquitin from its substrate could either lead to its inappropriate degradation or could interfere with the entry of the substrate into the narrow opening of the central proteolytic chamber of the proteasome.

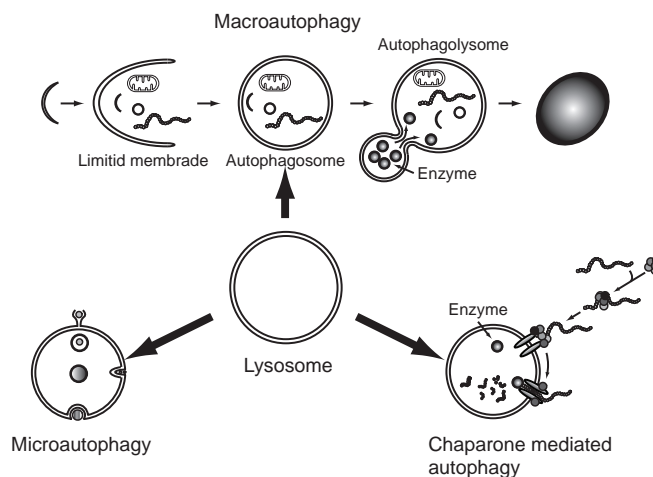
Ubiquitin is an important player in proteasomal degradation, but nevertheless, also key to the internalization of endosomes and the shuttling of vesicles between the membrane and different cellular compartments (e.g. lysosomes) through mono-ubiquitination. The DUBs can reshuffle ubiquitin from poly- to mono- or multi-ubiquitin chains making them important for protein half-life and cellular localization (Clague and Urbe, 2006).

Degradation is the end point for any protein and the control of degradation is critical to cell homeostasis (the UPS is summarized in Fig. 6). Through deubiquitylation, proteins can be rescued from degradation, making the proteolysis machinery a tightly regulated process.

## 1.6 Lysosome

### 1.6.1 General

The lysosome is a vesicular structure within mammalian cells that maintains an acidic pH (pH 4-5) by proton pumps and is core to the autophagy-lysosomal degradation system. The major targets for lysosomal degradation are organelles, plasma membrane and long-lived proteins. Lysosome recruits hydrolases, proteases, glycosidases, lipases, nucleases and phosphatases through the endoplasmic reticulum and Golgi elements which are the strategic cell centers for synthesis and trafficking of lysosomal enzymes. A particular enzyme family, the cathepsins, is transported to the lysosome in a pro-peptide form. The cathepsins are synthesized as a pre-pro-peptide, where the pre-peptide is cleaved during early translation, and where the pro-peptide becomes activated only after cleavage when it enters the acidic environment of the lysosome (reviewed in Bechet et al., 2005).



**Figure 7.** Schematic illustration of the three different lysosomal degradation routes, via microautophagy, macroautophagy and chaperone mediated autophagy.

Lysosomal degradation occurs through three pathways based on the substrate delivering mechanism. These are referred to as microautophagy, macroautophagy and chaperone mediated autophagy (CMA). Macroautophagy is the entrapment of large molecules and/or organelles between a double membrane, or autophagosome (Sneve et al., 2005; Terman and Brunk, 2004a). Later the autophagosome fuse with a lysosome (Terman and Brunk, 2004a). Microautophagy consists of the lysosomal membrane engulfing a portion of the cytosol through invagination later to be pinched off for its subsequent degradation within the lysosomal lumen (Ahlberg and Glaumann, 1985). The 3<sup>rd</sup> mechanism is through chaperone-mediated uptake. Several proteins act as chaperones and assist the entrance of proteins into the lysosome via an active process (Fig. 7). The lysosome appears to be involved in catabolism in conditions of starvation in order to free amino acids (Finn and Dice, 2006). Whether this process is involved and contributes to sarcopenia is not known, but should not be excluded.

### 1.6.2 Lysosome in aging

A common phenomenon of aging cells is the occurrence and subsequent build-up of lipofuscin or aging-pigment (introduced by Hueck 1912 (Hueck, 1912)), from the Greek *lipo* (for fat) and Latin *fuscus* (for dark). The first description of lipofuscin dates back to the year 1842 by Hannover (Hannover, 1842), and the relationship between lipofuscin and aging was first recognized in 1886 by Koneff (Koneff, 1886) who described lipofuscin to be being most abundant in post mitotic cells (i.e. neurons and myocytes) (Terman and Brunk, 2004a). Lipofuscin is a mixture of various components, but predominantly undegradable proteins and lipids. While the lipid components of lipofuscin are well-characterized, much less is known about its protein content. Other components of lipofuscin include carbohydrates and metals and are especially rich in iron (Brun and Brunk, 1970; Jolly et al., 1995; Terman and Brunk, 1998). Lipofuscin is localized mainly in lysosomes, but can also be detected in other cellular compartments. The formation of lipofuscin appears to relate to the production of ROS and it forms due to iron-catalyzed intralysosomal peroxidation (Kurz et al., 2007; Terman and Brunk, 2004). The accumulation in the lysosomal compartment of lipofuscin seems to set down the capacity of autophagy (reviewed in Brunk and Terman, 2002a).

The lysosome is affected by aging in several ways. One of these involves the accumulation of lipofuscin within the lysosome itself, causing newly synthesized enzymes entering the lysosome to come to a dead end. While these enzymes are fully functional, lysosomes that contain lipofuscin trap the enzymes from acting on degradable substrates (Brunk and Terman, 2002b; Kurz et al., 2007). Additionally, lipofuscin components can inhibit the proton pumps of the lysosome, that cause a shift in pH that is less optimal for proteolytic activity and degradation process (Bergmann et al., 2004). A decreased capacity of the lysosomes to degrade waste will cause a build-up of damaged organelles and proteins disturbing cell homeostasis. Disruption of lysosomal integrity which may occur in marked redox stress conditions triggers cell apoptosis (reviewed in Kurz et al., 2007) and a number of papers have recently reported on increased incidence of apoptosis in aged skeletal muscle (e.g. Dirks and Leeuwenburgh, 2002; Kujoth et al., 2005; Whitman et al., 2005; see however Rice and Blough, 2006). As mentioned above impediment of autophagy, or gene knock-out of cathepsins, associate with degeneration of long-lived cells (Felbor et al., 2002; Hara et al., 2006; Komatsu et al., 2006). As will be shown in this thesis there is a build-up of lipofuscin in old age skeletal muscle atrophy (**Paper V**).

## **2 Aims of the Thesis**

In comparison with other conditions of muscle atrophy, sarcopenia remains poorly understood. Over the years a number of theories aimed at explaining the mechanism that underpin old-age muscle atrophy have been proposed (see General discussion). These include concepts that sarcopenia is of neurogenic origin (i.e. due to loss of motoneurons); or caused by impoverished regenerative capacity; aging-related cell metabolic changes; or systemic alteration in aging (somatopaus). This thesis addresses changes in myofiber metabolism in old age with the specific aims:

- i. To explore growth pattern, basic behavior and mortality data of the laboratory rat with emphasis on alterations with advancing age and patterns of aging.
- ii. Biochemical characterization of aged skeletal muscle and sarcopenia.
- iii. To characterize changes in the ubiquitin-proteasome system in old-age and proteasome proteolysis in sarcopenia.
- iv. To study the effect of dietary (caloric) restriction on pattern of aging and old-age muscle atrophy.

### 3 Materials and Methods

#### 3.1 Cell lines

The rat muscle derived cell line L6 (in **Paper V**), was maintained under standard conditions using 37°C/5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; Gibco; Rockville, MD, USA) supplemented with 2mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% heat-inactivated FCS. The human cervix carcinoma cell line HeLa and mouse thymoma cell line EL-4 (**Paper IV** and EL-4 was used as a control in **Paper V**) were maintain under the same conditions, except that EL-4 was cultured in RPMI 1640 (Gibco; Rockville, MD, USA), supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% heat-inactivated FCS.

#### 3.2 Experimental animals

Animal data derive from analysis of laboratory rats and in **Paper I-III** and **V** we used outbred Sprague-Dawley (SD) rats (Harlan Sprague-Dawley, Houston, Texas, US) while in **Paper I** we also compare data from several different rat strains.

Animals were either delivered at an age of 2 months by a commercial breeder (SD, B&K or Scanbur, Stockholm, Sweden; CrI:SD (SD), Charles River, Germany) or bred in-house with founders from one of these stocks. Males were invariably virgins, while females were either virgins or retired breeders. Smaller groups of Lewis (inbred stock LEW/CrI; Charles River, Germany), Wistar (out bred WI, Wistar:Bkl, Wistar Institute, Philadelphia, US) and Fischer 344 (inbred F-344/DuCrI, Charles River, Germany) rats were used for comparison in **Paper I**. Several SD animal cohorts were kept on a dietary restriction (DR) corresponding to 70% of *ad libitum* (AL) intake of age-matched control animals. A smaller number of SD rats were housed in an enriched/complex environment (EE) consisting of large cages with ample play materials (tubes, running-wheel, plastic houses) and a housing group size of 9 (**Paper I**). All animals were kept under standardized barrier conditions (with 12/12 light/dark cycle in a climate-controlled environment). In addition, adults and aged AL rats in **Paper V** (deriving from the same colony) were treated with dexamethasone (DEX; 4 mg/kg i.p. for three days), seven adults were treated with chloroquine (Cq; 50 mg/kg i.p. for 16 days). Based on life-span expectancy of male and female SD rats (**Paper I**), the 30 month old rats were considered of old age (senescent). All experiments were approved by the Local Ethical Committee (Stockholm's Norra Djurförsöksetiska Nämnd; project no. N263/95; N90/97; N54/00; N221/03; N400/03; N122/03; N122/06 N123/06 and N124/06).

#### 3.3 Behavioral testing

*Open field activity* - Explorative behavior was examined with the open field test (Dorce and Palermo-Neto, 1994; Drago et al., 1996; Peng et al., 1994), using a square area with walls (70 × 70 × 30 cm) in gray colored plastic. The floor was subdivided into 25 equally large squares. In dim light the animal was placed in the center of the arena and allowed to freely explore the field for 180 sec. During this period the following behavioral characteristics were evaluated: a) ambulation frequency (number of squares entered with all four feet); b) rearing frequency (number of times animals stood on the hind limbs); c) immobility frequency (number of episodes of more than 3 sec without movement); d) the frequency of urination and defecation. In

between animals, the open field was carefully washed with a water-ethanol solution to eliminate possible bias due to odors left by previous subjects.

*Crossing a wire mesh screen* - A 70 cm long, 2.5 cm-wire mesh screen was used. In dim light, the animal was placed at one end of the path and a 60 W light source was directed to this spot. At the other end of the path, the “home cage” with littermates was placed. Each animal was given 90 sec to cross the path. Records included distance, time, and number of errors, i.e. instances of misplaced hind paws (slips). Hind limb performance was evaluated since this test, like the beam walking test (Alexis et al., 1995), presumably examines hind limb sensorimotor function.

