Protein Damage Control during Embryonic Stem Cell Differentiation:
Role of the Proteasome

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AKADEMISK AVHANDLING

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To Jens & Eira

ABSTRACT

During the lifespan of organisms ranging from yeast to humans, there is an accumulation of macromolecular damage. However, these organisms produce youthful progeny with low damage levels. This thesis focuses on how this is accomplished.

I have analyzed whether the levels of oxidatively damaged proteins change in mouse embryonic stem (ES) cells during the initial steps of cell specification (differentiation) from the pluripotent state. The results show that ES cells contain high levels of proteins modified by carbonyls and advanced glycation end products and that the identity of these damaged proteins, including chaperones and proteins of the cytoskeleton, are the same as those of aged tissues. However, early differentiation is accompanied by a dramatic drop in the damage of such proteins, both in cultured ES cells and in the blastocyst *in vivo*. In addition, differentiation of ES cells triggers production and assembly of the proteasomal complex PA28-20S/20Si. Experiments using proteasome inhibitors and RNAi technology suggest that both the 20S proteasome and its regulator PA28 are required for the clearance of damaged proteins during differentiation of ES cells.

The data point to previously unknown roles of PA28 both in protein homeostasis and during early embryogenesis. Moreover, the results support a model in which the restoration of low levels of protein damage at the start of each generation is achieved, in part, by a maintained capacity of the germ line to rid itself from such damage.

Similar to mouse ES cells, studies using *Drosophila melanogaster* indicate that the main reduction in protein damage at the start of a new generation may depend on the proteasome. However, in contrast to the situation in mice, this reduction of protein damage appears to take place prior to fertilization. The data also indicate that mating has a negative effect on protein damage control and highlight egg production as a potential culprit in the trade-off between somatic maintenance and reproductive success.

Key words: Embryonic Stem Cells, Proteasome, Proteasomal Activator PA28, Protein Carbonylation, Advanced Glycation End products, Cell Differentiation, Oxidative stress, Aging, RNAi

LIST OF PUBLICATIONS

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

I. *Elimination of damaged proteins during differentiation of embryonic stem cells.*
Hernebring M*, Brolén G*, Aguilaniu H, Semb H & Nyström T. (2006) Proc Natl Acad Sci U S A. 103(20): 7700–7705.
* contributed equally to this work

II. *Identification of Hsc70 as target for AGE modification in senescent human fibroblasts.*
Unterluggauer H, Micutkova L, Lindner H, Sarg B, Hernebring M, Nyström T & Jansen-Dürr P. (2009) Biogerontology. 10(3): 299-309.

III. *The proteasome activator PA28 is required for the removal of damaged proteins during differentiation of mouse embryonic stem cells*
Hernebring M, Fredriksson Å, Norrman K, Rivett J, Wiseman J, Semb H & Nyström T. Manuscript.

IV. *Effects of aging and reproduction on proteostasis in soma and gametes of* Drosophila melanogaster.
Fredriksson Å, Krogh-Johansson E, Hernebring M, Pettersson E & Thomas Nyström. Manuscript.

ABBREVIATIONS

ADP	Adenosine 5’-diphosphate
AGEs	Advanced glycation end products
ATP	Adenosine 5’-triphosphate
CEL	*Nε*-carboxyethyl-lysine
CML	*Nε*-carboxymethyl-lysine
CR	Caloric Restriction
DAPI	4',6-Diamidino-2-phenylindole
DHR	Dihydrorhodamine
EBs	Embryonic Bodies
ER	Endoplasmic Reticulum
ES	Embryonic Stem
ETC	Electron Transport Chain
HNE	4-Hydroxy-2-nonenal
ICM	Inner Cell Mass
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
iPS	induced Pluripotent Stem
LIF	Leukemia Inhibitory Factor
MCO	Metal-catalyzed oxidation
MDT	Mortality Doubling Time
MEFs	Murine Embryonic Fibroblasts
MHC-I	Major Histocompatibility Complex I
MS	Mass Spectrometry
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAGE	Polyacrylamide gel electrophoresis
POMP	Proteasome Maturation Protein
RAGE	Receptor for Advanced Glycation End products
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SSEA-1	Stage-Specific Embryonic Antigen 1
TE	Trophectoderm
TNFα	Tumor necrosis factor-α

CONTENTS

1. INTRODUCTION

PRELUDE

Cells and cell constituents of our bodies are constantly being replaced. Yet, various types of damage accumulate over time and contribute to the manifestation of what we call aging. The fact that the offspring is born without much damage raises an interesting question: How is this damage asymmetry between the progeny and the progenitor accomplished?

Germ cells are capable of immortality since they can potentially become part of an offspring, and thereafter the offspring's offspring, and so on. This quality makes them very fascinating from an aging perspective. Why do not the germ cells become damaged and aged like the somatic cells? What exceptional defense ability do they have?

The formation of a new individual begins with the fusion of gametes (mature sex cells) and subsequent advancement into embryonic stem cells. During early embryogenesis these undergo complex series of differentiation that will ultimately give rise to all the bodily cells types, including the germ cells capable of reproduction in another round. In this scenario, one can imagine that the germ line is kept isolated and protected from the damage the somatic cells are exposed to. This would demand a complete preservation strategy that sustains the integrity of the germ line in the course of its 'life cycles'. On the other hand, it is possible that there is some sort of damage clearing built-in in one of the developmental steps described above. In this latter case, immortality would be sustained by a maintained potential to get rid of whatever damage that may arise, either inherited or newly formed.

PURPOSE AND AIM

In order to better understand how youth can be ensured during reproduction, this thesis focuses on the protein damage control during early differentiation. Specifically, this thesis aims to answer the following questions:

(1) Are embryonic stem (ES) cells from mice essentially free of damage?
(2) Do the levels of protein damage change during early differentiation of ES cells?
(3) If so, how do they change and what causal mechanisms are involved?

DELIMITATIONS

As stated under *Purpose and aim*, I have chosen to focus on the differentiation of ES cells and the protein damage management during this stage. This approach will thus not cover all aspects of rejuvenation in relation to age-related damage. For example:

(1) If there is a riddance of damage it could take place at some other step than the onset of the ES cell differentiation process (e.g. during maturation of the germ cells or later on during development), and would in that case not be detected using this approach.
(2) The age-related damage in the scope of this thesis is limited to that of proteins as targets, while DNA damage is not covered.
(3) The mouse is used as a model organism in these studies, and the applicability to other organisms is not known.

2. WHAT IS AGING?

This chapter introduces a definition of aging and aging theories relevant for this thesis.

Aging can be defined as a progressive deterioration of bodily functions and fitness increasing the probability of death over time. The genetic code contains all the information needed to repair and/or replace any impairments formed with aging. So why don't the cells of our body do this? Medawar called this the paradox of aging (Medawar, 1952). That aging is not inevitable is also illustrated by the fact that we do have cells in our body that have the potential to live forever. Tumor cells can divide indefinitely and our germ cells can become part of a new and younger whole individual.

ULTIMATE AGING THEORIES: WHY AGING OCCURS

I will start off by addressing the ultimate question of what aging is, i.e. why aging occurs, as opposed to the proximate question of the mechanistic causes of aging, which will be addressed later on. In doing so, I will argue that aging is most likely not the result of an active aging program and that it is possible to prolong lifespan but at a certain cost. An important definition in considering aging is the Mortality Doubling Time that I will use when available.

The **Mortality Doubling Time (MDT)**[1] is a way to express aging that enables comparisons between populations (Gompertz, 1825). MDT is the time it takes for the probability of dying to double. For example, since women live longer than men[2] it is natural to presume that

[1] *The Mortality Doubling Time (MDT) is derived from Gompertz law of human mortality formulated in the 1820s (Gompertz, 1825). It describes the mortality as exponentially increasing over age. Displaying mortality with a logarithmical scale generates a straight line (generally between the ages of 40 to 80 in humans), the slope of which has been designated the rate of aging. Shifts in extrinsic or intrinsic mortality alter the position of this line (where it crosses the y-axis), as described by the addition of the Makeham parameter to the Gompertz equation (Makeham, 1860; Hallén, 2009).*

[2] *A child born in Sweden today is predicted to live around 80 years if it is a boy and 84 years if it is a girl (SCB, 2010), and a similar gender based distinction in life expectancy is seen throughout the world (UNSD, 2010). Of the 214 countries or areas present in the United Nations Statistics Division's Indicators of Health, only four (Botswana, Swaziland, Turks and Caicos Islands and Zimbabwe) show the opposite relation (UNSD, 2010). As pointed out by Steven Austad when he did this comparison in*

women age slower than men. There is however no difference in the speed by which men and women age. The MDT is 7-8,5 years for both sexes (Finch *et al.*, 1990; SCB, 2010); males just start off at a higher mortality. This increased risk of dying of males is the same throughout life and can be attributed to differences in the levels of **extrinsic mortality** (due to environmentally-imposed risks) and/or **intrinsic mortality** (caused by innate features, e.g. hormonal effects) between the male and female population.

Natural selection is unlikely to favor an aging program

The evolutionary explanation of aging boils down to whether aging is programmed or not. Aging as a genetic program is alluring because of its potential promise of the existence of a long life elixir. According to such a theory, it is possible to shut off the gene(s) that triggers aging and live forever. However, this is not likely to be the case.

The most important argument against an aging program is that it is difficult to understand how it would be selected for. Natural selection will favor genetic changes/programs that increase the offspring's chances to survive and reproduce. The force of natural selection is stronger at the time before and during the reproductive period than after. This is because features that promote survival after reproduction are not as likely to affect the odds of the progeny to live on and propagate (Medawar, 1952; Williams, 1957) and for an aging program to exist it needs to come with some advantage.

The pacific salmon is one of the most striking examples of a species that displays what seems like a typical programmed aging phenotype. It migrates back to its natal stream when sexually mature, spawns, and dies shortly thereafter. It has been argued that their remains supply nutrients to the often glacial-fed and energy poor rivers, and thereby increasing the probability that their offspring survive (Wingfield and Sapolsky, 2003). This would however be an advantage to the whole population reproducing in this area, and not specifically an energy source to the offspring of the "suicidal" parent. When the

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1997 (Austad, 1997) with data from 1988, the prediction of men to outlive women is so unexpected that one instantly suspects that women are valued differently in these countries/islands. Encouragingly, the number has decreased since then; there were six exceptions in his example.

salmon start this energetically demanding journey they stop feeding, and all energy left is devoted to migrating and gonad maturation. The gonads may actually account for more than 30% of their body weight in the end (Wingfield and Sapolsky, 2003). As expected, high levels of the stress hormone cortisol is found in the fish' circulating system during this period (Donaldson EM, 1968). This hormone is very likely to help the salmon to find its natal stream (Carruth *et al.*, 2002) while simultaneously causing death; since equal amounts of cortisol kill non-mated fish (Wingfield and Sapolsky, 2003).

For animals that are unlikely to achieve proper conditions for reproduction more than once, a fatal resource investment such as that of the pacific salmon is logical from an evolutionary perspective. However, there is no genetic program turned on with the single goal to altruistically kill the parent, and by extension there will not be a single gene or set of genes that can be turned off to avoid death.

Natural selection can promote longevity in species in which the survival of the offspring requires care from parents or other older relatives (Bourke, 2007). Thus, aging is probably an effect of the decreased need of the offspring for their parents. In other words, aging is the result of an absence of selection rather than a direct genetic program.

It is possible to alter the rate of aging

<u>How slower aging can be favored by natural selection</u>

If aging is not programmed – is it just random? The fact that the rate of aging can be modulated indicates that it is not just simply stochastic. Lowering the extrinsic mortality, by moving a population from a hazardous environment to a protected one, will over time (and many generations) lead to a slower rate of aging. This is linked to the fact that since fewer individuals will die from predators or accidents, they will live for a longer time. The force of natural selection can then operate over a longer period of the individuals' lifespans, favoring genes that promote maintenance later in life.

As had been the case for the marsupial Virginia opposum in a study of Austad in 1993 (Austad, 1993), in which two isolated populations in

environments of markedly different extrinsic hazard were compared with regard to their lifespan, litter size and growth rate etc. The study showed that the opossums that had a higher risk of dying young aged faster, demonstrated by a lower MDT. In other reports, artificial selection for late reproduction over many generations successfully generated long lived *Drosophila* strains (Rose and Charlesworth, 1980; Luckinbill, 1984; Partridge, 1999). In at least one of these, the effect was due to increased MDT (Partridge, 1999), while MDT data was absent from the other studies.

The cost of longevity

The examples above illustrate that is possible to extend lifespan. However, the obtained longevity comes with a cost. The opossums of lower extrinsic mortality and subsequent slower aging also produced less offspring early in life (Austad, 1993). Similarly, the lifespan extensions of late reproducing *Drosophila* were accompanied by a decline in early fecundity (offspring production). In fact, a recent comparative analysis of more than a hundred terrestrial vertebrate taxonomic families, demonstrated a positive correlation between MDT and the age at sexual maturity, as well as the duration of gestation period (embryo growth) (Ricklefs, 2010).

The idea of some kind of 'fixed quantity of vital vigor' that can be spent at different rates has been recognized for a long time (Pearl, 1928; Comfort, 1956; Williams, 1957). Initially, the distinction was made between growth and longevity and attributed to the rate of energy expenditure in the ***Rate of living theory*** (Pearl, 1928). While the rate of living theory is now considered an oversimplification, the concept of a compromise between growth and longevity of some sort may still be valid. There are two current theories to explain such compromises; the antagonist pleiotropy theory and the disposable soma theory.

The ***Antagonist pleiotropy theory*** (Williams, 1957) states that there are genes that promote early fitness and reproduction at the expense of late-life survival, as well as genes beneficial late in life with negative effects at young age. If extrinsic mortality rates are high, there is a strong positive selection for early-acting and "fast living" pleiotropic genes, resulting in young 'vigor' (and early reproductive success) but lower fitness at advanced age. The poor condition at old age will be

neutral for the survival of the species since very few specimens survive these hazardous conditions to reach this age. When individuals of a population increase their lifespan due to reduced environmentally-imposed deaths, genes with positive effects later in life give some advantage (as opposed to being neutral), and the pleiotropic effect is a subsequent reduction in fitness at young age.

According to the ***Disposable soma theory*** (Kirkwood, 1977; Kirkwood and Holliday, 1979) there are limited energy or metabolic resources that can be directed either towards maintenance/longevity or reproduction, rather than growth. August Weismann made the distinction between the immortal germ line (germ plasm) and the mortal soma in the late 1900s (Weismann *et al.*, 1893) and this may be argued to have laid the foundation of the Disposable soma theory. Animals that have a high reproductive fitness early in life are according to this theory predicted to have invested less in maintenance and hence age faster than those less efficient in reproduction. As opposed to the population-based evolutionary theory of Antagonist pleiotropy, the Disposable soma theory can operate at the level of the individual in one single lifespan.

Lifespan extensions based on a trade-off between reproduction and maintenance

In support of the Disposable soma theory, it is possible to prolong lifespan by shifting the trade-off of the undefined resources from reproduction towards maintenance. For example, keeping *Drosophila* females isolated from males generates a mean lifespan twice as long as those of mated females. Removing their ovaries increases longevity even further (Smith, 1958). In addition, mating of *Drosophila* males also experience a negative effect on lifespan, although not as pronounced as for the females (Partridge and Farquhar, 1981). This phenomenon has also been observed in the nematode *C. elegans*. Removing the germline by killing its precursor cells with a laser microbeam extended lifespan by 60% (Hsin and Kenyon, 1999).[3] In

[3] *However, targeting the C. elegans precursors of the somatic gonad as well as the germ line precursors, so that the entire gonad is missing, did not prolong life span (Kenyon et al., 1993), suggesting a more complex relation between reproduction and longevity in the nematode.*

addition, genetic alterations causing sterility by affecting the germ line also generated long lived strains (Arantes-Oliveira *et al.*, 2002).

Moving on to humans, an analysis of data from genealogies of the British aristocracy (with year of birth between 740 and 1875) demonstrated that longevity is negatively correlated to the number of children (Westendorp and Kirkwood, 1998). The same relation was found for women who lived to the age of 60 or longer, excluding pregnancy-related mortality. Surprisingly, male lifespan displayed similar inverse correlation to the number of children.[4]

In summary, there is a trade-off between offspring production and maintenance, and reducing reproduction can in many cases be considered as a means to prolong lifespan.

Caloric restriction: A means to push the trade-off towards maintenance at the expense of reproduction?

Since lowering the reproductive capacity can be regarded as a way to shift the trade-off towards survival and longevity, interventions that reduce offspring production and prolong lifespan may be an effect of this trade-off. One example is Caloric Restriction (CR).

As an attempt to examine the lifespan effects of delaying growth rate in rats, McCay performed the first successful lifespan extending CR experiment on mammals (McCay *et al.*, 1935). Previous studies had demonstrated positive correlations between longevity and amount of food, and McCay believed this could be caused by malnutrition of the less-eating test groups. Being a nutritionist, he realized the importance of including enough nutrients in the diet while cutting down on calories only. By doing so, he observed a 60% increase in mean lifespan of male rats.

The longevity effect of CR (without malnutrition) has since then been shown to be valid in organisms of highly diverse complexity, ranging from the yeast *Saccharomyces cerevisiae* (Lin *et al.*, 2002) to rhesus monkeys (Colman *et al.*, 2009) regardless of gender. The CR-induced

[4] *These results can be explained by both the Disposable soma theory and the Antagonist pleiotropy theory, illustrating that they are non-exclusive.*

lifespan extension is generally accompanied by a lowered offspring production, at least early in life (Marden *et al.*, 2003; Mair and Dillin, 2008; Flatt and Schmidt, 2009).

Thus, CR could exert its longevity effects through somehow shifting the focus of the trade-off towards maintenance, which would explain the associated decrease in reproductive fitness.

THE PROXIMATE QUESTION OF WHY WE AGE: THE MECHANISMS BEHIND AGING

There are many different possible approaches to the question of what aging is and what the causal mechanisms behind aging are. One is to establish correlations between aging and various types of events on the molecular, cellular and organ level. Another is to investigate features of premature aging diseases, i.e. diseases with symptoms that mimic aging (e.g. Hutchinson-Gilford progeria syndrome, Werner syndrome and Cockayne syndrome). In model organisms, specific genetic alterations causing increased lifespan can be studied and identified. The examination of human centenarians can be done to determine the distinguishing features of individuals surviving to a superior age. Long lived populations (described in the former section and others) can be studied in a similar manner; to find out which characteristics are linked to longevity.

Features that typify long lived populations

The *Drosophila* strains that had obtained a longer lifespan after selection of late reproduction over many generations displayed increased resistance to variety of stresses (Service *et al.*, 1985). In addition, caloric restricted animals exhibit decreased levels of macro-molecular oxidative damage and CR is thought to induce longevity by somehow lowering the detrimental effects of harmful processes and/or by boosting replacement and repair functions (Mair and Dillin, 2008 and references therein).

Mutations lowering the insulin/insulin-like signaling (IIS) pathway is known to extend lifespan in *C. elegans* (Friedman and Johnson, 1988; Kenyon *et al.*, 1993), *Drosophila* (Clancy *et al.*, 2001; Tatar *et al.*,

2001), and mouse (Selman *et al.*, 2008). These three organisms were subjected to a comparative analysis of the alterations in gene expression of long lived mutants with reduced IIS signaling (McElwee *et al.*, 2007). This study showed that the lifespan extension in all three organisms were associated with higher expression of genes encoding glutathione-S-transferases (GSTs). GSTs are involved in the detoxification of harmful metabolic byproducts and oxidative stress-induced breakdown products.

In summary, in these many examples, populations displaying extended lifespans exhibit features that would provide resistance against damages caused by life itself.

The general answer: life causes aging

As stated in the previous section, processes fundamental to life appear to be intimately linked to the progression of aging. There are more than 300 theories that aim to define the mechanistic causes of aging (Medvedev, 1990). Trying to generalize, one can say that aging is thought to occur due to two main reasons: (i) loss of cell cycle control and (ii) accumulation of metabolically generated damage.

Having cells able to divide is crucial for several bodily functions and a stringent cell division control is essential to avoid cancer. Cellular senescence can be regarded as a mechanism to reduce cancer occurrence, while in parallel contributing to the aging phenotype by limiting the regenerative potential of cells in the body (reviewed in Campisi and d'Adda di Fagagna, 2007; Liu and Sharpless, 2009). However, the incidence of cancer also increases with advanced age and genomic instability is a hallmark of both aging and cancer (Finkel *et al.*, 2007).

The second category is the accumulation of damage to cellular constituents. This is generally thought to occur as a byproduct of metabolism, and this damage accumulates over time with increasingly detrimental effects.

The two underlying reasons for aging described above are, however, not independent of each other. Rather, they are interdependent to at least some extent, because they can induce each other. Accumulation

of damage is most likely involved in both senescence and cancer, and both these events may generate more damage. In this thesis, I will focus on causes linked to damage accumulation, in particular damage to proteins.

3. PROTEIN DAMAGE

This chapter gives a background on various types of protein damage, how these are formed and what is known about their role in aging.

THE GENERATION OF PROTEIN DAMAGE

The Free radical theory of aging

In 1956, Harman proposed the ***Free radical theory of aging*** (Harman, 1956) which suggests that aging is the result of free radical attacks on cell constituents. Harman stated that reactions involving molecular oxygen in the course of cellular metabolism are likely to be the main source of these free radicals.

Although it took a few decades, the idea of a cellular and metabolic origin of free radicals is now universally accepted. Moreover, many studies have shown a correlative relation between aging and the damage these cause, though a causative relation is still highly debated.

Reactive Oxygen Species

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are reactive molecules which can have cellular origin and cause damage to proteins, lipids and nucleic acids. ROS include superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$); while nitric oxide ($\cdot NO$) and peroxynitrite ($ONOO^-$) belong to the family of RNS.

The mitochondria is a major source of cellular free radicals in the shape of $O_2^{-\cdot}$ (reviewed in Murphy, 2009), and this radical can serve as a precursor for several other ROS/RNS. $O_2^{-\cdot}$ is formed in the course of oxidative phosphorylation, during which electrons are transferred through the Electron Transport Chain (ETC) while generating a proton-motive force across the mitochondrial inner membrane. This energy potential, a combination of pH and electric charge, is used by ATP synthase to phosphorylate adenosine 5'-diphosphate (ADP) to adenosine 5'-triphosphate (ATP), the most important carrier of chemi-

cal energy in cells. The primary electron donors for the ETC are NADH (reduced form of NAD^+, Nicotinamide Adenine Dinucleotide) and $FADH_2$ (reduced form of FAD, Flavin Adenine Dinucleotide), reduced during glycolysis and the Tricarboxylic Acid (TCA) cycle, and the final electron acceptor is molecular oxygen (O_2) producing water (H_2O). The two major sites for $O_2^{-\cdot}$ formation are thought to be complex I (NADH dehydrogenase) (Kushnareva *et al.*, 2002) and complex III (ubiquinone-cytochrome c reductase) (Chen *et al.*, 2003).

During the past decade, the view that ROS is being an exclusively harmful and inevitable byproduct of aerobic respiration has changed. ROS has now been linked to a wide range of essential physiological responses, e.g. in cell proliferation, differentiation and migration (reviewed in Janssen-Heininger *et al.*, 2008).

Yet, the cell evidently requires many systems to protect itself from undesired effects of ROS. Superoxide dismutase (SOD) converts $O_2^{-\cdot}$ to H_2O_2 (McCord and Fridovich, 1969), which is then further neutralized by catalase (Radi *et al.*, 1991) or by the numerous types of glutathione peroxidases and peroxiredoxins present in the cell (reviewed in Fourquet *et al.*, 2008). Thus, it appears that cells are equipped with systems to keep the local concentrations of ROS within a physiological window; i.e. a range that does pass the threshold for generating damage but still allows useful functions, for example as signaling molecules.

Examples of protein damage

The reaction of ROS/RNS with proteins generates a plethora of oxidative modifications (reviewed in Stadtman and Levine, 2003; Stadtman, 2006), some of which are reversible and others irreversible.