*Beam balance* - A 2.5 cm-wide wooden beam was suspended 0.5 m above a soft surface. The rat was placed on the beam for a maximum of 60 sec, and the performance was ranked according to the following scale (adopted from Clifton et al., 1991):

1. Balances with steady posture; paws on top of the beam;
2. Grasps sides of beam and/or has shaky movement;
3. One or more paw(s) slip off beam;
4. Attempts to balance on beam but falls;
5. Drapes over beam and/or hangs on beam and falls off;
6. Falls off beam with no attempt to balance or hang on.

Each animal was subjected to three consecutive trials, and the mean score of these trials was calculated.

*Walking track analysis* - For this test, the animals feet were immersed in non-toxic acrylic color (fore paws with red and hind paws with black color) and they then had to walk through an 8.5 × 42 cm transparent Plexiglas tunnel with the “home cage” at the other end. A stripe of high-quality paper was placed on the runway floor and taken out for analysis after the animal had crossed the path. The following records were made from the walking tracks: a) stride length (distance between fore paw-fore paw and hind paw-hind paw); b) gait width (distance between left and right hind paws), c) placement of hind paw relative to fore paw (distance between hind paw-fore paw in each step cycle).

*Placing reaction* - Tactile placing was evaluated by supporting the animal’s trunk while allowing the limbs to hang freely. The animal was then brought towards a table edge, and the dorsal and plantar surface of each foot were gently touched. For each test a score of 1 was given for normal, immediate placing; a score of 0.5 was given if the placing was delayed or incomplete; a score of 0 indicated absent placing (Alexis et al., 1995; Gale et al., 1985).

*Righting response* - The rat was held in the examiner’s hand approximately 30 cm above a soft surface, and the righting reflex was elicited by turning the rat over on its back upon release. The rat’s attempt to right itself was studied and a score of 2 was given if the animal showed a normal righting response, i.e. counter to the roll direction; a score of 1 was given if the righting response was weak, delayed or in the direction of the roll; a score of 0 indicated no righting attempt (Gale et al., 1985; von Euler et al., 1996).

*Nociceptive hot plate test* - A plexiglas cylinder with a diameter of 20 cm was placed on a plate (30cm x 40cm), set to maintain a temperature of  $52 \pm 0.5^\circ\text{C}$ . The animal was placed on the heated surface until it licked paws, stamped, jumped or vocalized. The time lapse between placement and reaction was recorded as response latency (Espejo and Mir, 1993; Langerman et al., 1995). The cut-off time was set at 30 sec to avoid tissue damage.

The *LABORAS system* - For the automated recording of rodent behaviors in macrolon cages, this system was used to compare basic motor activities of middle-aged rats fed ad libitum to those of rats maintained on DR. The animals were placed in the macrolon cages individually, with the same bedding material and access to food and water as in their standard home cages. In our set up four cages were run in parallel and the recording time was set to 23 hrs for practical reasons (1h for was out between animal groups).

*Patterns of aging and stage ranking* - Gait cycle disturbance and weight bearing incapacitation (for example showing up as a decline in rearing behavior and impaired support of body weight) are characteristics of the old age phenotype. However, not all senescent rats disclose these stigmata. This is particularly evident in outbred rat strains. We therefore developed a simple stage-ranking protocol based on two parameters of hind limb function (Johnson et al., 1995; reviewed in Ulfhake et al., 2002):

- (a) Is a limb weight bearing? This was determined by the animals ability to extend the limb and use only the (sole of the) paw as supportive surface during a gait cycle. Infrequent errors to extend the leg were scored 1, while more frequent errors scored as 2. Complete failure to extend the limb during gait, leg and trunk resting on the supportive surface, was scored as 3.
- (b) Does a limb show a complete gait cycle coordinated with the other limb(s)? A score of 0 was given if a limb showed all 4 phases (stance, paw-off, swing, paw-on) coordinated with the movement of the other limbs. Infrequent error disrupting the stride rhythm was given a score of 1, while frequent errors resulting in a limping stride pattern was scored as 2. Severe limping and partial immobility (dragging the limb along) were scored as 3.

According to this scheme low symptom animals belong to stage 0 (no symptoms; score 0) or 1 (minor signs or infrequent errors; score 1) and high symptom animals belong to stage 2 (clear gait cycle aberrations, and decreased body weight support power in at least one limb, score 2) and stage 3 (advanced gait aberrations with signs of partial immobility of at least one limb, score 3).

### **3.4 Experimental regeneration of skeletal muscle**

In this thesis, data on hind limb skeletal muscle regeneration are presented for comparison (Altun, et al., unpublished data). In short, adult rats were under deep anesthesia subjected to nerve crush of the sciatic nerve at the mid-thigh level. After recovery from the surgery the animals were monitored for functional recovery of innervation and hind limb muscle strength. Different groups were allowed to survive for 4 up to 52 days post surgery and then sacrificed for analysis of different hind limb muscles and the nervous system.

### 3.5 Preparation of tissues

The triceps surae muscles (gastrocnemius and soleus) were removed from anesthetized (chloral hydrate 300 mg/kg, i.p.) rats, weighed and frozen in liquid nitrogen. All tissues were stored at  $-80^{\circ}\text{C}$  until processed.

The soleus muscle was used to evaluate the adaptation of hind limb muscle to body weight bearing demands. The rationale for using soleus is that this muscle is postural and steadily active in all types of locomotion (Hennig and Lomo, 1985). Based on this, we assumed that this muscle would adapt to everyday body weight bearing demands even in the very restricted environment of standard M4 macrolon cages. A ratio, referred to in the text and paper as muscle to whole body index (MBR, **Paper I** and **V**) or sarcopenia index (SI, **Paper III**), between soleus muscle weight (mg) and whole body weight (g) was created.

In addition to the soleus, gastrocnemius and plantar muscles, materials used here derive from related studies on muscle regeneration upon re-innervation (Altun, et al., unpublished data) and also includes then also the plantar muscle of the Achille's tendon group.

### 3.6 RNA isolation

Total RNA was isolated from tissues according to the Trizol protocol (Invitrogen). RNA amount and purity was measured in a spectrophotometer (Eppendorf, Hamburg, Germany), by running a denaturing agarose gel followed by analysis using a 2100 Bioanalyzer (Agilent Technologies, Kista, Sweden). RNA used in real-time PCR experiments was DNase treated (DNA-free<sup>TM</sup>, Ambion Inc., TX, USA), according to the manufacturer's protocol, to minimize protein and DNA contaminations, resulting in OD 260/280 values above 1.95.

### 3.7 Real-time PCR

Analysis of relative mRNA levels in gastrocnemius muscle was performed using reverse transcription (standard reagents, Applied Biosystems, Stockholm, Sweden) and real-time PCR. Real-time PCR was carried out on cDNA transcribed from 10 ng total RNA, with QuantiTect<sup>TM</sup> SYBR<sup>®</sup>-green mastermix (Qiagen, Stockholm, Sweden) and the appropriate primer pairs (see **Paper II, III** and **V**) in an ABI-Prism 7000 instrument (Applied Biosystems). Real-time analysis of SYBR green chemistry values was performed as previously described (Edstrom and Ulfhake, 2005). Correct melting temperature and size of the amplified products were confirmed using melting curves and agarose gel electrophoresis, respectively.  $\beta$ -actin was used as internal control (**Paper III** and **V**) to check for RNA integrity.

### 3.8 Gene chip array

#### 3.8.1 Microchip array Analysis

DNA chips were manufactured (CMM, KI) as described previously (Tollet-Egnell et al., 2000), from a collection of 6240 cDNA clones selected from the TIGR Rat GENE Index ([www.tigr.org](http://www.tigr.org)), Research Genetics and an in-house collection. In each hybridization, 30  $\mu\text{g}$  of the RNA from aged rats was compared to an equal amount of RNA from adults. Three sets of aged animals (grouped according to stage;  $n=2$  or  $n=3$ ) were run against adult controls ( $n=5$ ) and each set was run in triplicate to account for technical variability (in total 9 runs). Fluorescent-

labeled cDNA was synthesized with oligo-dT primer (New England Biolabs Inc.) by reversed transcription reaction using Superscript II (Life Technologies Inc.) in the presence of fluorescently labeled (Cy3-UTP and Cy5-UTP) nucleotides (Amersham Pharmacia Biotech). Dye swaps were used to label one of the three chips within each group. After hybridization, the chip was washed and dried before scanning with a GMS418 scanner (Affymetrix, CA).

### 3.8.2 *Microchip array image analysis*

GenePix Pro 5.0 software (Axon Instruments, CA) was used for fluorescent image analysis. The signal of each spot was calculated as the average intensity of the spot minus the background. Spots with intensities that were at least 1.6 times above the background were included in the study. Normalization between the two fluorescent images was performed using the "LOWESS" method in the SMA (Statistic for Microarray Analysis) package (Dudoit and Fridlyand, 2002; Yang et al., 2002). SMA is an add-on library written in the public domain statistical language R ([www.r-project.org](http://www.r-project.org)). The expression ratio for each group was calculated as the average  $\log_2$  ratio of replicated determinations. Genes with missing data in more than one of the three triplicates within each group were excluded from further analysis. The identification of genes with significant variation between old and young animals (disregarding the stage of the aged animals; see **Paper I**) was performed using SAM (significance analysis of microarrays) statistical technique (Tusher et al., 2001), with a false discovery rate threshold of 5%. This data analysis protocol has been used with the same microarray in numerous studies to identify expression changes that can be reproduced with other methods, such as Northern Blot, RNase protection and RT-PCR (Flores-Morales et al., 2001; Tollet-Egnell et al., 2001).

### 3.8.3 *Databank submission*

All cDNA genechip runs were uploaded and published on-line at NCBI's gene expression omnibus (GEO), [www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/), accession number GSE6229.

## 3.9 **Preparation of protein extract**

Muscle tissue was either extracted as total protein lysates with either RIPA buffer (**Paper III**; 50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mmol/L NaF, 1 mmol/L,  $\text{Na}_3\text{O}_4\text{V}$ , 1 mmol/L PMSF, 10  $\mu\text{g}/\text{ml}$  leupeptin, and protease inhibitors (Roche Molecular Biochemicals)) or Tris-buffer (**Paper II, IV and V**; 50 mmol/L Tris pH 7.4, 5 mmol/L  $\text{MgCl}_2$ , 250 mmol/L sucrose, and fresh 2 mmol/L ATP, 1 mmol/L DTT, and in **Paper II** 1 mmol/L NaF and 1 mmol/L,  $\text{Na}_3\text{O}_4\text{V}$  was supplemented). Membrane fractions, nuclei, and cell debris were removed by centrifugation at  $12,000 \times g$  for 15 minutes for all extractions (**Paper II, III, IV and V**) and an additional ultracentrifugation (**Paper II and V**) of  $100,000 \times g$  for 1 hour at  $4^\circ\text{C}$  (Beckman, Fullerton, CA, USA) for proteins extracted with tris-buffer. Enrichment of large protein complexes (**Paper V**) was performed by ultracentrifugation for 5 hours at  $100,000 \times g$  at  $4^\circ\text{C}$  (5 hours pellet fraction). The protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, USA).