Reversible oxidative modifications

Reversible oxidation in an ordered manner is vital for protein function; having important roles in protein folding (reviewed in Raina and Missiakas, 1997) and regulation of proteins activities (Sullivan *et al.*, 1994; Ciorba *et al.*, 1997). Such modifications can also serve as a buffer during transient periods of oxidative stress (Luo and Levine,

2009; Requejo *et al.*, 2010) preventing the oxidation from targeting more critical sites (Reddy *et al.*, 1994).

Reversible oxidative modifications affect sulphur-containing amino acids, i.e. cysteine and methionine. These alterations include disulphide bridges, reduced by the glutaredoxin/glutathione/glutathione reductase system and thioredoxin/thioredoxin reductase system (reviewed in Holmgren, 2000); and methionine sulphoxide, reversed by methionine sulphoxide reductase (Msr) in a thioredoxin dependent reaction (reviewed in Brot and Weissbach, 1983; Toda *et al.*, 2010).

Irreversible oxidative protein damage: Carbonylation

Protein carbonylation is thought to be the most abundant irreversible protein oxidative modification (Stadtman and Levine, 2003). Its presence is a sign of damage produced by multiple types of ROS (Stadtman and Levine, 2003) and it can impose a change in the structure of the protein, leading to impaired enzymatic function (Levine, 1981; Yan *et al.*, 1997; Yan and Sohal, 1998). Protein carbonylation is commonly used as a biomarker for oxidative protein damage.

In biological samples, glutamic and aminoadipic semialdehydes are the main variants of carbonyl derivatives. These are formed by metal-catalyzed oxidative (MCO) attacks on arginine and proline, generating glutamic semialdehydes, and lysine, producing aminoadipic semialdehydes (Requena *et al.*, 2001). MCO attacks involve the reaction between protein-associated Fe(II) and H_2O_2, generating a free radical (·OH or ferryl ion etc) which then attacks the surrounding side chains (Stadtman, 1990). This has been described as a caged process and therefore not accessible to free radical scavengers (Stadtman, 1992).

In addition to the generation through MCO attacks, protein carbonylation can be formed by secondary reactions with reactive carbonyl moieties of lipid peroxidation products, oxidized carbohydrates and advanced glycation/lipoxidation end products (AGEs/ALEs) (Stadtman and Levine, 2003; Nystrom, 2005; Dalle-Donne *et al.*, 2006).

Irreversible protein damage: Advanced glycation end products (AGEs)

Advanced glycation end products (AGEs) are the result of a complex set of reactions between reduced sugars and amino acid side chains, specifically lysine and arginine, that may include lipid peroxidation (Baynes, 2001; Rahbar and Figarola, 2003; Thorpe and Baynes, 2003; Semba *et al.*, 2010). The first stages of these reactions are often reversible non-enzymatic protein glycation to Schiff bases and Amadori rearrangements. These early glycation products can then go through further complex reactions ultimately categorizing them as the heterogeneous group of AGEs (Baynes, 2001; Thorpe and Baynes, 2003). AGE modified proteins cannot be repaired and are often functionally deficient (Ahmed *et al.*, 2005; Dobler *et al.*, 2006).

Identical AGEs can be derived from different pathways and carbohydrates. For example, *N*ε-carboxymethyl-lysine (CML), a well categorized and a widely used marker for AGEs, can be generated through three main pathways: i) Hodge pathway (from Amadori compound), ii) Wolff pathway (via glyoxal from autoxidative glycosylation) and iii) Namiki pathway (via glyoxal from Shiff base). In addition, CML can be formed through several alternative pathways, e.g. via glyoxal from ascorbic acid or lipids and autoxidation of serine (Baynes, 2001; Thorpe and Baynes, 2003 and references therein).

Most AGEs require oxidative modifications to be formed, but there are exceptions. One of them is *N*ε-carboxyethyl-lysine (CEL) which may be derived through non-oxidative routes from triose phospates or methyl glyoxal, intermediates in anaerobic glycolysis (Thorpe and Baynes, 2003; Desai KM, 2010).

As already mentioned, early glycation products can be reversed. For example, fructosamine, a common early glycation product, may be dismantled by fructosamine 3-phosphokinase (Delpierre *et al.*, 2000; Szwergold *et al.*, 2001). In addition, several enzymes are involved in the detoxification of reactive intermediates, i.e. glyoxal and methylglyoxal, thereby reducing the formation of AGEs. One important such detoxifier is the glutathione dependent glyoxalase system (reviewed in Thornalley, 2003), overexpression of which have been shown to lower AGE formation (Shinohara M, 1998; Morcos *et al.*, 2008; Brouwers *et al.*, 2011).

AGEs and reactive intermediates, such as methylglyoxal, may themselves induce ROS (reviewed in Baynes, 2001; Desai KM, 2010; Semba *et al.*, 2010). Addition of methylglyoxal has been shown to increase ROS/RNS levels of various cell types (Kikuchi *et al.*, 1999; Chang *et al.*, 2005; Rondeau *et al.*, 2008). The means by which methylglyoxal/AGEs cause elevated levels of ROS/RNS have been attributed to: i) inactivation of glutathione peroxidase and glutathione reductase (Blakytny R, 1992; Park YS, 2003), ii) AGE-modification of mitochondrial proteins (Rosca *et al.*, 2005; Morcos *et al.*, 2008), iii) AGE binding to the receptor for advanced glycation end products (RAGE) activating a variety of signaling transduction cascades contributing to the complications of inflammation, diabetes (Yan *et al.*, 2009), neurodegeneration (Miranda and Outeiro, 2010) and cancer (Sparvero *et al.*, 2009).

PROTEIN DAMAGE AND AGING

Protein damage increases during aging

The load of protein damage increases with aging in numerous tissues and organisms including humans. Specifically, the human brain accumulates protein carbonyls (Smith *et al.*, 1991) and AGEs (Kimura *et al.*, 1996) during aging. There is an age-dependent increase in the load of carbonyls of human fibroblasts (Oliver *et al.*, 1987), and senescent human fibroblasts exhibit elevated levels of AGEs and carbonyls (Paper II). In addition, AGEs have been shown to increase with age in human cartilage (Verzijl *et al.*, 2000), lens proteins (Ahmed *et al.*, 1997), arteries (Nerlich and Schleicher, 1999) and skeletal muscles (Haus *et al.*, 2007).

In rodents, carbonylation has been demonstrated to accumulate during aging in rat hepatocytes (Starke-Reed and Oliver, 1989) as well as in various tissues of the Mongolian gerbil (Sohal *et al.*, 1995). The level of AGEs in skeletal muscles of rats are also dependent on their age (Snow *et al.*, 2007). *Drosophila* aging is associated to increased protein carbonyls (Sohal RS, 1993) and AGEs (Oudes *et al.*, 1998), and *C. elegans* display a similar aging pattern regarding these types of irreversible damages (Adachi *et al.*, 1998; Morcos *et al.*, 2008).

In the examples recapitulated above the relation between age and damage load is not linear. Rather, the elevation of protein damage during aging has some resemblance to the exponential increase in mortality over age which Gompertz used in his definition of aging (as outlined in chapter 2). Alternatively, the elevation in protein damage over age might be two-phased, displaying one slope in the first 60% of lifespan and a steeper one thereafter (Levine, 2002).

The exponential or biphasic increase in protein damage could be explained by the vicious circle of protein damage generation. As already mentioned, protein damage impairs important cellular processes and some of these processes are themselves linked to damage prevention, repair (reviewed in Dröge and Schipper, 2007) and removal (see chapter 4). For example, reduced translational or transcriptional fidelity has been shown to increase the protein products' susceptibility to oxidative damage (Dukan *et al.*, 2000; Ballesteros *et al.*, 2001). In addition, damaged proteins can form aggregates which can generate more damage by producing ROS (reviewed in Tabner BJ, 2005), which may be linked to the previously discussed induction of ROS by AGEs.

There are reports demonstrating that old mitochondria produce more ROS (Shigenaga MK, 1994; Head *et al.*, 2009) although some of these results have been questioned (Maklashina and Ackrell, 2004). Comparative studies of animals with different aging rates have shown ROS production to be negatively correlated to longevity (Barja, 2004; Lambert *et al.*, 2007), though some exceptions exist during modulation of aging in, for example, *Drosophila* (Miwa *et al.*, 2004; Sanz A, 2010) and *C. elegans* (reviewed in Van Raamsdonk and Hekimi, 2010).

The increase in protein damage load with aging has also been correlated to deteriorating functions. For example, there is a negative correlation between the levels of carbonyls and fitness in *Drosophila* (as in being an active or passive fly) (Sohal RS, 1993). Furthermore, the amount of AGEs in the blood plasma of older adults (>65 years of age) is associated with an increased risk of death of any cause (Semba *et al.*, 2009). However, these correlations could be linked to disease rather than aging. AGEs are particularly associated with numerous age-related diseases, and serve as a biomarker for several disorders (reviewed in Semba *et al.*, 2010).

Modulations of ROS/RNS and protein damage levels

As stated above, aging is associated with an accumulation of ROS/RNS-induced damage (e.g. AGEs and carbonyls). However, these observations are merely correlative and do not necessarily concern the causes of aging. Numerous studies have been carried out aimed at pinpointing the causal players in aging. One of these took advantage of the detoxifying properties of glyoxalase-1 and showed that its overexpression lowered AGE levels and prolonged *C. elegans* lifespan (Morcos *et al.*, 2008). Many reports have concentrated on the direct modulation of ROS/RNS levels, which have led to conflicting results.

For example, an extensive review of data from human randomized clinical trials demonstrated that antioxidant supplements do not correlate with reduced mortality (Bjelakovic G, 2008). In addition, some studies even indicated that antioxidant supplements correlate with a shorter lifespan. In accordance with the unpredictability of antioxidants in human clinical trials, genetic alterations affecting antioxidant capacity in mice is also a complicated and conflicting issue in relation to aging (Salmon *et al.*, 2010). Lowering superoxide activity has been shown to either have no effect on lifespan (Van Remmen *et al.*, 2003; Elchuri *et al.*, 2004), reduce lifespan (Elchuri *et al.*, 2004; Sentman *et al.*, 2006), and cause embryonic lethality (Li *et al.*, 1995; Huang *et al.*, 2001). Similarly, deletion of MsrA (methionine sulphoxide reductase) has been shown not to affect lifespan in one study (Salmon *et al.*, 2009) and to impose a negative effect in another (Moskovitz *et al.*, 2001). Overexpression of superoxide can increase longevity (Hu *et al.*, 2007), or be ineffective (Jang *et al.*, 2009). Overexpressed catalase localized to the peroxisome or nucleus did not affect lifespan significantly, whereas targeting catalase to the mitochondria increased lifespan (Schriner *et al.*, 2005). In addition, studies on the effects of antioxidant enzymes on lifespan in *Drosophila* and *C. elegans* are equally conflicting (reviewed in Zimniak, 2008; Salmon *et al.*, 2010).

Evidently, drawing conclusions from antioxidant experiments is a challenge, if not to say a gamble (see also Murphy *et al.*, 2011). One of the reasons for this is most likely the role of ROS in signaling, which requires appropriate cellular localization of the scavenger. In addition, important cellular processes like these often have redundancies covering up some defects. When approaching factors that may limit

lifespan, one can argue lifespan extensions to be generally more 'relevant' than reductions, simply because the latter could be caused by malfunctions unrelated to aging. Moreover, since ROS is important for signaling in cellular functions, disturbing ROS signaling should be more likely to shorten, rather than prolong, lifespan.

Thus, the information from antioxidant treatments is inconclusive regarding whether ROS/RNS have a causative role in aging and the discrepancy illustrates the complexity of both ROS homeostasis and the aging phenomenon. However, the examples above demonstrate that it is possible to extend lifespan by reducing ROS/RNS levels in some cases. Why this intervention sometimes has this effect and sometimes not remains to be elucidated.

4. REMOVAL OF PROTEIN DAMAGE: THE PROTEASOME

Here an introduction to the proteasomal system is presented, including its regulators, its degradation of damaged proteins, and role in aging.

THE PROTEASOME AND ITS REGULATORS

The 20S proteasome

20S is the catalytical core of the proteasome (also referred to as Core Particle, CP or Multicatalytic protease, MCP). It is a hollow cylindrical structure composed of one ring of α-subunits on each side of two inner β-subunit rings (Lowe *et al.*, 1995; Groll *et al.*, 1997). In eukaryotes, the seven subunits in each ring have functionally important differences in their inward-facing N-termini. The α-subunits regulate substrate entry and binding of regulators. In the absence of a regulator the N-terminal ends of α-subunits form a closed gate into the inner proteolytic β-subunit chamber (Wenzel T, 1995; Groll *et al.*, 2000). Proteolysis is carried out by the β1 (Psmb1/Lmpc5/delta), β2 (Psmb2/Z), and β5 subunits (Psmb5/X) (Seemuller *et al.*, 1995). β1 exhibit caspase-like activity (peptidyl-glutamyl-peptide bond hydrolase), i.e. cleavage after acidic amino acids (Loidl *et al.*, 1999). β2 and β5 display trypsin-like (cleavage after basic amino acids) and chymotrypsin-like activities (cleavage after hydrophobic amino acids), respectively (Fenteany *et al.*, 1995).

In higher eukaryotes, there are alternative proteolytically active β-subunits, replacing the regular ones under certain conditions. When β1i (Psmb9/Lmp-2/RING12), β5i (Psmb8/Lmp-7/RING10) (Glynne *et al.*, 1991; Kelly *et al.*, 1991; Ortiz-Navarrete *et al.*, 1991) and β2i (Psmb10/Mecl1) (Groettrup *et al.*, 1996; Hisamatsu *et al.*, 1996; Nandi *et al.*, 1996) are in the 20S structure, the complex is called 20Si or the immunoproteasome, because of its role in antigen presentation (see below). The βi-subunits are not substituted in preexisting 20S, rather they are incorporated in *de novo* synthesized 20S proteasomes (Griffin *et al.*, 1998) and may result in intermediate variants of 20S and 20Si,

containing β5i or β5i and β1i (Guillaume *et al.*, 2010). In addition, the thymus-specific β5t-subunit (Psmb11) can replace β5 forming thymoproteasomes, which is thought to be important in the generation of MHC class I restricted T cells (Murata *et al.*, 2007).

Regulators of the 20S proteasome

The 19S regulator

The most studied regulator of the proteasome is the 19S (also known as PA700/Regulatory Particle, RP/ATPase complex, AC) which binds one or both ends of 20S to form the 26S proteasome (Fig. 1). The proteins targeted for 26S degradation are covalently linked to a polyubiquitin chain, which is accomplished by a cascade of the enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase). The 26S proteasome recognizes the polyubiquitin tags and degrades the protein in an ATP-dependent process. Major targets include proteins controlling cell cycle, transcription, DNA repair and apoptosis etc (reviewed in Finley, 2009).

The crystal structure of the 19S regulator has not yet been resolved, which may be due to its unstable conformation and/or variable composition. However, cryo-electron microscopy (Nickell *et al.*, 2009; Bohn *et al.*, 2010) supports a model in which there are two sub-assemblies of the multi-subunit complex of 19S, called the base and the lid (Glickman *et al.*, 1998). The base contains six AAA+ ATPases (Rpt1-6) (Tomko Jr *et al.*, 2010) and proteins involved in scaffolding as well as ubiquitin recognition and processing. The lid is composed of nine non-ATPase subunits, of which at least one exhibits deubiquitinylation activity (Rpn11) (reviewed in Kim *et al.*, 2011; Stadtmueller and Hill, 2011).

The PA28 regulator

PA28 (11S/PA28αβ/REG) is a cytosolic regulator of 20S and potently activates ATP-independent degradation of oligopeptides, but reportedly not whole proteins (Yukawa *et al.*, 1991; Dubiel *et al.*, 1992; Ma *et al.*, 1992). The expression of genes encoding PA28α (Psme1) and PA28β

(Psme2) is induced by the same cellular signals as the βi-subunits, and the PA28 regulator is, similar to the βi-subunits, thought to be important in the generation of peptides for antigen presentation (see below).

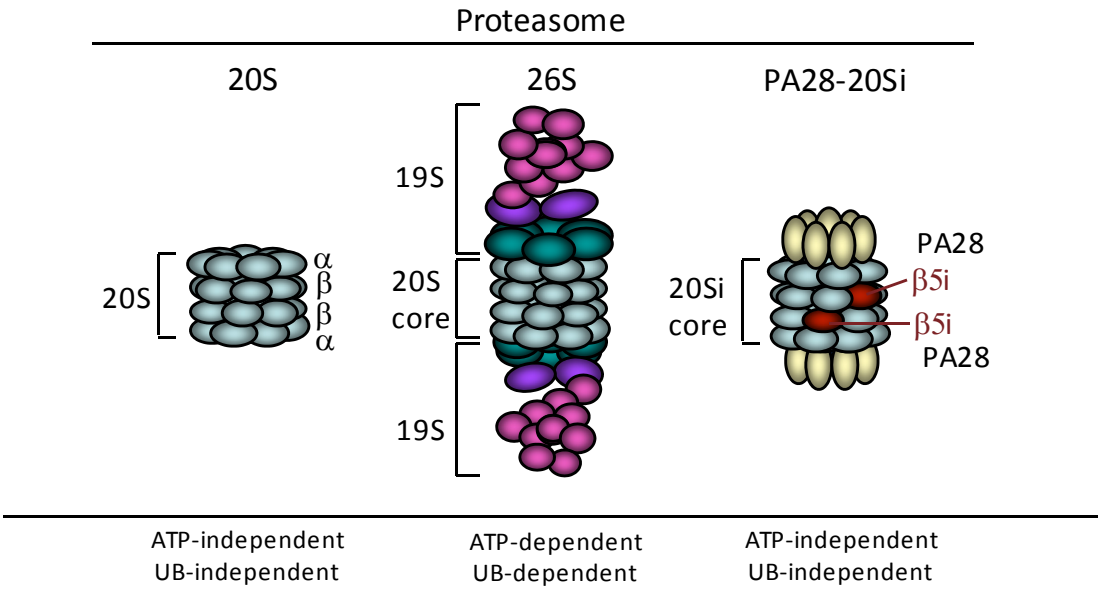

Figure 1. *The proteasome 20S/20Si and its two cytosolic regulators 19S and PA28. There are also hybrid proteasomes containing both 19S and PA28.*

The subunits PA28α and PA28β, together, make up PA28, most likely by forming a PA28$\alpha_3\beta_4$ heptameric ring with alternating α and β subunits (Rechsteiner *et al.*, 2000) (Fig. 1). The central channel of the ring is aligned with that of 20S (Knowlton *et al.*, 1997; Whitby *et al.*, 2000) and PA28 C-terminal residues are important for binding to the 20S proteasome (Ma et al., 1993) by insertion into pockets situated between the 20S α-subunits (Whitby *et al.*, 2000). Internal activation loops of PA28α and PA28β are critical for the stimulation of peptide hydrolysis (Zhang *et al.*, 1998) inducing a conformational change in the α-subunits leading to an open gate 20S conformation (Whitby *et al.*, 2000).

PA28 can bind on one side of 20S while 19S is occupying the other (Hendil *et al.*, 1998). This complex is referred to as a hybrid proteasome (Tanahashi *et al.*, 2000), although the specification PA28-20S-19S is necessary since another hybrid variant also exists (i.e. PA200-20S-19S, see below). A combination of immunoprecipitation

and Western blotting analysis of HeLa cells estimated that as much as 25% of the 20S core might be occupied in hybrid proteasomes (Tanahashi *et al.*, 2000), strongly indicating a biological relevance of this complex. The prevalence of 20S to form PA28-20S-19S hybrid was also demonstrated in a study on rabbit reticulocytes (Shibatani *et al.*, 2006). The function of the PA28-20S-19S hybrid proteasome is still unknown, but there are speculations of a role as an antigen processor that can denature target proteins (Hendil *et al.*, 1998). Accordingly, the hybrid formation is strongly induced in IFN-γ-stimulated cells (Hendil *et al.*, 1998; Tanahashi *et al.*, 2000).

Other regulators: REGγ and PA200

REGγ (PA28γ) is built up by PA28γ (Psme3, Ki), an isoform of PA28α and PA28β, forming a heptamer structurally similar to PA28. However, in contrast to PA28, its binding primarily induces the trypsin-like activity of β2 (Realini *et al.*, 1997). REGγ is localized to the nucleus and is involved in cell cycle regulation, some cell cycle regulators likely being exclusively degraded by REGγ-activated 20S (e.g. Chen *et al.*, 2007). In addition, REGγ may be important for the degradation of proteins that contain few lysines, which are required for ubiquitin-labeling and 19S dependent proteolysis (Chen *et al.*, 2007).

The PA200 (Blm10 in yeast) regulator activates proteasomal hydrolysis of peptides, but not entire proteins (Ustrell *et al.*, 2002). It is nuclear and involved in DNA repair (Ustrell *et al.*, 2002), forming hybrid PA200-20S-19S proteasomes that accumulate on chromatin in response to ionizing radiation (Blickwedehl *et al.*, 2008). In addition, PA200 has been shown to be important for spermatogenesis (Khor *et al.*, 2006) and maintenance of yeast mitochondrial function (Sadre-Bazzaz *et al.*, 2010).

The function of PA28 and 20Si

The role of PA28 and 20Si in antigen presentation

Antigen peptides are presented on the cell surface by the major histocompatibility complex I (MHC-I) (Rock and Goldberg, 1999). Cells with MHC-I carrying foreign, or in other ways aberrant, antigens are

recognized by cytotoxic T cells and stimulated to go through apoptosis (Berke, 1995). Proteasomes produce the major part of antigen peptides for MHC-I. The immunomodulatory cytokine interferon-γ (IFN-γ) (reviewed in Schroder et al., 2004) induces gene expression of βi-subunits, PA28α/β, and genes encoding MHC-I molecules (Akiyama et al., 1994; Joon Young et al., 1995). IFN-γ also enhances the expression of the proteasome maturation protein (POMP) important for efficient 20S/20Si biogenesis (Heink *et al.*, 2005).

The 20Si proteasome generates antigen peptides more optimal for MHC-I presentation, both in length and cleavage site. Although the three βi-subunits (β1i, β2i and β5i) are very similar to their β counterparts, the peptides produced are slightly different (Gaczynska *et al.*, 1993; Boes *et al.*, 1994). This is partly the result of β1i, in contrast to β1, harboring chymotryptic activity rather than caspase-like activity (Groll *et al.*, 1997). In addition, 20Si has an increased efficiency in the production of peptides compared to 20S (reviewed in Sijts and Kloetzel, 2011). Moreover, the existence of 20S βi intermediate variants mentioned above (Guillaume *et al.*, 2010), indicates that the generation of a wide spectrum of peptides may be of major importance in an appropriate response to the introduction of foreign proteins.

PA28 also contributes to the antigen peptide heterogeneity, as well as increasing the antigen peptide formation efficacy. However, PA28 affects the production and presentation of only certain epitopes (reviewed in Sijts and Kloetzel, 2011). The effects of 20Si and PA28 appear not to be cooperative or additive with regard to the cellular immune response or antigen processing, supporting the notion of PA28 and 20Si as functionally complementing each other in generating a wide range of antigenic peptides (Strehl *et al.*, 2005). Notably, PA28 activates 20S hydrolysis of the fluorogenic chymotryptic substrate suc-LLVY-AMC (Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin) to a larger extent than that of 20Si (Strehl *et al.*, 2005; Hernebring unpublished result).

PA28 could also function as a chaperone that transfers formed antigen fragments to the class I peptide-loading complex in the endoplasmic reticulum (ER) membrane (Yamano *et al.*, 2002; Rechsteiner and Hill,

2005). In such scenario, PA28 in the hybrid PA28-20S-19S proteasome may bind to the peptide-loading complex in the ER and channel the antigen directly to empty class I molecules (Rechsteiner and Hill, 2005). The role of PA28 as a chaperone is supported by the finding that PA28 assists in the Hsp90-mediated refolding of denatured luciferase (Minami *et al.*, 2000).