## 3.10 **Immunoblotting**

Equal amounts of protein from tissue lysates were separated by SDS-PAGE, transferred to a PVDF membrane (Amersham Biosciences, Buckinghamshire, UK) and blotted with pri-

mary antibody containing 5% milk and 0.05% tween20 or Odyssey blocking buffer and 0.1% tween20 (see **Paper II-V**). Immunodetection was performed using enhanced chemiluminescence detection (ECL-plus), enhanced chemifluorescence detection (ECF) or IR-Dye detection according to the manufacturer's protocol (Amersham Biosciences or LI-COR Biosciences, Cambridge, UK) and the membranes were exposed to XOMAT-AR film (Kodak) for visualization, when using ECF detection on a Storm<sup>TM</sup> Instrument (Amersham Biosciences) or an Odyssey infrared scanner (LI-COR Biosciences) when IR-dyes were used.

### 3.11 2D gel analysis of protein extracts

Sample (10  $\mu$ l), Urea (10 mg) and NEPHGE (nonequilibrium pH gradient and SDS-PAGE) sample buffer (10  $\mu$ l sample buffer; 9.5 M Urea, 2% (w/v), NP-40, 2% pH 3.5-10 ampholines, 5%  $\beta$ -mercaptoethanol) was mixed. The sample was loaded into the tube where the separation gel was casted and overlaid with NEPHGE overlay buffer (6 M Urea and 1% pH 3.5-10 ampholines). The upper chamber, held running buffer I (0.01 M  $H_3PO_4$ ) and the lower chamber running buffer II (0.02 M NaOH). Reverse polarity was used and the run was set for 400 V for 12 h, 800 V for 2 hours and finally 1000 V for another 2 hours. The gels were gently removed from the glass tubes and incubated with 2X sample buffer (0.125 M Tris pH 6.8, 20% (w/v) glycerol, 10%  $\beta$ -mercaptoethanol, 4.6% SDS) for a minimum of 30 minutes. Gels were then placed on a SDS-Page gel, sealed with 1% agarose in running buffer and run the second dimension.

### 3.12 Proteasome activity measurement

#### 3.12.1 Proteasome active site labeling

Labeling of proteasomal  $\beta$  subunits using active site-directed probes was prepared and performed essentially as reported previously (**Paper IV**; Berkers et al., 2005). In brief, the radio-labeled active site-directed probe AdaY(<sup>125</sup>I)Ahx<sub>3</sub>L<sub>3</sub>VS (0.5x10<sup>6</sup> cpm) or the Dansylated probe DansylAhx<sub>3</sub>L<sub>3</sub>VS (1  $\mu$ M) was added to 25 $\mu$ g of tissue lysate and incubated for 2 hours at 37°C. Proteins were then separated by SDS-PAGE gel and visualized by either autoradiography or immunoblotting using anti-Dansyl antibodies. Two-dimensional NEPHGE SDS-PAGE analysis was performed as described in **Papers IV** and **V**. Autoradiograms were scanned as 16-bit tiff images and quantified by densitometry using the ImageJ software (<http://rsb.info.nih.gov/ij/>).

#### 3.12.2 Fluorogenic substrate assay

Ten  $\mu$ g of lysates was incubated with the fluorescent substrate Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumaric acid (suc-LLVY-AMC; 100  $\mu$ mol/L) in 100  $\mu$ l lysis buffer to assay for chymotryptic-like activity. To measure 20S proteasome activity, lysates were prepared in buffer without ATP and with 0.02% SDS as described in (Kisselev et al., 1999). The samples were analyzed in a fluorescent plate reader (SPECTRAMax<sup>®</sup> GeminiXS, Molecular Devices, Sunnyvale, CA, USA) at zero and every 5 minutes for three hours using an excitation/emission ratio of 380/460 nm at 37°C. One  $\mu$ g enriched proteasome fraction (5h pellet) was assayed for chymotryptic-like (Suc-LLVY-AMC; 100  $\mu$ mol/L), tryptic-like (Boc-Leu-Arg-Arg-AMC (Boc-LRR-AMC); 100  $\mu$ mol/L) and caspase-like activity (Suc-Tyr-Val-Ala-Asp-AMC (Suc-

YVAD-AMC); 100  $\mu\text{mol/L}$ ). Measurements were performed with extracts derived from individual animals and displayed as units of fluorescence or units of fluorescence per  $\mu\text{g}$  lysate per minute. All experiments were repeated three times. For each value, background subtraction was performed.

### 3.12.3 Casein assay

One  $\mu\text{g}$  of proteasome enriched fractions (5h pellets) were incubated with 20  $\mu\text{g}$  casein (Sigma/Aldrich, St. Louis, USA) for the indicated times. The reaction was then stopped by acidification with formic acid. Samples were subsequently analyzed by reversed phase chromatography using an Agilent (St. Clara, CA, USA) 1200 HPLC system with a C18HT column (2.1mm x 2.5cm, Agilent, St. Clara, CA, USA) with the following gradient conditions: 0-50% B, solvent A ( $\text{H}_2\text{O}$  + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid) at a flow rate of 0.5 ml/min. Casein input material and peptide fragments were detected using UV (214 nm). Alternatively, 1/20 of the material was subjected to liquid chromatography tandem mass spectrometry analysis (nanoLC-MS/MS) using a Nano-Acquity UPLC coupled to a QTOFpremier (Waters, Milford, MA, USA) tandem mass spectrometer. Data was acquired in  $\text{MS}^E$  mode, processed with ProteinLynx Global Server (version 2.2.5) and searched against the SwissProt database using Mascot (Matrixscience, London, UK) to identify casein peptide fragments.

### 3.13 Immunoprecipitation of proteasomes

Anti-proteasome immunoprecipitations were carried out on 500  $\mu\text{g}$  pooled muscle lysate prepared as above. Lysates were pretreated with or without DansylAhx<sub>3</sub>L<sub>3</sub>VS (as described above). Samples were diluted in NET buffer (0.5% NP-40, 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA) containing protease inhibitor cocktail (Roche, East Sussex, UK). Samples were pre-cleared and immunoprecipitated with 3  $\mu\text{l}$  of anti-proteasome serum (a kind gift from Dr. Monaco (Brown et al., 1991)); immune complexes were recovered with proteinA-agarose (Invitrogen). Pellets were washed three times with 1 ml NET buffer before the addition of SDS-PAGE sample buffer, and followed with analysis by reducing SDS-PAGE and immunoblotting (as above).

### 3.14 Profiling active deubiquitylating enzymes

Labeling of active DUBs using the ubiquitin-based probe HA-Ub-VME was prepared and performed essentially as described in (Borodovsky et al., 2002). In brief, 0.5  $\mu\text{g}$  of HA-Ub-VME probe was added to 25  $\mu\text{g}$  of tissue lysates and incubated for 1 hour at 37°C. Samples were denatured, proteins were then separated on a 8% SDS-PAGE gel and individual active DUBs were visualized by anti-HA immunoblotting (see above).

### 3.15 Protein identification by mass spectrometry

Individual gel spots were excised and subjected to trypsinolysis (Hanna et al., 2000). Protein digests were analyzed using a matrix-assisted laser desorption ionization time-of-flight/ time-of-flight mass spectrometer (MALDI-TOF/TOF; Ultraflex, Bruker Datonics, Bremen, Germany). Unidentified proteins were further analyzed by MALDI-tandem mass spectrometry (MALDI-MS/MS; laser induced fragmentation technology, LIFT) and by electrospray ionization liquid chromatography-tandem mass spectrometry (ESI-LC-MS/MS) using a QTOF-

micro (Waters, Micromass, Manchester, UK), or a high-capacity iontrap (HCTplus, Bruker Daltonics) tandem mass spectrometer. Proteins were identified by peptide mass fingerprinting (PMF), in which mass spectrometry (MS) spectras were searched against Swissprot and NCBIInr (National Center for Biotechnology Information non-redundant) using Mascot (Matrixscience, London, UK, (Perkins et al., 1999)) or Protein Prospector (MS-fit, UCSF; <http://128.40.158.151/mshome4.0.htm>). Individual MS/MS spectra were searched against Swissprot/NCBIInr using Mascot. Identification was based on the presence of at least two peptides, and on Mascot scores higher than 50.

### 3.16 Lipofuscin analysis

Serial transverse or longitudinal sections of fresh frozen soleus muscle were cut at 14  $\mu\text{m}$  on a cryostat, mounted in glycerol and cover slipped. Using epifluorescence illumination (Nikon Optiphot), representative tissue fields were captured with a Hamamatzu CCD camera (16 bits of grey scale resolution) and adjusted for brightness and contrast using Adobe Photoshop CS2.

### 3.17 Statistics

All statistics were performed using Statistica 6.1 (Statsoft, Tulsa, USA). In **Paper I**, comparisons of experimental groups were mainly carried out with nonparametric analysis of variance (Kruskal-Wallis test) and a post hoc test for pair wise comparisons (multiple comparison of mean rank for all groups; two-tailed test). Comparison of two dependent samples was accomplished using a Wilcoxon matched-pairs test. Series of repeated measurement were analyzed by analysis of variance (ANOVA) for repeated measurements and Bonferoni's post hoc test. Kaplan-Meier plots were used to analyze survival records and the log-rank test was used to compare samples (Lee and Go, 1997). Variance in body weight among litter mates fed ad libitum and on dietary restriction, respectively, were compared by calculating the coefficient of variance (CV, standard deviation divided by mean weight per cage) followed by student t-test. Correlation of two parameters (interval scale) was accomplished using the Spearman Rank correlation test with significance testing of the rank correlation coefficient ( $r_s$ ). Statistical significance levels were set to: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

In **Paper II, III, and V**, comparisons of experimental groups were carried out with ANOVA, and when variance was significant Bonferroni's post hoc test was used for pair wise comparisons. Statistical significance levels were set to: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . In **paper V**, test of correlation was accomplished with least-square regression using untransformed data and calculation of the coefficient of correlation,  $r$ .

All box-plots have the following definitions: box limits represent upper and lower quartile values, and are separated by the median (crossbar within box). The interquartile distance thus contains 50% of the data. Maximum and minimum values, which are not defined as outliers, are illustrated using error bars. Outliers (circles) are defined as values deviating from the quartile borders by more than 1.5 times the interquartile distance. In all other cases, error bars represent standard deviation.

### 3.18 Methodological considerations

The solidity of any result in science depends on the technique(s) used. Each technique has advantages and disadvantages. The techniques used in this thesis were selected for being specific and sensitive, while the samples subjected to analysis derive from living solid tissues and thus are not as well well-characterized. A tissue is typically a mosaic of different cell types and variable amounts of ground matrix; the integrity of which is shredded in the extraction of RNA or protein. Studying aging in skeletal muscle a tissue that normally is continuously remodeled causing shifts in cell numbers and cell types, amount of fat and connective tissue, poses a problem when not based on morphological techniques. The standard procedure to work-around this problem with techniques based on homogenates, as well as flaws in the preparation techniques, is to express the mRNA species, or protein, under study normalized to a so-called “house keeping gene/protein” in the same extract. This “internal control” for loading is widely accepted as a justification to compare samples quantitatively, e.g. adult vs. aged skeletal muscle. A prerequisite is then that the internal control, being a protein or an mRNA species, has a stable expression level. There is a number gene products such as GAPDH (energy production), tubulin and  $\beta$ -actin (both cytoskeletal component/structural proteins) and 18S rRNA (ribosomal RNA) widely used for this purpose. However, more recent studies profiling of the transcriptome and proteome in different tissues reveal that most of the classical internal controls are regulated in different conditions such as aging (see **Paper II**).

In a review Bustin (Bustin, 2002) the problem of normalizing mRNA is discussed, and it is concluded that it not safe to use a single-gene product. Bustin argues that in analysis of a smaller number of gene products the most important step is to reassure the purity and integrity of the RNA samples, while the deployment of a “house-keeping gene” may be of little value. Minimal or no contamination (of protein and genomic DNA) can be analyzed by running a denaturing RNA gel, an Agilent bioanalyzer and OD measurements. In the papers of this thesis we have worked along the guide-lines given by Bustin, using extracts treated with DNAase, high OD values (showing that the amount of remaining protein is small an about equal among samples) and, in addition, we run the Agilent chip based analysis of RNA integrity. The information retrieved from Agilent chips are rRNA (5S, 18S and 28S) integrity, mRNA integrity and the level of DNA contamination. We observed that the profiles of the rRNA of all the samples were comparable. Degradation of RNA and contamination of DNA was not detectable and the samples used were of the same comparable quality.

Chip-based profiling of the transcriptome poses less of a problem since besides the above mentioned sample quality controls, normalization is being made towards a major part, or most, of the mRNA species expressed (see **Paper II**). Still, a remaining issue with this technique being used on extracts from solid tissues is the number and type of the contributing cells and the transcriptional “status” of these cells (since the starting amount is a weighed quantity of the tissue or the whole organ). An attractive strategy would be to co-extract DNA and total RNA and then separate mRNA from total RNA. The mRNA could then be expressed related to the amount of DNA (i.e. number of cells) and as a fraction of total RNA. However, at least in our hands, co-extraction of DNA and RNA proved unreliable and this was also true when mRNA, in a second step, was to be separated from total RNA. We therefore refrained from doing this awaiting a more refined extraction technique to become available.

A similar line of reasoning applies to protein analysis, albeit the numbers of steps from DNA to mature protein are many, therefore making it more difficult to pin-point the mechanism underpinning a protein shift than a mRNA shift. As loading control we have separated the protein lysate on a gel and stained for all the proteins (using coomassie-, silver- or flourogenic staining) and by integrating the staining signal of the full lane we showed that there are minimal variations between samples (**Paper III** and **V**).

## 4 Results and Discussion

### 4.1 A Rodent model

*Old-age muscle atrophy and behavioral impairments in the aging rat.* Rodents are widely used as models for many human diseases as well as in basic life-science research. To serve as a model of aging, it is prerequisite that the age-related changes correspond to those encountered in humans and that these alterations can be consistently determined by behavioral tests. Motor disturbances and old age-associated muscle atrophy (senile atrophy or sarcopenia) are typical phenotypic alterations encountered during normal aging in humans. Similar age-dependent changes are evident in the rat thereby allowing the rat to serve as a useful model (Alliot et al., 2002; Cowen et al., 2005; Gutman and Hanzlikova, 1972; Ulfhake et al., 2000; Ulfhake et al., 2002).

In **Paper I** of this thesis, we have compiled and analyzed a large collection of life-span data, including growth curves, behaviors and loss of muscle mass from both sexes of several rat strains. The majority of the data is derived from groups of outbred female Sprague–Dawley (SD) rats however, groups of outbred male SD and outbred male Wistar (Wi), inbred male Fisher 344 (Fi) and female Lewis rats were included for comparison. In addition, data were also collected from animals in which the normal pattern of aging was challenged by environmental parameters (i.e. enriched environment (EE) and dietary (calorie) restriction (DR)). In aging-studies, the chronological age of the animals used is not enough, the expected life-span of the strain and stock used must also be known (Burek and Hollander, 1980). For several of the strains and stocks studied, the median survival age was 30 months (Fig. 1, **Paper I**) (**Paper I**; Berg, 1967; Burek, 1978; Burek and Hollander, 1980; Coleman et al., 1977; Masoro, 1980; Ryle et al., 1995). This determined age, or life-span, was later used to define whether animals were “old-aged” (see **Papers II-III** and **V**). Considering the shorter life-span of rodents in their natural setting, (Berry and Bronson, 1992) it can be concluded that a protected environment (like animal housing facilities) is a major modulator of life-span. Furthermore, no distinct effects on median life span expectancy within a protected environment were observed with regard to gender or between virgins and breeding females. However, DR was found to increase and EE exhibited a tendency to increase life-span.

In contrast to humans, rodents continue to grow throughout the major part of their life-span. Therefore, we used total body weight as a measure of growth (Fig. 2, **Paper I**). Growth patterns were similar among the strains studied, albeit inbred strains were lighter than outbred strains. Importantly, all strains showed a drop in body weight in senescence. This senile loss of body mass noticeably affects striated muscle witnessed by a significant decrease in muscle weight relative to whole body weight (Fig. 8, **Paper I**; see also Fig. 1, **Paper V**). While EE challenge failed to significantly improve body composition, animals under DR revealed, as early as middle-aged animals, an improved body composition (muscle to body weight ratio) that lasted throughout senescence (Fig. 11, **Paper I**; see also Fig. 1, **Paper V**).

To understand the consequences of an altered cell function at the level of the organism, behavioral tests serve as a high-end read-out. The battery of sensorimotor tests used in this thesis recorded basic phenotype characteristics and are compliant with tests recommended by both Eumorphia (EMPRESS) and SHIRPA for basic phenotyping of rodents. It is evident from our results that some behaviors changes gradually during aging (from maturity to death),

such as alterations in explorative behavior, whereas other behaviors, like reactions to noxious stimuli, are maintained until old age (Figs. 3-4, and S2-S3, **Paper I**). Common to most of the behavioral tests was the functional decline became accelerated towards the end of the expected life span. The loss of muscle mass, therefore muscle strength, exhibited in old age is probable key factor to the outcomes of several tests. These include the marked decrease in rearing behavior and poor performance in a beam balance test. Rather unexpectedly, animals under EE challenge did not significantly perform better in the behavioral tests than animals in standard-housing conditions (Fig. S4, **Paper I**). As predicted, animals on DR performed better than their aged-matched controls in several, although not all, of the tests (Fig. 9 and see also Fig. 10, **Paper I**).

An important finding was that rats, like humans, show different patterns of aging. Some animals were more successful while others were less successful in maintaining body composition and behaviors in old age. (cf. Figs 3-4, 8, S2-S3, **Paper I**). We developed a simple stage-ranking protocol (for details see **Paper I**) aimed at evaluating and grouping cohort members to either a low symptomatic (successful aging) or a high symptomatic (unsuccessful aging) group (Figs. 6 and S4, **Paper I**). Behavioral data was back analyzed using this new system of grouping to explore if stage-ranking could predict an individual animal's outcome in the behavioral tests. As depicted in Fig. 7 of **Paper I**, the stage-ranking assessment can predict outcome in many, but not all, of the behavioral tests. Furthermore, stage-ranking varied with the degree of old-age muscle atrophy (Fig. S6, **Paper I**). Given that the living conditions are strictly standardized, the difference in the pattern of aging may reflect the genetic make-up of cohort members. Consistent with this hypothesis, the inbred rat strains Lewis and Fisher 344 showed less variability than the outbred SD and Wistar rats. Outbred rat strains may thereby more closely model human variability in patterns of successful/unsuccessful aging and are therefore more valuable to pin-down factors that contribute to the pace and extent of aging-induced impairments. In addition, the fact that inbred rats also show variability indicates that epigenetic modifications may influence the pattern of aging. Together, using both inbred and outbred stocks and strains of rats will aid in the determination of contributions to aging from genetic background, epigenetic modifications and other environmental influences.

Restricted access to food is the only general paradigm known to improve both the health and life span expectancy of animals. Dietary restriction probably slows down the pace of aging by influencing basic cellular metabolism and this is reflected in modulation of motor activity on the level of the organism. It should be stated that in the present studies, significant improvements were made in body composition, performance in behavioral tests and survival, although the degree of food restriction was less than the degree of dietary restriction most commonly used, 70% of *ad libitum* intake versus 50-60%, respectively. It should also be noted that rats fed *ad libitum*, over-eat due to the boredom produced by caged-housing (giving a heightened interest for this animal model since it may reflect concurrent medical problems in humans). A very interesting observation in this context was a reduction in body weight variability among cage-mates on DR compared to rats fed *ad libitum* (**Paper I**).

In aging research, appropriate controls pose a particular problem and thereby increase the demand for studies involving more than two age groups. A multiple age-grouped study is less laborious and therefore usually favored over a longitudinal study design. Nevertheless, the latter design has several advantages since it eliminates the risk of drift within the genetic background as well as subtle differences in husbandry while also decreasing the number of

animals per study. Its shortcoming, however, is the very limited possibility to correlate alterations observed in behavior to cellular changes at the tissue level. Furthermore, inherent to the longitudinal design is the fact that many behavioral tests are not well-suited for longitudinal measurements. We addressed this issue by comparing longitudinal data collected from smaller groups of female SD rats to those obtained using a multiple age group design (Fig. 5, **Paper I**). Although this comparison is only descriptive, the results demonstrate that there is a fair degree of consistency between the two study designs.