20Si and PA28 may have other (antigen independent) tasks

20Si and PA28 are thought to have yet unknown functions, not related to their role in antigen presentation. A main reason for this is that 20Si and PA28 are present at immune privileged sites (Noda *et al.*, 2000; Singh *et al.*, 2002; Díaz-Hernández *et al.*, 2003; Kapphahn *et al.*, 2007), i.e. tissues that do not elicit an immune response upon the introduction of an antigen (Medawar, 1948). For example, βi-subunits are found in the eye (Singh *et al.*, 2002) and in the brain (Díaz-Hernández *et al.*, 2003). PA28 is also present in the eye (Kapphahn *et al.*, 2007), while only PA28α but not PA28β was detected in the brain (Noda *et al.*, 2000), suggesting that the PA28α may act alone. PA28α can form a homoheptamer, although it is not as stable as the PA28αβ heteroheptamer (Realini *et al.*, 1997).

In summary, 20Si and PA28 are important for the generation of antigen peptides in the immune system. However, their presence at immune privileged sites suggests parallel functions, which may for example include a chaperone function of PA28.

DEGRADATION OF DAMAGED PROTEINS BY THE PROTEASOME

Irreversibly damaged proteins cannot be repaired and need to be degraded (or exported) to be removed from the cell. Irreversibly aggregated proteins are generally considered to be degraded in the lysosome through autophagy (reviewed in Tyedmers *et al.*, 2010). The Lon protease is thought to account for the major part of the degradation of unfoldable damaged proteins in the mitochondria (reviewed in Ugarte *et al.*, 2010) while the proteasome acts in the cytosol.

The proteasome in protein damage degradation

Several reports have demonstrated that oxidatively damaged proteins are selectively degraded (Levine, 1981; Rivett, 1985a; Tarcsa *et al.*, 2000; Rodgers *et al.*, 2002) and that mild oxidative stress increase intracellular proteolysis (Fagan *et al.*, 1986; Davies and Goldberg, 1987; Grune *et al.*, 1995). In an early study, the 20S proteasome was isolated as a protease (one out of four) able to preferentially degrade oxidatively modified gluthamine synthetase from *E. coli* (Rivett, 1985a).

The importance of the proteasome for the degradation of oxidized proteins was further emphasized by the observation that the H_2O_2-induced elevation in overall protein turnover of rat liver epithelial cells was abrogated by lowering proteasomal subunit levels (Grune *et al.*, 1995). Moreover, the levels of oxidatively damaged proteins increased upon treatment with the proteasome inhibitor lactacystin in human lung fibroblasts (Sitte *et al.*, 1998) and in a mouse macrophage cell line (Rodgers *et al.*, 2002), while overexpression of the β5 subunit of 20S (Chondrogianni *et al.*, 2005) or the proteasome maturation protein (POMP) (Chondrogianni and Gonos, 2007) lowered the protein carbonyl load.

Does 20S act alone?

The 20S proteasome alone is frequently considered to be responsible for the proteasomal degradation of oxidized proteins. The arguments behind this notion are that this process does not appear to require ATP (Rivett, 1985b; Fagan *et al.*, 1986; Davies and Goldberg, 1987; Pacifici *et al.*, 1989) or ubiquitin (Tarcsa *et al.*, 2000; Shringarpure *et al.*, 2003). In addition, purified 20S can degrade oxidatively modified proteins *in vitro* (Rivett, 1985b; Pacifici *et al.*, 1989; Bulteau *et al.*, 2001; Ferrington *et al.*, 2001).

As stated earlier in this chapter, when a proteasomal regulator is not bound to 20S, the 20S α-subunits form a closed gate into the inner proteolytic chamber (Wenzel T, 1995; Groll *et al.*, 2000). It has been proposed that hydrophobic patches exposed on the surface of oxidized protein substrates (Cervera and Levine, 1988; Chao *et al.*, 1997) may interact with α-subunits in a similar manner as the regulators and

thereby activate 20S proteolysis (Davies, 2001; Grune *et al.*, 2003; Liu *et al.*, 2003).

Inhibition of the proteasome upon severe oxidative stress

While mild oxidant treatments increase proteolysis (see above), acute oxidative stress has been shown to reduce protein turnover. This is thought to be because: i) the proteolytic systems themselves becoming oxidatively damaged and, ii) inhibitory clogging of the proteolytic machinery by cross-linked and aggregated protein substrates. For example, in contrast to its oxidized form (Bulteau *et al.*, 2001), severely damaged glucose-6-phosphate dehydrogenase (G6PDH) (cross-linked by the lipid peroxidation product 4-Hydroxy-2-nonenal, 4-HNE), was not only resistant to proteolysis by the 20S proteasome, but also inhibited 20S activity (Friguet *et al.*, 1994). In another study, addition of lipofuscin/ceroid pigments (fluorescent age-related aggregated and cross-linked proteins and lipids) inhibited the proteolytic activity of both purified 20S proteasomes and proteasomes in human lung fibroblast cells (Sitte *et al.*, 2000a).

THE PROTEASOME AND AGING

Proteasomal activity has been shown to decrease with aging in various human cell types and tissues, e.g. lens (Viteri *et al.*, 2004), epidermal cells (Petropoulos *et al.*, 2000), lymphocytes (Carrard *et al.*, 2003; Ponnappan *et al.*, 2007), and both cultured fibroblasts (during replicative senescence) (Sitte *et al.*, 2000b; Chondrogianni *et al.*, 2003), and fibroblasts from donors of various age (Hwang *et al.*, 2007). In addition, similar age-associated reduction in proteasomal activity has been observed in cardiac tissue (Bulteau *et al.*, 2002), the spinal cord from rats (Keller *et al.*, 2000), and brain tissue from both rat and mouse (Zeng *et al.*, 2005). Impairments in proteasomal activity have also been reported during aging of *Drosophila* (Vernace *et al.*, 2007; Tonoki *et al.*, 2009) and in some cells of C. elegans (Hamer *et al.*, 2010).

In certain examples listed above, the decrease in proteasomal activity with aging is correlated to a reduction in specific subunits of 20S/20Si (Bulteau et al., 2002; Chondrogianni et al., 2003; Ponnappan et al.,

2007) and 19S (Chondrogianni *et al.*, 2003; Tonoki *et al.*, 2009). Accordingly, overexpression of the 20S β5 subunit extended the replicative lifespan of cultured human fibroblasts (Chondrogianni *et al.*, 2005), and overexpression of 19S Rpn11 prolonged *Drosophila* lifespan (Tonoki *et al.*, 2009). Yet, other reports have shown 20Si subunits to accumulate during aging, namely in rat muscle (Ferrington et al., 2005) and human hippocampus (Mishto et al., 2006).

As already mentioned, the proteasome itself is a target of damage which can reduce its proteolytic activity. This was observed to occur in rat spinal cord in which 20S β-subunits were increasingly HNE-modified upon aging (Keller *et al.*, 2000). Also subunits of 20S/20Si proteasome in human lymphocytes displayed enhanced glycation and/or HNE conjugation with age which correlated with reduced proteasomal activity (Carrard *et al.*, 2003). Moreover, as stated previously, heavily oxidized and cross-linked proteins inhibit the proteasome and this is thought to be a major factor in aging and age-related pathologies (Grune *et al.*, 2004; Friguet, 2006; Dunlop *et al.*, 2009).

Thus, the age-related decrease in proteasomal activity could be due to i) direct inhibition of subunits by damaging modifications, ii) increased amounts of inhibitory aggregated substrates, and iii) lowered or altered subunit levels.

5. EMBRYONIC STEM CELLS

This chapter gives an introduction to the main model system, mouse embryonic stem cells.

During the preimplantation development of the mouse embryo, the fertilized egg undergoes a series of cleavage divisions, without changing its overall size. At the 8-cell stage, a process called compaction occurs in which intercellular adhesion increases (around 2.5 days after fertilization, E2.5), leading to the formation of the morula. Three days after fertilization of the egg, the outer cells of the morula are committed to the trophectoderm (TE) lineage. The TE cells allow water to enter the embryo and a fluid filled cavity, the blastocoel, forms. The embryo is now, at E3.5, considered a blastocyst and this stage will last until E4.5 when the embryo is ready for implantation into the uterine wall.

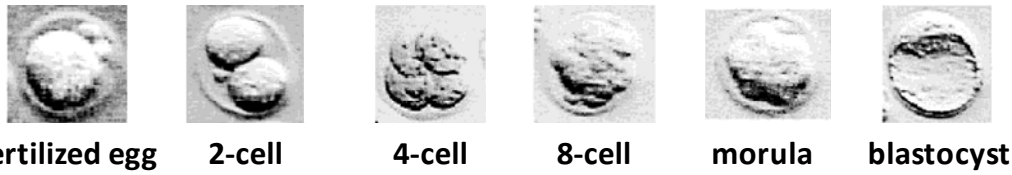

Figure 2. *The preimplantation period from the fertilized egg to the blastocyst lasts around four days in mice and involves cleavage divisions, compaction of the 8-cell embryo, and the formation of a fluid filled blastocoel inside the embryo defining it as a blastocyst. Reprinted and modified (from Hamatani et al., 2004) with permission from Elsevier.*

The blastocyst contains two cell types, TE and the inner cell mass (ICM) resulting from the first fate decision of the mouse embryo, i.e. the first step of differentiation that takes place *in vivo*. The TE cells will form part of the placenta, while the ICM will segregate into two new lineages; the primitive endoderm, and the epiblast. The epiblast will give rise to the embryo itself and the primitive endoderm will contribute to the yolk sac. (The period of preimplantation is thoroughly reviewed in Zernicka-Goetz *et al.*, 2009; and Cockburn and Rossant, 2010).

ES cells are derived from the ICM of the blastocyst and mouse ES cells were first isolated in 1981 (Evans and Kaufman, 1981; Martin, 1981) followed by human ES cells in 1998 (Thomson *et al.*, 1998). Differentiated mammalian cells exhibit a finite proliferative lifespan; referred to as the Hayflick limit (Hayflick, 1965), which makes them stop dividing after a certain number of cell divisions when cultured *in vitro*. This feature is not shared by the ES cells, which, along with adult stem cells and cancer cells, have the potential to proliferate indefinitely. In addition, ES cells exhibit a unique ability to differentiate into all cell types of the body (pluripotency). Injection of ES cells into a mouse blastocyst which is then transplanted into a uterus of a surrogate mother, will lead to chimeric offspring. However, if directly implanted into a uterus they are unable to generate an embryo and are therefore not categorized as totipotent (as are the fertilized egg and the morula) (De Miguel *et al.*, 2010).

A number of transcription factors are important in the lineage specification between ICM and TE. Specifically, expression of Oct4, Sox2 and Nanog is maintained in the ICM cells and lowered in the cells destined to become TE (along with an increase in another transcription factor, caudal type homeobox 2, Cdx2) (Cockburn and Rossant, 2010). Oct4 and Sox2 are two of the four transcription factors (c-Myc and Klf4 are the other two) whose combined overexpression can convert somatic fibroblasts to pluripotent cells (induced pluripotent stem cells, iPS cells) (Takahashi and Yamanaka, 2006), thus confirming their significance for the pluripotent state (reviewed in Hanna *et al.*, 2010).

In order to remain undifferentiated, ES cells require non-confluent conditions and the presence of the cytokine Leukemia Inhibitory Factor (LIF) (reviewed in Burdon *et al.*, 2002; and De Miguel *et al.*, 2010). Differentiation of mouse ES-cells can therefore be induced by allowing the cell culture plates to become confluent and/or withdrawing LIF from the media. The effect of LIF removal is reversible up until 36 h after withdrawal (Boeuf *et al.*, 2001).

6. RESULTS

This chapter reviews major findings included in this thesis.