#### **4.2 The superficial muscle groups of the rat calf**

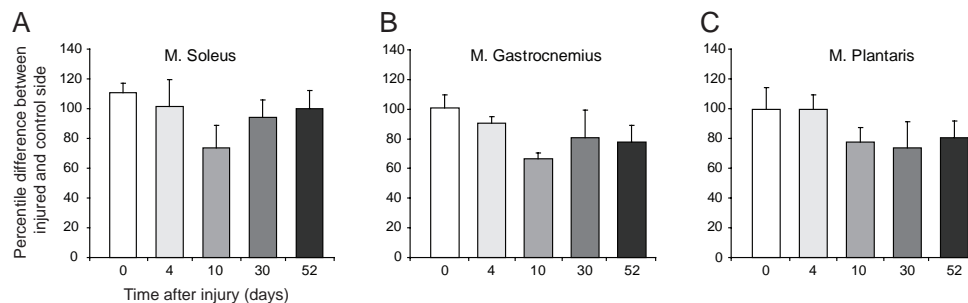
This group of muscles is comprised of the plantar, gastrocnemii and soleus muscles that share a common site of insertion on the calcaneus bone. While the plantar muscle is not well-developed in humans, it is a powerful and fast muscle in many other mammals like rodents used for jumping and essentially consists of anaerobic (glycolytic/fast/white) fibers. The gastrocnemii, whose lateral and medial heads join in the muscle belly, has a common insertion on the heel by the Achilles tendon and is classified as a “mixed” muscle consisting of fast-fatigue/white fibers, intermediate fibers and slow (red/aerobic fibers/fatigue resistant) fibers (the latter, approximately 20-30% of the fiber population). The soleus muscle consists mainly (~90%) of slow acting myofibers.

Muscle fibers are arranged in motor units (MUs), where a MU is a functional muscle unit innervated by only one alpha-motoneuron (MN) in adults. The motoneurons that innervate the calf muscles are situated in the lumbar spinal cord (L4-L6). In addition to alterations in MN firing-rate (impulse frequency), recruitment of additional motor units (set by a MUs activation threshold) provides a second mechanism to modulate muscle-force output. A MU holds only *one* fiber type, thereby making MUs fast, intermediate or slow according to the myofiber-type characteristics. Hence, the velocity of muscle force-output can be varied through modulating the recruitment of fast versus slow MUs. Both the recruitment pattern and duty cycles of MUs in the calf muscles of rats have been explored using freely-moving rats (Hennig and Lomo, 1985; Hennig and Lomo, 1987a; Hennig and Lomo, 1987b). While slow units are frequently recruited and demonstrate long duty-cycles, large fast units are seldom recruited except for short bursts during ballistic movements (jump/escape) (*idem*).

Although the number of fibers per MU varies among different muscles, slow MUs hold fewer myofibers than fast MUs. In other words, this means that fast MN have a more elaborate terminal axonal network than slow MN. It is well known that impulse activity and muscle contractions are essential for the maintenance of MUs, but it is not clear through which mechanism(s) large, fast and infrequently used MUs are maintained. Fast MUs may, due to their organizational complexity and infrequent usage, be more vulnerable to aging than slow MUs. If such a selective vulnerability exists, it could, in part, explain why fast myofibers appear to be more affected by aging than slow myofibers (Larsson, 1995). However, the situation is complicated by the fact that fiber-type fidelity is not as rigid as once believed. Fiber transitions and hybrid fibers (I+II or IIa+IIc etc.; quite common in human masticatory muscles (Kirkeby and Garbarsch, 2000; Korfage et al., 2005)) occur in adult skeletal muscle and appear to increase in frequency with advancing age (Andersen et al., 1999b; Edstrom and Ulfhake, 2005; Klitgaard et al., 1990b; Williamson et al., 2000). There are conflicting reports concerning the effects of disuse versus exercise on the incidence of myosin heavy chain co-expression (Andersen et al., 1999a; Klitgaard et al., 1990a; Short et al., 2005; Williamson et al., 2000) but it seems

that inactivity promotes dedifferentiation, whereas endurance training decrease the number of hybrid fibers and increase the expression of myosin heavy chain I (*idem*).

In the studies of the present thesis, the soleus muscle was used to evaluate muscle to whole body weight ratios during life-span; the rationale behind using this muscle is it is used not only posture but is also steadily active in all types of locomotion (Hennig and Lomo, 1985). Based on this, we assumed that this muscle would adapt to everyday body weight bearing demands even in the very restricted environment of standard M4 cages. A ratio, muscle weight (mg) to whole body weight (g) was created (MBR). Unfortunately since the soleus muscle is rather small, it does not provide enough material for the biochemical analyses to be performed on the individuals level, instead we used the gastrocnemius muscles which contain all three MU types (see above) for these analyses. We and others have shown that old-age muscle atrophy affects the gastrocnemius as well as the plantar and soleus muscles (Gallegly et al., 2004; Gutman and Hanzlikova, 1972; Mosoni et al., 2004). However, since aging seems to selectively affect fast over slow myofibers, the dynamics of atrophy and regeneration may differ among muscles due to their fiber type composition. In related work (Altun, et al., unpublished data), we studied the recovery of innervation and muscle mass in a sciatic nerve crush model. A standardized crush was applied to the mid-thigh, thereby affecting the innervations of all the distal calf and foot-sole muscles. Groups of animals were allowed to recover for up to 52 days post crush and monitored for functional recovery through the behavioral testing of pain, touch, balance and gait pattern. By the end of the fourth week, all animals displayed a functional recovery without further improvements to the end of the study even though muscle mass increased during the entire period (Fig. 8). It is interesting to note that the soleus muscle, in particular, displayed an almost complete recovery of its muscle mass by day 52, while both the gastrocnemius and plantar muscles do not. These observations support the idea that atrophy and recovery, to some extent, are fiber-type specific.



**Figure 8.** Changes in muscle mass in response to a sciatic nerve crush and the subsequent reinnervation and functional recovery. (A) m. soleus, (B) m. gastrocnemius and (C) m. plantaris. Note that while there is an almost complete recovery of soleus muscle mass, while both the gastrocnemius and the plantar muscles lag behind in recovery during the observation period.

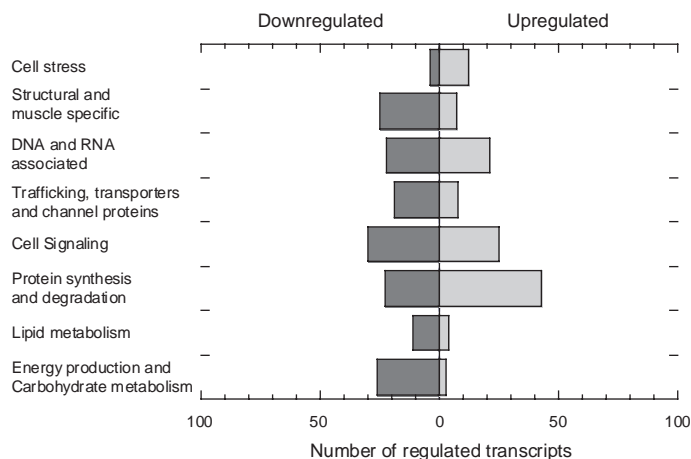
### 4.3 Muscle profile and iron load

*Proteomic and transcriptional profiling of hind limb skeletal muscles of 30-month old rats.* In **Paper II** we used two-dimensional gel electrophoresis (Fig. 1, **Paper II**) and mass-spectrometry to screen for changes in proteins combined with cDNA profiling to assess transcriptional regulations in the gastrocnemius muscle of adult (4 months) and aged male Sprague-Dawley rats (30 months). Our general objective was to unmask proteomic and transcriptional phenotype characteristics of sarcopenia.

The microchip array data, covering 6240 cDNAs, are based on three independent data sets per animal group and three animal groups (GEO, accession no. GSE6229). Only changes present in at least two of the three experiments in each animal group (i.e. six of the nine runs) were included in the set of 444 significantly regulated mRNAs. Although the false discovery rate was 5% using the threshold described in materials and methods in each experiment, the likelihood that the same error would show up in six experiments is significantly smaller. Since we observed marked age-dependent differences for the 100 most abundant proteins in crude extracts, we decided to optimize staining procedures for this set to achieve an adequate quantification. Analysis of the less abundant proteome clearly requires the prefractionation of muscle extracts that can then be characterized separately. For an independent experimental and additional validation of the differential expression of proteins and mRNA, we used Western blot (Figs. 2 and 3, **Paper II**) and real-time PCR (Supplementary Fig. S1, **Paper II**).

A number of studies on aged skeletal muscles from humans, primates, and rodents have been performed using genomic techniques (Kayo et al., 2001; Lee et al., 1999; Pattison et al., 2003; Welle et al., 2001; Zhang et al., 2002; **Paper II**). These studies, although performed on different types of hind limb muscle, have provided a partly coherent framework for phenotyping aged mammalian skeletal muscle on the mRNA expression level. From these data sets, a sarcopenic phenotype may be extracted, which includes a marked down-regulation of glycolytic and mitochondrial oxidative enzymes, extracellular matrix proteins, and mRNAs encoding mature contractile proteins concomitant with increases in mRNAs encoding for transcription factors involved in muscle cell differentiation, scavengers of free radicals, DNA damage induced proteins, and RNA/DNA repair/editing molecules (Fig. 9 summarizes transcriptional changes found in **Paper II** that are also available at [www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/), GEO, series accession no. GSE6229).

Enzymes involved in protein turnover are also modulated (Bardag-Gorce et al., 1999) and evidence has accumulated for the conclusion that muscle atrophy in disease or disuse is driven by



**Figure 9.** Functional category grouping of transcripts regulated ( $\log_2$  ratios) in aged muscles (30 months old) as compared to adult (4 months old), based on annotations in EGON and DAVID, from the gene chip array analysis in **Paper III** (a full annotation of the results is available at: [www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/), GEO series accession number GSE6229).

increased proteolysis of myofibrillar proteins via the ubiquitin proteasomal pathway (Glass, 2005). It remains unclear whether a similar mechanism is in operation in sarcopenia, and available data are to a certain extent conflicting (Bardag-Gorce et al., 1999; Ferrington et al., 2005). The transcriptional profile of aged muscle in **Paper II** does not support the hypothesis that sarcopenia is associated with an induction of proteasomal proteolysis. This evidence is consistent with the results presented in **Paper III** and **V**, that demonstrate a down-regulation of atrophy-induced muscle specific E3-ligases in old age. From this it may be concluded that sarcopenia is driven by mechanisms different from those governing muscle atrophy in disease or disuse and as described in **Paper V**, old-age muscle atrophy may depend on an increased proteolysis via the proteasome but through a completely different mechanism.

Although evidence suggests that protein handling (translation, post translational modifications, and degradation) may change with increasing age, relatively little is known about the aged muscle proteome or proteins expressed by the genome (Chang et al., 2003; Cottingham, 2006; Gelfi et al., 2006). We found 35 proteins to be differentially expressed. Of these differentially expressed proteins, 11 were increased and 24 were decreased (Table 1, **Paper II**). In addition, 9 proteins appeared in multiple spots (the same protein with different isoelectric focusing points) indicating that at least one or several of its post translational modified forms were regulated (Supplementary Table S2, **Paper II**). The most significant findings are being discussed below.

According to the free radical theory of aging, oxidative stress is the driving mechanism in cell and tissue impairment (Finkel and Holbrook, 2000) and stipulates that oxidative damage accumulates with increasing age. Regulated molecules (compiled in Tables 2 and 3, **Paper II**) include heat shock proteins, molecules involved in redox processes and scavenging of free radicals, as well as those involved in iron metabolism and homeostasis. However, there was no concerted increase in free radical defense molecules; instead, our data indicate a specific pattern of regulations. The differential expression of SOD1 (Cu/Zn SOD) and SOD2 (MnSOD) was re-examined by immunoblot (Fig. 3C and D, see also Table 2, **Paper II**), and confirmed a downregulation of SOD2 and an increase of SOD1 in senescent skeletal muscle. SOD2 is important for the capturing of superoxide radicals released during oxidative phosphorylation in the mitochondria, whereas SOD1 is considered primarily a cytosolic scavenger. The mechanism for the differential expression of the SODs is unclear. Although both SODs are associated with oxidative stress (Kirby et al., 2002; Parkes et al., 1998; Sun et al., 1999), transcription of SOD1 and SOD2 are controlled by different pathways and transcriptional machineries (Chang et al., 1999; Scandalios, 2005). In **Paper III** we show that forkhead box O 4 (FOXO4) transcription is down-regulated in the rodent model of sarcopenia, which may explain the decreased levels of SOD2 transcript and protein in aged skeletal muscle. Piec and colleagues (Piec et al., 2005) on the other hand found that SOD1 decreased from adolescence to middle age but underwent no further change in old age. Although the increase of SOD1 noted in our experiments using Sprague-Dawley rats may be a strain-specific feature, it is also possible that the groups of aged rats utilized here, and by Piec and colleagues (Piec et al., 2005), do not match when adjusted to the strain-specific expected lifespan.

A novel finding was that molecules involved in iron-binding/transport (serotransferrin precursor) and heme metabolism were found to be expressed at increased levels in aging. Elevated transferrin content was confirmed in independent immunoblot experiments (Fig. 2, **Paper II**) and the measurement of iron in muscle lysates showed an accumulation of iron in aged rats

(Fig. 2, **Paper II**). Also the results from the transcriptional analysis were consistent with iron loading during aging in skeletal muscle (Table 2, see also GEO, accession no. GSE6229, **Paper II**). The level of aconitase, a key protein for cellular iron homeostasis, was also increased in aged muscle (Tables 1 and 2, **Paper II**). It is known from previous studies on aged liver, kidney, and brain that tissue iron content accumulates in senescence (Cook and Yu, 1998; Focht et al., 1997; Schipper, 2000) and that ferritin, the major mobile iron-binding protein carrier, increases with age (Piec et al., 2005; Rikans et al., 1997). Iron is an indispensable metal-ion necessary for cell respiration (mitochondrial complex IV) as well as other enzymatic processes; however, free  $\text{Fe}^{2+}$  is also a key element in Fenton chemistry and produces OH through the Haber–Weiss reaction (Fig. 4, **Paper II**). Moreover, iron load in hepatocytes has been associated with mitochondrial dysfunction (Schipper, 2000) and has also been implicated in the formation of lipofuscin, the polymerization of lipid and protein residues. Lipofuscin accumulates with increasing age in most postmitotic cells as a nondisposable waste trapped in secondary lysosomes (Terman and Brunk, 2004a). As presented in **Paper V**, this also occurs in aged skeletal muscle. Lipofuscin may act as a repository for redox active iron, which may drive cellular oxidative stress in senescence. The data obtained indicate increased iron transport (transferrin), binding (ferritin), and possibly increased iron response element binding protein-1 activity (increased protein levels of aconitase; see Table 2 and Fig. 4, **Paper II**). In parallel, we found increased levels of hemoxygenase and biliverdin reductase (Table 2, **Paper II**) in aged skeletal muscle. Increased levels of hemoxygenase have been reported in the brains of individuals with various neurodegenerative diseases as well as during aging (Zecca et al., 2004). Iron load increases the risk for common hallmarks of aging such as DNA damage, protein oxidation and misfolding, and lipid peroxidation (Fig. 4, **Paper II**), as well as iron homeostasis has been described to influence the lifespan in simpler organisms such as yeast (Desmyter et al., 2004).

#### 4.4 Muscle specific E3-ligases are decreased in old age muscle atrophy

Muscle mass is under constant remodeling, the net effect of myofibrillar protein synthesis or breakdown cause myofibers into a hypertrophic or atrophic state, respectively. In a range of conditions including cancer, diabetes, Cushing’s syndrome, denervation, uremia, sepsis, disuse, and fasting, skeletal muscles atrophy through degradation of myofibrillar proteins mainly via the ubiquitin–proteasome pathway (Attaix et al., 1998; Lecker et al., 2004; Lecker et al., 1999; Mitch and Goldberg, 1996; Solomon and Goldberg, 1996). The notion of a general mechanism underlying all skeletal muscle atrophy is appealing and recent advances in this field have identified the FOXO-regulated ubiquitin E3-ligases, Atrogin-1 and MuRF1, as a common mechanism for muscle atrophy caused by a range of etiologies (Bodine et al., 2001; Gomes et al., 2001; Jagoe and Engelen, 2003; Lecker et al., 2004; Lecker et al., 1999; Rommel et al., 2001; Scheck et al., 2004; Sandri et al., 2004; Stitt et al., 2004). However, whether this mechanism is in operation in muscle wasting during aging remains unresolved (Fig. 2, Thesis Introduction). Atrogin-1 and/or MuRF1 messenger RNA (mRNA) levels in aged muscle are either reportedly unchanged in the human (Welle et al., 2003; Whitman et al., 2005), slightly increased (Pattison et al., 2003), or decreased in the rat (Deruisseau et al., 2005). To address whether Atrogin-1 and MuRF1 are regulated in sarcopenia, we used female SD rats, to which the degree of hind-limb sarcopenia was ascertained through the relationship between the weights of the postural hind-limb soleus muscles to the total body weight (MBR, Fig. 2, **Paper III**). As controls, 4-month-old *ad libitum* (AL) fed adult rats (4 mo-AL) and 12-month-old AL

adult rats (12 mo-AL) were used and were found not to be statistically different with respect to MBR scoring. However, aged AL animals (30 mo-AL) had significantly reduced muscle weights and a lower muscle to body ratio compared to the adult control groups. Furthermore, aged rats on a DR (30 mo-DR) were determined to have soleus muscle weights and MBR values in-between those of the 30 mo-AL group and the adult control groups (Fig. 2, **Paper III**). Real-time PCR quantifiably showed a significant reduction in the levels of Atrogin-1 and MuRF1 mRNAs in the sarcopenic 30 mo-AL group versus controls (Fig. 3A and B, **Paper III**), whereas there was no statistically significant difference in the mRNA levels of these two E3-ligases between either of the two adult control groups and the 30 mo-DR group. In **Paper V** we confirmed these findings in male SD rats (Fig. 6A, **Paper V**) and, in addition, found no significant change in the expression of E2-14K, a conjugase that also has reported implications in muscle atrophies (Glass, 2005; Whitman et al., 2005). Moreover, after treatment of adult and aged male AL animals with dexamethasone, known to induce all three of these ubiquitin conjugating enzymes (Bodine et al., 2001; Glass, 2005), we observed an increase in mRNA expression of all three molecules only in adult but not in aged rats (Fig. 6B, **Paper V**). From our findings, we can conclude that muscle atrophies in old age are mechanistically different from acute atrophies induced by disuse, disease, or denervation.

Additionally, our results suggest that an AKT (protein kinase B) mediated inactivation of FOXO4 underlies this suppression. In skeletal muscle, FOXO4 is claimed to be the most abundantly expressed member of the FOXO family of transcription factors (Furuyama et al., 2002). Our data demonstrate that FOXO4 transcription is suppressed in sarcopenic muscles of *ad libitum*-fed rats whereas neither the transcription of FOXO1 or FOXO3 significantly change during aging beyond 12 months of age in the gastrocnemius muscle.

#### 4.5 Tools for studying the proteasome

The proteasome, a multi-catalytic protease complex, is involved in many important cellular functions. In order to analyze proteasome contents and their role in different biological processes, a number of tools have been developed. A straight-forward, but not very informative since the proteasome must be fully assembled to function, approach is to measure the amounts of different proteasomal proteins by immunoblot (see Introduction). A better approach is to enrich for proteasomes through centrifugation or to isolate proteasomes by column exchange purification. Additional analysis of the various proteasomal assembly-chaperone proteins (Fig. 4) or a transcriptional analysis of specific proteasomal subunits (Fig. 3) and their associated enzymes (Fig. 5) may provide important data with regard to proteasome induction and upstream signaling pathways (Fig. 2).

The most common method to assay proteasomal activity is by the use of small fluorogenic peptides that normally contain three to four amino acids coupled to a fluorophore. This is a quick and easy technique for use in cell extracts that allows for the measurement of the chymotryptic proteolytic activity specific to the proteasome. To probe for other proteolytic sites (i.e. tryptic-like or caspase-like), proteasomes must first be isolated, or enriched, in order to eliminate other proteolytic enzymes that may potentially cross-react with these substrates. Since these approaches require much larger amounts of starting material (up to 200 g, (see Kisselev et al., 1999)), this is not always a feasible approach. Moreover, the enrichment process is more time consuming and potentially interesting molecules associated with the proteasome (for example protein interaction partners and substrates) in such preparations may be lost.

An alternative approach for the measurement of proteasomal proteolysis is the use of a full-length protein (i.e. casein, catalase or GAPDH) as a substrate. This approach is more biologically relevant compared to the use of short fluorogenic peptide substrates, due to the short peptides is easier to degrade for the proteasome and does not always reflect the degradation speed. However, a disadvantage of this method is the requirement of pure proteasome preparations in conjunction by column exchange purification analysis to detect any break down products. This latter method is both time consuming and needs large amounts of starting material.