EARLY EMBRYONIC DEVELOPMENT IN MICE INCLUDES A CLEARANCE OF DAMAGED PROTEINS

Proteins damaged by carbonylation or AGEs diminish during ES cell differentiation *in vitro*

To understand how the protein damage load is set to zero at the start of each new generation, we measured the levels of protein damage in the course of the early stages of ES cell differentiation. ES cells cultured *in vitro* were differentiated using various protocols, of which the first approach was spontaneous differentiation by removal of the cytokine LIF in combination with contact-inhibition by confluent conditions. Immunohistochemical (IHC) detection of protein carbonyls and AGEs demonstrated that cells that had embarked on differentiation displayed significantly lower levels of carbonyls and AGEs than undifferentiated ES cells (Paper I, Fig 2 and Fig 4B-D). Elimination of carbonyl and AGE modified proteins upon differentiation was confirmed by analysis of protein extracts (Paper I, Fig 3A and Fig 4A). In the second approach, ES cells were allowed to go through spontaneous differentiation in adherent cultures for 5 and 8 days in the presence of LIF. The cells generated displayed similar remarkable reduction in carbonyl levels (Paper I, Fig. 3B) excluding the possibility that LIF itself may act as an oxidant. In addition, differentiation of ES cells into embryonic bodies (EBs) and retinoic acid-induced differentiation into neuronal and/or primitive endoderm cells resulted in the same characteristic clearance of carbonylated proteins both in the absence and presence of LIF (Paper I, Fig. 3B).

The elimination of protein damage triggered by differentiation could, in principle, be a result of dilution of damage by increased growth rate (shorter doubling time). However, ES cells in culture exhibit a vast duplication potential, both by their unlimited proliferative capacity and short doubling time (Udy et al., 1997) and the doubling time increased rather than decreased upon differentiation (Paper III, Fig. 1B).

Therefore, the observed damage drop cannot be attributed to dilution effects.

Protein carbonylation in biological samples is mainly formed by a metal catalyzed oxidation that involves a reaction with hydrogen peroxide (Requena et al., 2001) (see chapter 3). In order to investigate whether a reduced rate of damage formation could be the basis for the elimination of protein carbonyls, we determined if differentiation resulted in a diminished cellular concentration of peroxides. Un-differentiated ES cells and cells that had been differentiating for three days were stained with Dihydrorhodamine (DHR), a peroxide indicator, and analyzed by flow cytometry. This demonstrated that undifferen-tiated ES cells displayed slightly higher peroxide levels than their differentiated counterparts (Paper III, Fig 1D). However, the difference was not statistically significant and the modest reduction in peroxides is not likely to cause the abrupt reduction in protein carbonyls observed.

The differentiating embryo rids itself of damaged proteins *in vivo*

At the stage when the embryo is ready for implantation into the uterine wall, it is referred to as a blastocyst, which occurs around four days after fertilization in mice (see chapter 5). The blastocyst contains two cell types, TE and ICM resulting from the first fate decision of the mouse embryo, i.e. the first step of differentiation that takes place *in vivo*.

If differentiation of ES cells is accompanied by elimination of damaged proteins during normal development *in vivo* we would expect to observe differential carbonyl levels in cells within a blastocyst. Specifically, a higher load of damage would be expected in ICM, from where the pluripotent ES cells originate, than in the outer and differentiated cell layer, TE. This was indeed the case (Paper I, Fig. 5). We confirmed that protein carbonyls were predominantly associated with pluripotent cells in the blastocyst by co-staining with SSEA-1 (Stage-Specific Embryonic Antigen 1) (Paper I, Fig. 5A-D). Similar to carbonyl damage, AGE modification was primarily observed in the inner cell mass of blastocysts (Paper I, Fig. 5E-G). The data demonstrate that eradication of damaged proteins is an integral part of early embryonic development.

Carbonyl and/or AGE modified proteins are specifically eliminated

Differentiation is accompanied by alterations in the cellular proteome. Therefore, it is possible that the reduction in protein carbonyls could be due to a decrease in the total concentration of specific proteins rather than specifically the damaged form of the proteins. A proteomics approach based on two-dimensional (2-D) immunodetection and mass spectrometry (MS) of proteins modified by carbonyls and AGEs established that a discreet number of proteins are targeted by these modifications in undifferentiated ES cells (Paper I, Fig. 1C). SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) followed by Western analysis demonstrated that the overall concentration of the affected proteins analyzed (HSP90, HSP70, and β-actin) did not change appreciably during differentiation (Paper I, Fig. 3C). In other words, the carbonylation load of the affected proteins is reduced upon differentiation not the levels of the proteins *per se*.

Carbonyl and AGE modifications in ES cells are similar to that of cells in adult tissues

Proteins damaged by carbonylation, identified as described above, included the chaperones HSP90, GR75, and HSC70, α and β chains of tubulin, and β-actin (Paper I, Fig. 1C). Interestingly, several of these proteins have been shown to be highly carbonylated in aged organisms ranging from bacteria to plants and mammals (Ikeda *et al.*, 1996; Castegna *et al.*, 2002; Desnues *et al.*, 2003; Johansson *et al.*, 2004; Reverter-Branchat *et al.*, 2004). Undifferentiated ES cells displayed AGE modification of one major protein, Hsc70 (Paper I, Fig. 4A). 2-D and MS analysis of senescent human fibroblasts and endothelial cells also identified Hsc70 as the main target of AGE modifications (Paper II). Moreover, carbonyl levels in undifferentiated ES cells correspond to those of differentiated tissues of adult mice; as determined by the protein carbonyl load of protein extracts from 6-months-old brain and liver (Paper I, data not shown). Thus, the protein damage status regarding carbonyl and AGE modifications in adult tissues are akin to that of undifferentiated ES cells.

THE PROTEASOME ACTIVATOR, PA28, IS ESSENTIAL FOR THE ELIMINATION OF DAMAGED PROTEINS DURING MOUSE ES CELL DIFFERENTIATION

The Clearance of Protein Damage in ES Cells Requires Active Proteasomes

Both carbonylation and AGEs are irreversible protein modifications which need to be degraded or exported to be removed from the cell (e.g. Friguet, 2006) and the proteasome accounts for the major part of the degradation of unfoldable damaged proteins in the cytosol (Dunlop et al., 2009; Jung et al., 2009) (see chapter 4).

To test if proteasome activity affects protein carbonyl levels in ES cells, the cells were treated with the proteasome-specific inhibitor epoxomicin at concentrations causing a modest inhibition of activity and the ES cells were concomitantly induced to differentiate. The concentration of epoxomicin used (20nM) led to a 67% inhibition of the proteasome (Paper III, Fig. 1E), and increased the level of ubiquitinated proteins (Paper III, Fig. 1F) and carbonylated proteins (Paper III, Fig. 1G) without inducing apoptosis (Paper III, data not shown). This data suggest that proteasomal activity during early ES cell differentiation is required to keep carbonyl levels at bay and indicate that an increase in protein damage removal may be the cause of the differentiation-induced damage elimination.

Differentiation of ES Cells Triggers Production and Assembly of the PA28-20S/20Si Proteasomal Complex

To elucidate the dependence on proteasomal activity for the differentiation-induced clearance of protein damage we started by analyzing the absolute levels of proteasomal subunits. Since the proteins targeted by carbonylation and glycation are mainly cytosolic (Paper I, Fig. 1C) we focused on the 20S/20Si core and the two known cytosolic regulators of proteasome activity, 19S and PA28 (see chapter 4). SDS PAGE followed by Western blotting demonstrated that subunits of the 20S core (β5 and a mixture of α-subunits) and 19S (Rpn7) are present to the same extent in undifferentiated and differentiated cells (Paper III, Fig. 2B). However, the levels of the PA28

subunit PA28α and the 20Si immunoproteasomal subunit β5i were markedly induced upon early ES cell differentiation.

We next determined whether the increased levels of the specific proteasomal subunits leads to the formation of mature proteasomal complexes in differentiating ES cells. The conditions of cell lysis dictates which proteasomal regulator will interact with the 20S/20Si core in the lysate (Rivett et al., 2002). Using protocols optimizing 20S/20Si interaction with either 19S or PA28 followed by separating complexes on native gels demonstrated that the PA28-20S/20Si complex is barely detectable in undifferentiated cells, but increases dramatically upon early ES cell differentiation (Paper III, Fig. 2C). In contrast, the levels of the 26S proteasome displayed no major change with differentiation. Probing for 19S subunit in the extracts favoring PA28-20S/20Si complex confirmed that it does not bind 20S/20Si core during these extraction conditions (and 26S is accordingly absent). While these data do not necessarily reflect the exact situation inside the cell regarding proteasomal complex interactions or activities, they are consistent with an accumulation of PA28-20S/20Si proteasomes in differentiating ES cells.

To establish whether the differentiation-associated PA28-20S/20Si proteasomes are functionally active, we used the same conditions as above in proteasomal activity assays using fluorogenic peptides as substrate. Conditions that favor the formation and maintenance of the PA28-20S/20Si complex generated a specific peptide hydrolyzing activity (pmol/μg protein/min) that is induced 5-fold upon early differentiation of ES cells (Paper III, Fig. 2D). In contrast, the 26S activity was not induced upon differentiation, and the specific activity of the PA28-20S/20Si complex surpassed that of 26S in the differentiated samples.

PA28 is required for the reduction in protein damage during early ES differentiation

The observation that the clearance of protein damage upon differentiation of ES cells is proteasome-dependent and coincides with the formation of functional PA28 complexes, suggests a role for PA28 in the degradation of oxidatively damaged proteins. Since the results

are purely correlative, we next aimed to elucidate whether there is a causative relation between PA28 formation and the elimination of protein damage. In order to do so, RNAi was used to target and lower PA28α levels during ES cell differentiation. Targeting PA28α (Paper III, Fig. 3, lane 3) abolished the reduction in protein carbonyls upon differentiation of ES cells (Paper III, Fig. 3, lane 1 and lane 3), while the RNAi control LacZ (Paper III, Fig. 3, lane 4) did not. Hence, the proteasomal regulator PA28 is essential for the differentiation-associated decline in protein damage.

PROTEIN DAMAGE CONTROL DURING REPRODUCTION OF AN ARTHROPOD: *Drosophila melanogaster*

We were interested in investigating the asymmetry between the germ line and the soma regarding proteostatic features known to change upon aging in order to shed light on the bearing of theories of preservation and trade-offs. The fruit fly *Drosophila melanogaster* has been widely used as a model organism historically and vast amounts of data about its biology have been collected throughout the years. Practical advantages in the usage of this animal in research includes that it is small, has a short life cycle and simple diet, and more importantly for aging research: it has a relatively short lifespan. These advantages made us extend our studies into this model system.

Brief introduction to *Drosophila melanogaster*

Drosophila melanogaster has four distinct life stages: egg, larva, pupa and adult. The life cycle is completed within 10 days and the mean life span of the adult fly is around 5 weeks. As for all sexually reproducing animals, it begins with oogenesis and spermatogenesis; of which oogenesis is of special interest with regard to protein damage because it harbors the cytosol which will be passed on to the next generation.

During oogenesis, ocytes are produced in tube like structures called ovarioles which constitute the fly ovary. Egg chambers bud off the germarium at the anterior end, and mature as they descend through the ovariole. When the mature oocyte/egg has reached the end of the ovariole it is released into the oviduct where it can be fertilized by

sperm stored from a previous mating (oogenesis is thoroughly reviewed in Lodish, 2000; Bastock and St Johnston, 2008; Roth and Lynch, 2009).

Protein carbonyls increase with aging in both soma and germ line in *Drosophila*

The levels of carbonylated proteins is known to rise during aging in *Drosophila* (Sohal RS, 1993). We confirmed this and observed that this increase is predominantly a result of damage accumulation in abdomen and thorace, while protein carbonyl levels decreased in the head (Paper IV, Fig. 1A). The abdomen was the body part most affected by aging with regard to protein carbonyls, which is interesting because it accommodates the fly germ line.

Since the abdomen not only contains the germ line, but also somatic tissues, we isolated the eggs from the abdominal tissues by dissection. This enabled a separate analysis of protein carbonyls in eggs and egg-depleted flies, which demonstrated that eggs from both young (5 days) and old flies (30 days) have lower carbonyls than the fly they originate from (Paper IV, Fig 2A). However, protein carbonylation increases upon aging to the same extent in both the egg-depleted flies and the eggs (Paper IV, Fig 2A). Thus, data on protein carbonyls in soma versus dissected eggs in *Drosophila* suggests that the germ line is not preserved with regard to protein damage during aging.