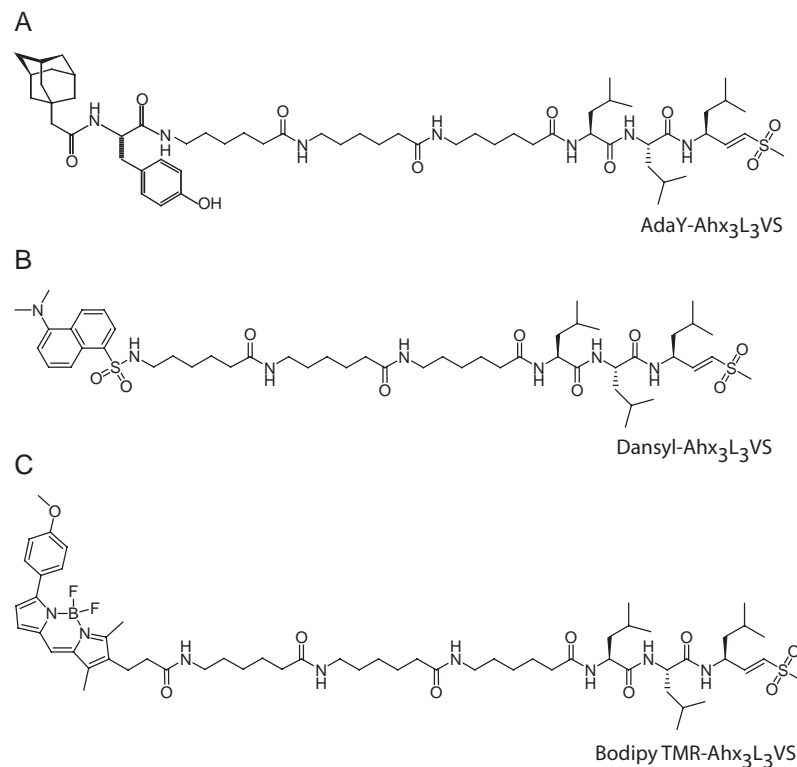
Proteasomal proteolysis can be mechanistically analyzed by utilizing chemical “suicide” inhibitors that will bind covalently to the active site residue, a threonine located within the N-terminus each of the catalytic beta subunits  $\beta 1$ ,  $\beta 2$  and  $\beta 5$ . These types of inhibitors, when equipped with a tag allowing for their detection, are referred to as active site-directed molecular probes (**Paper IV**; Bogoyo et al., 1997; Bogoyo et al., 1998). When used correctly, these probes will not only reflect activity, but also indicate which catalytic subunits are affected since more than one of the subunits has to be inhibited for inhibition of proteasomal degradation to take place (Kisselev et al., 2006). The use of these probes to visualize the active subunits of the proteasome has turned out to be of great value in determining if any of the catalytic sites are occupied or modified.

In **Paper IV**, we modified the inhibitors NLVS and Z-L<sub>3</sub>VS, initially synthesized by Bogoyo and coworkers (Bogoyo et al., 1997; Bogoyo et al., 1998), aiming to create derivatives with increased potencies. Specifically, a vinyl sulfone moiety that covalently binds the catalytic subunits was employed as chemical “warhead” (Bogoyo et al., 1997; Bogoyo et al., 1998). We demonstrated that peptide based inhibitors containing this chemical moiety can be effectively used to measure proteasome activity and derived results closely correlating with results obtained from fluorescent substrate based assays. We also observed that through the elongation and modification of the Z-L<sub>3</sub>VS molecule, it acted more like a poly-peptide substrate (**Paper IV** and **V**; Bogoyo et al., 1997; Bogoyo et al., 1998). Based on these results, we generated several new tools, such as adamantane-acetyl-(6-aminohexanoyl)<sub>3</sub>-(leucyl)<sub>3</sub>-vinyl-(methyl)-sulfone (Ada-Ahx<sub>3</sub>L<sub>3</sub>VS) or its iodotyrosyl residue modified version with or without a biotin label, AdaY-Ahx<sub>3</sub>L<sub>3</sub>VS and AdaK(Bio)-Ahx<sub>3</sub>L<sub>3</sub>VS respectively. The AdaY-Ahx<sub>3</sub>L<sub>3</sub>VS modification that enabled <sup>125</sup>I-radiolabeling, lost its cell permeability compared to its precursor, Ada-Ahx<sub>3</sub>L<sub>3</sub>VS (**Paper IV**), but retained its specificity and sensitivity thereby allowing for its use only in lysates. However, the Ada-Ahx<sub>3</sub>L<sub>3</sub>VS derivative appeared to be cell permeable and was found to inhibit with increased potency compared to ZL<sub>3</sub>VS (**Paper IV**). Taken together, Ada-Ahx<sub>3</sub>L<sub>3</sub>VS and AdaY-Ahx<sub>3</sub>L<sub>3</sub>VS are useful tools since they bind all active  $\beta$ -subunits of the proteasome including those within the immuno-proteasome (**Paper IV**; Altun et al., 2005).

In comparison to the commonly used inhibitors MG-132, lactacystin and epoxomicin, Ada-Ahx<sub>3</sub>L<sub>3</sub>VS has some advantages. Both the non-peptide inhibitor lactacystin and MG-132 (LLnL) have high affinity for the chymotryptic-like activity site and lower or no affinity at all for the caspase-like or tryptic-like sites. Moreover, MG-132 does not covalently attach to the catalytic site therefore making it a reversible inhibitor. Ada-Ahx<sub>3</sub>L<sub>3</sub>VS on the other hand, is irreversible thereby allowing for a more accurate determination of available catalytic sites. With regard to the peptide exo- $\alpha$ -keto epoxomicin, it inhibits the chymotryptic-like site in an irreversible manner but unfortunately shows inhibition of caspase-like and tryptic-like activity only at much higher concentrations (Kisselev and Goldberg, 2001). Both Ada-Ahx<sub>3</sub>L<sub>3</sub>VS and AdaY-Ahx<sub>3</sub>L<sub>3</sub>VS are excellent for blocking all the catalytic  $\beta$ -subunits and

the corresponding activities efficiently and irreversibly. Furthermore, AdaY-Ahx<sub>3</sub>L<sub>3</sub>VS has the advantage of being used as a tool for visualizing the availability of the subunits (**Paper IV**).

Following the generation of Ada-Ahx<sub>3</sub>L<sub>3</sub>VS and its modified versions (AdaY-Ahx<sub>3</sub>L<sub>3</sub>VS and AdaK(Bio)-Ahx<sub>3</sub>L<sub>3</sub>VS), these probes were further engineered by exchanging the detection groups (tags) from iodotyrosinyl or biotin, to a dansyl-residue (Berkers et al., 2005). Even more recently, proteasome probes containing a bodipy-tamra moiety as a detector have been synthesized (Fig. 10, Verdoes et al., 2006). One advantage of these novel probes is that they are not radiolabeled (Berkers et al., 2005; Verdoes et al., 2006). The latest version, a fluorophor derivative, has the further advantage of being able to be used in PAGE-based assays without the need for immunoblotting (Verdoes et al., 2006) and may be further developed for use as an *in situ* probe, thereby allowing for the examination of proteasomal proteolysis without distorting tissues complexity. Probes derived from the work in **Paper IV** were used in **Paper V** of the thesis.



**Figure 10.** Chemical structures of the proteasome active-site labeling probes (A) AdaY-Ahx<sub>3</sub>L<sub>3</sub>VS (B) Dansyl-Ahx<sub>3</sub>L<sub>3</sub>VS and (C) Bodipy-tamra-Ahx<sub>3</sub>L<sub>3</sub>VS.

#### 4.6 The proteasome in old age muscle atrophy

A large body of evidence has linked the proteasome and its associated enzymes in the ubiquitin-proteasomal pathway to skeletal muscle atrophy in a range of systemic diseases including cancer, diabetes, Cushing's syndrome, uremia, and sepsis, as well as in disuse, fasting and

muscle denervation (reviewed in Glass, 2005; Lecker et al., 2004; Lecker et al., 1999; Mitch and Goldberg, 1996). The role of the UPS in old age muscle atrophy, however, is controversial and conflicting results have been reported regarding alterations in proteasome expression and activity (Attaix et al., 2005a; Bardag-Gorce et al., 1999; Ferrington et al., 2005; Husom et al., 2004; Martinez-Vicente et al., 2005; Whitman et al., 2005). Several groups, including ourselves, have reported (**Paper III**; Deruisseau et al., 2005; Whitman et al., 2005) that Atrogin-1 and MuRF1 are not induced in aging. In fact, these muscle-specific E3 ligases are actually down-regulated in the aged rat (Fig. 3 in **Paper III** and Fig. 6 in **Paper V**) and can then not even be induced by corticosteroid treatment (Fig. 6, **Paper V**). A transcriptional based analysis of muscle tissue in a range of species (see above and **Paper II**) have not identified upregulation of proteasomal mRNA transcripts as part of the sarcopenia profile; findings that we confirmed with qPCR in **Paper V** (Fig. S5). While the lysosomal pathway, the second major pathway for cellular protein degradation, appears to be under considerable stress during aging as well as associated with several manifestations of aging in post mitotic cells (Brunk and Terman, 2002b; Terman and Brunk, 2004a), transcriptional profiling has yet to associate old-age muscle atrophy with increased activity of the autophagosome-lysosomal pathway, (see references cited in Paper II, Attaix et al., 2005a; Attaix et al., 2005b). The aim of **Paper V** was to re-examine the proteasome and its associated enzymes in old-age muscle atrophy. For this study we used 30-month old male SD rats and 4-month old males as controls; moreover, we used dietary restriction (DR) to challenge the normal pattern of aging in SD rats.

We observed a two- to three-fold increase of proteasomal proteins in crude lysates from aged sarcopenic skeletal muscle (Figs. 1 and 2, **Paper V**) associated with a corresponding increase of proteasomal particles (Fig. 3, **Paper V**). These increases were reduced under DR (*idem*). An increase of proteasomal protein in aged muscles has been previously been reported (Bardag-Gorce et al., 1999; Ferrington et al., 2005; Husom et al., 2004), however, Ferrington and coworkers (Ferrington et al., 2005; Husom et al., 2004) claimed that this was due, in part, to an upregulation of the immunoproteasome rather than the 26S proteasome (see introduction) and, in part, to the decreased proteolytic activity of the 26S proteasome in aging. Combined, the results established an over-all decrease in proteasomal proteolysis in aged skeletal muscle according to their results (*idem*).

In **Paper V**, we were not able to reproduce the findings of Ferrington and colleagues. In agreement with their results, we found a small increase in the components of the immuno-proteasome in the aged muscle; however, we determined that the overwhelming increase of proteasome in aging concerns the 26S species (Figs. 2-3, S1 and S3, **Paper V**). We also assayed the activity of the proteasome utilizing all 3 techniques described in section 4.5 above (active-site labeling; small fluorogenic substrates and full length protein substrates) and found an increased level of proteasomal proteolysis matching the increased level of proteasomal proteins and particles (Figs. 2-3, **Paper V**). The discrepancy between our results and those of Ferrington and coworkers are not easily explained but difference in study design and in the assaying of proteasomal activity may have contributed. We used SD rats using a strict age-cohort design while the old Fisher-344/BN hybrid rats used by Ferrington and colleagues varied considerably in age when adjusted to expected life-span (Nadon, 2004). This may indeed be relevant, since (Bardag-Gorce et al., 1999) in an earlier of their studies on aging, using the LOU rat, increases in the proteasome were reported until a very old age when both activity and content had dropped. Our data are consistent with those found in LOU rats up to 29 months

of age. Another explanation for the difference may be the three independent techniques used to assay proteasomal activity in crude lysates and in preparations enriched for proteasomal particles. Therefore from our results, we conclude that increased proteasomal proteolysis may very well contribute to muscle atrophy during aging. A DR regime reported to retard aging in a large number of species including rats, also impeded the accumulation of proteasomes and the increase of proteolytic activity in muscle extracts compared to aged-matched controls (Figs. 2-3, S1, **Paper V**).