The germ line displays an elevated 26S proteasomal activity that is preserved during *Drosophila* aging

In order to find out more on the lowered levels of protein carbonyls in the dissected eggs, we turned to the proteasome. For practical reasons, we used newly fertilized eggs (0-1h after fertilization), permeabilized them and treated them with two different proteasome inhibitors. This treatment increased their carbonyl load suggesting a role of this proteolytic complex in the lowered levels of protein damage in eggs (Paper IV, Fig 2E).

Drosophila does not have a PA28α/β regulator, so 19S is the only known cytosolic regulator of the 20S proteasome, forming the 26S

proteasome. In line with the results from proteasomal inhibition of newly fertilized eggs, 26S proteasome activity was higher in the eggs than in the soma (Paper IV, Fig 2C). Moreover, as shown previously by others (Vernace *et al.*, 2007; Tonoki *et al.*, 2009), 26S activity decreased further in the soma with aging of the fly (Paper IV, Fig 2C). However, whereas the levels of protein carbonyls increased in the eggs of old flies, 26S activity of eggs from young and old was equivalent to each other (Paper IV, Fig 2C).

Trade-off effects of reproduction is age dependent

To investigate the potential trade-off between reproduction and aging, virgin and mated flies were compared with regard to lifespan, protein carbonyls and 26S activity. Virgin flies displayed a prolonged life span compared to their mated counterparts (Paper IV, data not shown), as also shown by others (e.g. Smith, 1958). In addition, and also in line with theories concerning negative effects of mating, both soma and eggs of mated flies exhibit a higher carbonyl load than that of virgin flies (Paper IV, Fig 3B).

We also analyzed the immediate effects of mating by letting young (0 days) and old virgin flies (30 days) mate (samples taken 5 days after mating). Interestingly, in young flies, 26S activity decreased upon mating while carbonyl levels increased, whereas there were no effects of mating in old flies (Paper IV, Fig 3C and 3D). This age dependence of the mating effect could be linked to the fact that egg production goes down during aging (Rogina *et al.*, 2007). Specifically, mating of young flies results in a boost in egg laying which is not observed in old flies (Rogina *et al.*, 2007).

Summary of results from *Drosophila*

In summary, aging of *Drosophila* is accompanied by increased levels of protein carbonyls and decreased 26S proteasomal activity. The 26S activity appears to be preserved in the germ line upon aging while the levels of protein carbonyls go up, so that the asymmetry between soma and germ line increases with aging regarding 26S activity but stays the same with respect to carbonylated proteins. Mating in *Drosophila* is accompanied by age-related traits such as reduced 26S activity and

increased protein damage, at least in young flies, and this mating response is abrogated in old flies.

7. DISCUSSION

In this chapter, the results included in this thesis are discussed and put into a broader context.

ELIMINATION OF DAMAGED PROTEINS DURING EARLY EMBRYONIC DEVELOPMENT IN MICE

Results indicate that the mouse germ line is not preserved but rather harbors an ability to rid itself from damaged proteins

Data presented in this thesis suggest, based on experiments with ES cells, that there is a clearance of protein damage during early embryonic development in mice, which requires the proteasome and its activator PA28. Before the elimination of damaged proteins, the carbonyl load in undifferentiated ES cells is comparable to that of adult tissues; both in relative levels and damage targets (Paper I). The unexpectedly high levels of protein damage are evidently not a problem to these proliferative and pluripotent cells. Moreover, the analogous damage load of ES cells and adult somatic tissues implies that the germ line has not been kept free from protein damage up until this point, and, if the 'aging clock' needs to be reset for a new generation regarding protein damage, there must be some other mechanism that accomplishes this. In this context, the boost in PA28 dependent proteasomal activity, which reduces protein damage during these conditions, is a major candidate for this protein damage control. Figure 3 schematically describes two models which can explain how youth at the start of each new generation concerning protein damage can be achieved: through preservation or rejuvenation of the germ line.

The trigger of 'rejuvenation', with regard to protein damage, appears to be accomplished at the very earliest stages of differentiation, as observed both in cultured ES cells and the developing embryo. In cultured ES cells, the reduction is seen already after a few days of differentiation-inducing treatments. In the blastocyst, of the two cell types present (ICM and TE; see chapter 5), proteins carrying carbonyl and AGE modifications are localized to the undifferentiated cells in the ICM, while the TE cells are virtually damage free. These cells are the result of the very first step of differentiation that takes place *in vivo*,

pointing to a link between protein damage clearance and the initiation of differentiation from the pluripotent state. Following this line of reasoning, multipotent progenitor cells and adult stem cells are expected to exhibit minimal amounts of protein damage; the elimination enabling them to start off with low levels in a new individual.

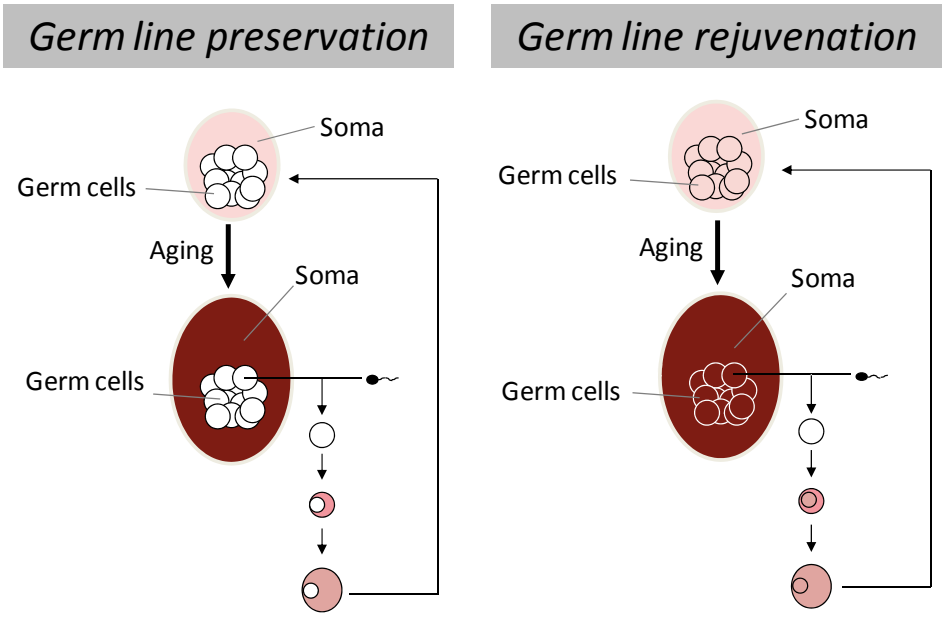

Figure 3. *Models of germ line preservation and rejuvenation. According to the Germ line preservation model (to the left), the cells that will become part of the offspring are kept isolated and maintained in a way so that they are protected from the damage (red) the cells of the parent are exposed to. Therefore, the cells of the parent age while the germ cells do not. One can also imagine a maintained potential to get rid of the damage that build up over time, as in the model of Germ line rejuvenation (to the right). In this model, damage might accumulate as it does in the cells of the soma. However, at some point during maturation of the germ cells or early embryogenesis the potential of damage clearing is set into action and the damage load is reset so that it corresponds to that of a young individual.*

One may argue that maintaining a capacity to rid oneself from something could be more resource efficient than using efforts to never acquire it in the first place. This may be because in the latter case, resources need to be continuously invested over a long time, while the

former method settles with once per generation. However, as we shall see, preservation mechanisms do exist.

Other mechanisms by which protein damage may be set to zero in newborn offspring

Although the data presented in this work solely support the model of germ line rejuvenation, it needs to be stressed that these two models are not exclusive and there are mechanisms which do preserve the germ line, as hypothesized by Kirkwood and Holliday (1979). For example, spermatogenic cells are kept protected from circulating toxic substances by a blood-testis barrier and from heat-induced damage by the lowered temperature of the testis due to its extraperitoneal location (reviewed in Walter et al., 2003). Certain DNA repair pathways have been shown to be more active in germ line than adult tissues, at least in spermatogenic germ cells from mice (Intano *et al.*, 2001), and the mutation frequency is lower in the mice germ line than in somatic cells (Walter et al., 1998; Xu et al., 2008; McCarrey, 2009).

Another mechanism by which low damage may be obtained at the start of each new generation is through a selection process that rids the organism from germ cells exhibiting high levels of damage (Holliday, 1975; Medvedev, 1981). If such a process is preceded by cell divisions that asymmetrically segregate macromolecular damage, it is another means of germ line rejuvenation. If not, this mechanism cannot be categorized as either preserving or rejuvenating. The idea of a selection mechanism is quite straight-forward regarding traits that can be classified as either 'good' or 'bad'; such as the presence or absence of certain DNA damages, but not in the situation of differing protein damage concentrations. However, one can imagine a threshold level of damage or the presence of protein aggregates as a potential trait sentencing a germ cell to be discarded. During development and maturation of the germ line there are quality-control mechanisms involving apoptosis (Johnson *et al.*, 2004; Suh *et al.*, 2006; Beyer *et al.*, 2011) which can be considered as such a process of selection. This selective mechanism is generally thought to mainly concern damage to or aberrancies in DNA, but may include protein damage as well.

How PA28 may exert its effects on protein damage

Early differentiation of ES cells triggers, as shown in this thesis, production and assembly of the proteasomal regulator PA28. Expression of the genes encoding PA28α during mouse embryogenesis from the unfertilized egg to the blastocyst stage (along with e.g. PA28β and β5i) was also observed in an analysis of global expression profiles (Hamatani *et al.*, 2004). Thus expression of PA28 is not specific for ES cells cultured *in vitro*.

When we lowered the levels of PA28α by RNAi the dramatic drop in carbonylated proteins associated with differentiation was abolished. Thus, the PA28 dependent boost in proteasomal activity during early ES cell differentiation is most likely the cause of the succeeding protein carbonyl clearance. In line with this reasoning, PA28 has recently been shown to reduce the levels of protein oxidation in rat cardiomyocytes (Li *et al.*, 2011) and to protect against H_2O_2-induced oxidative stress in MEFs (murine embryonic fibroblasts) (Pickering *et al.*, 2010).

The PA28 regulator may exert its functions together with 19S and 20S in the hybrid proteasome. However, the degradation of oxidatively damaged proteins has been shown to be independent of ATP (Rivett, 1985b; Fagan *et al.*, 1986; Davies and Goldberg, 1987; Pacifici *et al.*, 1989) and ubiquitin (Tarcsa *et al.*, 2000; Shringarpure *et al.*, 2003). Moreover, the proteasomal regulator REGγ is a heptamer structurally similar to PA28 (built up by PA28γ, an isoform of PA28α and PA28β; see chapter 4). Since REGγ does not form hybrids with 19S and 20S and is able to degrade whole proteins in an ubiquitin-independent manner (e.g. Chen *et al.*, 2007), it seems plausible that PA28 would also be able to do so. Although there have been several reports suggesting that PA28 was unable to degrade protein substrates *in vitro* (Yukawa *et al.*, 1991; Dubiel *et al.*, 1992; Ma *et al.*, 1992), it is possible that there is some hitherto unidentified cofactor required, or, alternatively, that the right protein substrate has not yet been found. The identity of potential cofactors and/or substrates, and whether the recognition involves carbonylation of the protein targets, remains to be elucidated.

It has recently been suggested that the indicated role of PA28 in antigen presentation may be side effects from "a not yet resolved true physiological function of PA28" (Sijts and Kloetzel, 2011). The arguments behind this statement include the observation that PA28 is important for the processing of only a few epitopes, as well as the presence of PA28 (and 20Si) at immune privileged sites (see chapter 4). One could speculate that PA28 has a role in unfolding damaged proteins prior to degradation and thus the chaperone functions of PA28 (Minami *et al.*, 2000) would be of major importance.

In an immune response, the cytokine IFN-γ is not only responsible for the induction of PA28; a part of its antimicrobial strategy is also to activate a respiratory burst (also called oxidative burst) in macrophages, neutrophils and phagocytes (reviewed in Schroder et al., 2004). The protective role of PA28 upon oxidative stress described above may be important to limit the detrimental effects in the surrounding tissues during an immune response. In this context, it is relevant to note that an oxidative burst is involved in the activation of sea urchin eggs at fertilization and this may also be part of the activation of the embryo in other species (Ris-Stalpers, 2006).

The levels of PA28 regulators IFN-γ, TNFα and NF-κB are all increased upon aging in mammals (reviewed in Kovacs *et al.*, 2009; Salminen and Kaarniranta, 2010). NF-κB can be activated by various events associated with aging: e.g. DNA damage, oxidative stress (redox) sensitive upstream kinases and AGEs binding to RAGEs. An over-activated NF-κB signaling leads to age-related maladies such as cellular senescence, inflammation, atherosclerosis and neuronal degeneration. On the other hand, a functional NF-κB signaling is important for development, innate immunity, and cellular survival processes (Salminen and Kaarniranta, 2010; and references therein). The opposing effects of NF-κB on 'fitness' (or performance) during early and late life makes it a potential target for genetic alterations causing antagonist pleiotropy; the theory of Williams (1957) (see chapter 2). It is not known whether PA28 is formed upon over-activation of NF-κB signaling, or by the increased presence of its other two regulators IFN-γ and TNFα. Observations concerning the coregulated 20Si have given differing results depending on the tissue; increasing levels upon aging in rat muscle (Ferrington et al., 2005) and human hippocampus

(Mishto et al., 2006), but decreasing levels in human lymphocytes (Ponnappan *et al.*, 2007) (as stated in chapter 4).

NON-MAMMALIAN MODELS OF PROTEIN DAMAGE CONTROL: *Drosophila melanogaster*, *Caenorhabditis elegans* and yeast

Germ line management of protein carbonyls in *Drosophila melanogaster*

As demonstrated in this thesis, protein carbonyl levels are higher in the soma than in the germ line (eggs) of *Drosophila* (Paper IV, Fig. 1A). Subsequent measurements have shown that there are no further major reductions in protein carbonyls later on during egg development (unpublished observation, personal communication with Åsa Fredriksson). These results indicate that the main decrease in protein damage at the start of a new generation, in contrast to the situation in mice, takes place prior to fertilization in *Drosophila*. Therefore, protein damage clearance during germ line maturation and/or mechanisms of preservation may be of higher importance in the fly than in mice.

The proteasome might be a central player in preserving the germ line from obtaining high damage load, as indicated by its ability to degrade protein carbonyls in fertilized eggs (Paper IV, Fig. 2E). In addition, apart from accelerating aging, mating is accompanied by reduced 26S proteasomal activity and increased protein damage in young flies (Paper IV, Fig 3C and 3D) further linking proteasomal activity to the levels of carbonylated proteins. In contrast, this effect of mating was absent in aged flies (as already discussed in chapter 6), potentially due to the deficiency in egg production observed in old flies. These results indicate that the trade-off between maintenance and reproduction is linked to egg production in *Drosophila*, and this is in line with the previously mentioned longevity effects of isolating females flies from males or removing their ovaries (Smith, 1958) (see chapter 2 and will be further discussed later on).

The observation of increasing protein carbonyl levels in the germ line during *Drosophila* aging indicates that the preservation of the germ line is not sufficient to maintain a low steady state level of damage for extended periods of time. The levels of protein carbonyls increase in

spite of a sustained 26S proteasomal activity, which is just as high in germ line of old flies as in young. This indicates that the proteasome is not the resource that is failing upon aging in the germ line of *Drosophila*. However, it needs to be emphasized that only proteasomal 'capacity' was measured for the 26S and not 20S, and the latter has been implicated in the degradation of oxidized proteins (see chapter 4). Elevated damage levels may instead be due to, for example, an increased rate of protein damage generation by enhanced ROS levels during aging (Cochemé *et al.*, 2011).

Elimination of protein carbonyls in *C. elegans* during oogenesis

A recent study demonstrated that carbonylated proteins are eliminated also during early development of *C. elegans* (Goudeau and Aguilaniu, 2010). During oocyte maturation, shortly after cellularization and before fertilization of the embryo in this hermaphrodite, there is an abrupt reduction in protein carbonyls. While this elimination is, similar to the damage clearance in ES cells, dependent on the 20S proteasome, it also requires the proteasomal regulator 19S. This indicates that the 19S regulator is responsible for the proteasome dependent protein damage elimination in animals which do not possess a PA28 proteasomal regulator, at least in *C. elegans*.

Asymmetric inheritance of aggregated carbonylated proteins in yeast

The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* display another method than damage clearance to generate an offspring with a reduced damage load. Being a unicellular organism, cell division is the equivalent of reproduction and the means used include an asymmetric division in which damaged protein is retained within the mother cell (Aguilaniu *et al.*, 2003; Erjavec *et al.*, 2008; Liu *et al.*, 2010). In the budding yeast *S. cerevisiae*, the mechanism behind this segregation involves transport of protein aggregates along actin cables from the emerging daughter cell into the mother (Liu *et al.*, 2010).

TRADE-OFF THEORIES AND THE RESETTING OF PROTEIN DAMAGE IN OFFSPRING

How can lowering the reproductive capacity shift the trade-off towards survival and longevity?

As stated in chapter 2, some theories highlight that there is a compromise between the allocation of resources towards either reproduction or maintenance (in accordance with the Disposable soma theory; Kirkwood and Holliday, 1979). This is illustrated by the fact that reducing fertility or breeding tends to extend life span, and that interventions which cause longevity often, but not always, also restrict reproduction (Flatt and Schmidt, 2009).

At the cellular and organismal level, there are an immense number of processes constantly requiring resources, often on-demand. These processes compete with each other and have done so during the evolutionary history of any organism. This has affected the biological traits of that organism, such as how fast it grows and when it becomes sexually mature etc. During these complex events, there is, as already stated, generally a trade-off between processes involved in reproduction and processes contributing to the maintenance of the existing organism.

A prolonged life span is often accompanied by lower levels of macromolecular oxidative damage (see chapter 2). Because ROS may function as a signal in proliferative pathways, there may be a link between the intensity of growth and the accumulation of ROS mediated damage. Thus, if mounting levels of oxidative damage is a cause of aging, the combination of proliferative and damaging effects of ROS may be at the very core of trade-off theories. In accordance with this reasoning, as already mentioned, ROS levels generally correlate negatively to longevity (Barja, 2004; Lambert *et al.*, 2007). However, there are reports suggesting some exceptions exist during modulation of aging (Miwa *et al.*, 2004; Sanz A, 2010; Van Raamsdonk and Hekimi, 2010). These latter examples could illustrate the presence of other aging causes not related to ROS levels or the complexity of ROS as pleiotropic effector molecules.

The results presented in this thesis on how *Drosophila* responds to mating include the observation that the shortened lifespan of mated flies is accompanied by increased levels of protein carbonyls in both soma and eggs (Paper IV, Fig. 3B). This could be explained by an increased ROS production during proliferative activities such as egg production.

Because a relatively high 26S activity may be considered as a maintenance investment, the observed reduction in 26S activity in the immediate mating response in young flies may be the result of two parallel course of events: i) upon mating, resources are allocated towards the burst in egg production from the maintenance factor 26S, and/or ii) egg production involves ROS generation of which 26S is a susceptible target (Reinheckel *et al.*, 1998). Both of these routes could in theory result in enhanced carbonyl levels. In accordance, mating of aged flies does not lead to a boost in egg production, characteristic of mating in young flies, which may be the reason why mating did not affect 26S activity and protein carbonyls in old flies.

CONCLUSIONS AND PERSPECTIVES

This chapter summarizes the thesis' major conclusions and future considerations.

CONCLUSIONS

Results presented in this thesis demonstrate that undifferentiated ES cells harbor a protein damage load equivalent to that of adult tissues, both in absolute carbonyl levels and in the identity of proteins targeted by the damaging AGE and carbonyl modifications. Moreover, early embryonic differentiation in mice is accompanied by a clearance of such protein damage, both *in vitro* and *in vivo*. This elimination of damaged proteins may be an integral part of embryonic development as a means to ensure an offspring carrying a low damage load. Using mouse ES cells, we show herein that early differentiation triggers production and assembly of the PA28-20S/20Si proteasomal complex, which is responsible for the concomitant riddance of protein damage. Transcripts encoding these proteins are present in the developing mouse embryo (Hamatani et al., 2004), indicating that the PA28-20S/20Si proteasomal complex is produced upon early differentiation also *in vivo*. These results point to the PA28-20S/20Si proteasomal complex as a proteostatic 'rejuvenation' factor and the importance of this complex for protein damage control during embryogenesis *in vivo* and aging is awaiting future clarification.

FUTURE CONSIDERATIONS

The potential of induced pluripotency

As described in chapter 5, forced simultaneous expression of four transcription factors (Oct4, Sox2, Klf4, and c-Myc) can transform differentiated somatic fibroblasts into pluripotent iPS cells. Besides giving immense potential to regenerative medicine, this makeover raises questions of high interest from an aging perspective: Are these cells as 'young' as the ICM cells are? If so, how were these cells rejuvenated from an aged state previously thought irreversible?

One 'rejuvenated' trait that iPS cells have obtained is elongated telomeres. Telomeres are protective caps at the ends of chromosomes, which cannot be maintained in their full length during DNA replication, and are therefore becoming progressively shorter with each cell division. Shortening of telomeres is tightly linked to induction of senescence, genomic instability and cancer (reviewed in: Finkel et al., 2007; Liu and Sharpless, 2009). The telomere elongating enzyme telomerase is turned off in most somatic cells, but is active during early embryonic development, in ES cells and in iPS cells (reviewed in Donate and Blasco, 2011). Cloned embryos obtained by nuclear transfer from an adult donor cell into an enucleated oocyte, also display normal telomere length (Hochedlinger and Jaenisch, 2003).

Results in this thesis suggest that a clearance of damaged proteins is an integral part of early embryonic development. To elucidate whether iPS cells display such damage elimination upon differentiation is of major interest. If they do, the question of iPS cells regain the capacity to rid themselves from damage is of special interest. If they don't, is there an effect on the damage control and/or life span of the offspring obtained from iPS cells?

The potential of PA28

As shown herein, early ES cell differentiation encompasses an induction of the PA28-20S/20Si proteasomal complex and both proteasomal activity and PA28 are required for the observed elimination of damaged proteins. However, whether this damage clearance is *de facto* a mechanism of rejuvenation and longevity assurance of the progeny is yet to be defined.

PA28 is not essential for survival and its absence does not appear to hamper the generation of seemingly healthy mice – such mice are, however, unable to produce a certain antigen epitope but are not generally defective in antigen processing (Murata et al., 2001). The levels of protein damage of these mice are of major interest in determining the importance of PA28-induced damage clearance during embryogenesis. Because of the link between inflammation and aging (discussed briefly in chapter 7), the most optimal approach to study aging effects of embryonically acting PA28, would be a system in which PA28 could be briefly turned off during mouse embryogenesis *in vivo*.

As mentioned, overexpressing PA28 has been shown to reduce protein carbonylation levels in differentiated rat cardiomyocytes (Li et al., 2011). If an increased level of protein damage is one contributing factor of aging, and if PA28 can decrease these levels without other deleterious effects, then PA28 overexpression might prolong lifespan. Such a finding would provide vast opportunities for reversing the negative health effects associated with aging and enable aging decelerating treatments.

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This chapter summarizes the people to whom I am grateful.

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When love beckons to you, follow him,
Though his ways are hard and steep.
And when his wings enfold you yield to him,
Though the sword hidden among his pinions may wound you.
And when he speaks to you believe in him,
Though his voice may shatter your dreams
as the north wind lays waste the garden.

For even as love crowns you so shall he crucify you. Even as
he is for your growth so is he for your pruning.
Even as he ascends to your height and caresses your
tenderest branches that quiver in the sun,
So shall he descend to your roots and shake them in their
clinging to the earth.

(…)

But if in your fear you would seek only love's peace and love's
pleasure,
Then it is better for you that you cover your nakedness and
pass out of love's threshing-floor,
Into the seasonless world where you shall laugh, but not all
of your laughter, and weep, but not all of your tears.
Love gives naught but itself and takes naught but from itself.
Love possesses not nor would it be possessed;
For love is sufficient unto love.

(…)

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