With this in mind, we then set out to identify the mechanism by which proteasomes increase in aging muscle. As discussed above proteasomal induction does not occur through the common mechanism of induction of muscle-specific E3 ligases and the subsequent transcriptional up-regulation of the proteasome subunits (for references see above and **Papers III** and **V**). Therefore we looked at other aspects of the complex protease kinetics: complex assembly and degradation. The assembly of the proteasome complex is assisted by several chaperones (see Introduction section 1.5.1 for details and Figs. 3-4). Through real time PCR we could not detect any transcriptional induction of these chaperone-assembly proteins (Fig. S6, **Paper V**). Therefore we turned our direction to examining the elimination of proteasomes. Although proteasomal degradation is not fully understood, the lysosomal pathway has been implicated in hepatocytes (Cuervo et al., 1995). Lysosomes have been reported to be under stress during aging and appear to become impaired by the accumulation of lipofuscin, at least in aging cardiomyocytes (Brunk and Terman, 2002a; Cuervo et al., 2005; Terman and Brunk, 2004b). By sectioning muscle tissue, we observed a conspicuous accumulation of lipofuscin in aged skeletal muscle indicating lysosomal load (Fig. 4, **Paper V**). To further investigate this, we used chloroquine to block the lysosomal pathway, achieved by elevating lysosomal pH, in both in tissue culture in a rat-derived muscle cell line (L6) as well as in adult rats. An increase in proteasome levels and activity was clearly observed in both (Fig. 4, **Paper V**). Thus, at least in rat myofibers, the blocking or impairment of lysosomes will cause an accumulation of active proteasomes. Based on this, we propose that impairment of the lysosomal/autophagy pathway represents a mechanism behind the accumulation of proteasomes that occurs during aging (**Paper V**). If this holds true, increased degradation by proteasomes would occur by an impairment of cell homeostasis mechanisms. The increased degradation through the proteasome will also demand increased levels of ubiquitin and/or rates of ubiquitin turn-around (Fig. 6) since proteins are tagged for this degradation machinery through polyubiquitylation (see Figs. 5-6). There is existing evidence for the increase of ubiquitin and ubiquitylation during aging (Cai et al., 2004; **Paper II** and references therein). In **Paper V**, we provide evidence for a marked increased activity of several members of the DUB family indirectly providing the evidence for increases in deubiquitylation (Fig. 5, **Paper V**).

Of special interest in this regard, is the down-regulation of the muscle-specific E3-ligases Atrogin-1 and MuRF1 during aging; this observation not only indicates that myocytes are equipped with means to selectively suppress these enzymes but also that this suppression is in operation in sarcopenic muscles. This previously not recognized option may allow muscle fibers to save myofibrillar proteins in a situation of increased protein degradation. A further piece of evidence for this suppression is the finding that not even treatment with steroids was able to induce these E3 ligases in the aged sarcopenic muscle (Fig. 6, **Paper V**).

#### 4.7 Conclusions

- In this thesis we have characterized a Sprague-Dawley based rat model as a useful tool to study old-age muscle atrophy (**Paper I**). The loss of muscle mass is part of a gender indifferent general drop in body mass that occurs in late life and affects skeletal muscle conspicuously. The pace of aging reflected in behavioral disturbances, decreased motor activity and loss of muscle mass, can be retarded by modest dietary (caloric) restriction while other environmental factors explored here had less of a clear impact on the aging parameters studied.
- Profiling of the transcriptome and proteome of sarcopenic muscles (**Paper II**) reveal that these tissues are in a situation of distressed repair with overt signs of redox stress, increase in iron load, DNA damage and efforts to regenerate myofibers. This profile is compatible with some of the theories on aging such as the “disposable soma theory” (Kirkwood and Austad, 2000) and the “Free radical theory of aging” (Harman, 1981; Harman, 1994).
- In contrast to many clinical conditions involving muscle atrophy, sarcopenia is not driven by the induction of muscle-specific E3 ligases of the UPS or by a transcriptional induction of the proteasome. In old-age muscle atrophy, these ligases are suppressed at the transcriptional level and are not inducible by corticosteroids (**Paper III and V**). A tentative mechanism for this old-age-suppression of E3 ligases is the translocation of FOXO from the nucleus to the cytosol via an AKT-mediated phosphorylation and is supported by evidence provided in **Paper III**.
- In the re-examination of the role of the proteasome in sarcopenic rat muscle, in which the probes developed in **Paper IV** were part of the toll-box, we found a two to three fold increase of assembled proteasome particles in muscle, and a corresponding increase in proteolytic activity was shown (**Paper V**). In aged animals, the increase in proteasomes correlated well with the degree of muscle wasting. Combined with the finding that DR retards aging and impedes the increase of proteasomes, this argues that loss of muscle mass in old-age, at least in part, is due to increased proteolysis by the proteasome. Although the mechanism by which active proteasomes accumulate in senescence awaits conclusive evidence, we provide data suggesting the impairment of the lysosomal pathway as a candidate mechanism.

#### 4.8 General Discussion

In this thesis we provide evidence that the common pathway activated in a range of clinical conditions with muscle atrophy is suppressed, rather than induced, in aging. Yet sarcopenic muscle contain increased levels of proteolytically active proteasomes, we conclude that increased proteasomal proteolysis probably contributes to the muscle wasting in old-age, but through a different mechanism that remains to be fully elucidated. In the final section of the thesis we would like to briefly place our findings into a broader context of present theories on sarcopenia. 35 years ago, Gutman and Hanzlikova (Gutman and Hanzlikova, 1972) wrote an excellent review on sarcopenia; then referred to as senile muscle atrophy. The majority of this review remains valid today.

Early on, a distinction was made between those that considered sarcopenia to be of a neurogenic origin and those that considered it a process originating in the target muscle. The former theory is based on the notion of a successive “drop-out” of motoneurons with advancing age, initially compensated for by the collateral innervation of vacated myofibers by the surviving motoneurons (reviewed in Larsson, 1995; Lexell, 1995). Awaiting compelling evidence, obtained using unbiased quantification techniques to demonstrate that we loose spinal motoneurons during aging, the neurogenic origin theory is still missing this critical piece of support (reviewed in Ulfhake et al., 2000). However, there are several lines of evidence that indicate that sarcopenia is associated with an impaired innervation of myofibers (see **Paper II**; Edstrom and Ulfhake, 2005). Of particular interest in this context are the results from cross-transplantation experiments (Carlson et al., 2001; Carlson and Faulkner, 1989) that show that the age of the recipient host had the strongest impact on successful re-innervation when skeletal muscle from young and aged donors were transplanted. This result indicates the decreased innervation capacity of aged motoneurons, for which there is additional support, and would explain why regeneration of myofibers is halted at the step of innervation in sarcopenic muscle (Edstrom and Ulfhake, 2005).

On the other side, we have the concept that impairment of myofiber maintenance and regeneration are at the origin of sarcopenia. A theory that attracted considerable attention was “the programmed aging theory” where adenopaus (review in e.g. Binnerts et al., 1992; Lamberts, 2000; Toogood, 2004) causes systemic levels of growth hormone to decline and as a consequence, liver production of IGF-1 will drop in senescence. IGF-1 is a critical growth and differentiation factor for skeletal myofibers (for Discussion and references see **Paper III**). Supporting this, the transgenic overexpression of IGF-1 in myofibers induce myofiber hypertrophy and impede aging-related myofiber atrophy (Barton-Davis et al., 1998; Musaro et al., 2001). However, myofibers appear not to be dependent on systemic IGF-1 (selective knock-down of IGF-1 in hepatocytes left myofibers unaffected; (Yakar et al., 1999)) rather myofibers rely on locally produced IGF-1 (autocrine and paracrine signaling). Available data does not indicate the down-regulation of IGF-1 or its cognate receptor in skeletal muscle during aging (**Paper III**; Edstrom and Ulfhake, 2005; Hamilton et al., 1995). On the contrary, animals with a less successful pattern of aging had the highest levels of IGF-1 transcript (Edstrom and Ulfhake, 2005), an observation consistent with the longevity of the growth hormone receptor mutated mouse (Coschigano et al., 2003). Another theory argues that loss of myofibers and fiber atrophy are due to myofiber degeneration caused by an accumulation of cellular damage exhibiting increased frequency of myonuclei apoptosis (Dirks and Leeuwenburgh, 2002; Kujoth et al., 2005; Whitman et al., 2005; see however Rice and Blough, 2006); the scientific evidence for this notion remains still a bit scanty, but deserves further exploration. More recently, there has been some focus on stem (satellite) cell depletion/senescence, making replenishment of myonuclei a bottle neck in aging. Data from studies on stem cell depletion vary but it remains an attractive hypothesis to explain loss of myofibers and myofiber atrophy (Abedi et al., 2005; Carlson and Conboy, 2007; Collins et al., 2007; Dezawa et al., 2005 and references therein; Verdijk et al., 2007).

Other theories focus on cell metabolism and, as discussed on several instances through-out this thesis, there is a common signaling pathway to several conditions involving the loss of muscle mass and is triggered by corticosteroids or circulating cytokines (TNF $\alpha$ ; INF $\gamma$ , IL-1). There are certainly changes to levels of circulating cytokines during aging (review in Bru-

unsgaard et al., 2001), however, the evidence for this hypothesis as a mechanism behind sarcopenia is currently not very strong (see **Paper III** and **V**). Changes with advancing age in the metabolism of carbohydrates and amino acids have also attracted a lot of attention as a possible mechanism in old-age muscle atrophy (Attaix et al., 2005a; Combaret et al., 2005; Dardevet et al., 1995) and may in part relate to altered nutritional status of the organism. It is here appropriate to recall that fasting is a powerful inducer of myofiber atrophy and that starvation (at the cell level) is capable of inducing not only the UPS but also the lysosomal system. If these systems now are interrelated, in which evidence now exists and is also provided in this thesis, the homeostasis mechanism regulating anabolic and catabolic signals may become upset in old-age myofibers.

Although we tend to look for *one* key mechanism underlying a biological phenomenon or a clinical condition, several of the above discussed mechanisms may operate in parallel to produce sarcopenia as the consequence of a slowly progressing accumulation of non-repaired damages in postmitotic cells; this explanation would satisfy those heralding the “disposable soma theory”.

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