Aus dem Institut für Medizinische Psychologie der
Ludwig-Maximilians-Universität München

Vorstand: Prof. Martha Merrow, Ph.D

The Interaction between the Circadian Clock, Temperature Entrainment and the Insoluble Protein Content

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät
der Ludwig-Maximilians-Universität zu München

vorgelegt von

Nora Melina Sofie Rubbe

aus

München, Deutschland

2021

Gedruckt mit Genehmigung der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

Berichterstatter: Prof. Dr. Martha Merrow, Ph.D.

Mitberichterstatter: Prof. Dr. med. Jan Rémi
Dr. Dominic Landgraf

Mitbetreuung durch den promovierten Mitarbeiter:

Dekan: Prof. Dr. med. dent. Reinhard Hickel

Tag der mündlichen Prüfung: 30.09.2021

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation

The Interaction between the Circadian Clock, Temperature Entrainment and the Insoluble Protein Content

selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation

The Interaction between the Circadian Clock, Temperature Entrainment and the Insoluble Protein Content

is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den 04.10.2021 Nora Rubbe

Table of Contents

1. SUMMARY **7**

ZUSAMMENFASSUNG **9**

2. INTRODUCTION **11**

2.1. Circadian Clock, Temperature entrainment and the link to Neurodegenerative diseases **11**
2.1.1. System of the circadian clocks in mammals 11
2.1.2. Link between circadian rhythm and neurodegenerative diseases in aging mammals 16
2.1.3. Features of neurodegenerative diseases in aging mammals 21

2.2. Proteostasis in aging cells **24**
2.2.1. Proteostasis network and chaperone system 24
2.2.2. Changes of the proteostasis network in aging cells 28

2.3. Senescence, an aging cell **31**
3.3.1. Definition of Senescence 31
3.3.2. Features of a senescent cell 32

3. AIM OF THE PROJECT **34**

4. MATERIALS **35**

4.1. Buffers and media for cell culture **35**
4.1.1. Culture medium for routine 35
4.1.2. Experimental medium for senescence experiments 35
4.1.3. Buffers 36

4.2. Gels **38**
4.2.1. Hand cast 10 % Tris-glycine gel for protein aggregation assay 38
4.2.2. Gel for protein aggregation assay (after normalization) 39
4.2.3. Hand cast 7.5 % Tris-glycine gel for Western Blot 39

4.3. Antibodies **39**
4.3.1. Primary antibodies 40
4.3.2. Secondary antibodies 40

5. METHODS 41

5. 1. Cell culture 41
5.1.1. Cell lines and maintenance 41
5.1.2. Subculturing cells 42
5.1.3. Cell counting 43

5.2. Senescence induction 43
5.2.1. Senescence induction 43
5.2.2. Adaption of FBS concentration 43

5.3. Beta-galactosidase staining 44
5.3.1. Cell seeding and maintenance 45
5.3.2. Cell staining 45

5.4. Protein aggregation assay 47
5.4.1. Cell culture and senescence induction 47
5.4.2. Temperature cycles 47
5.4.3. Cell harvesting 48
5.4.4. Protein extraction 48
5.4.5. Gel electrophoresis 49
5.4.6. Normalization 50

5.5. Western Blot 52
5.5.1. Time course 52
5.5.2. Protein extraction 53
5.5.3. Determination of protein concentration 54
5.5.4. Tank Blotting 55
5.5.5. Ponceau staining 55
5.5.6. Immunolabeling 55

5.6. Heat shock experiment 56

5.7. Statistical analyses 56

6. RESULTS 57

6.1. Establishing a model for senescence-induced aggregation in neuroblastoma cells 57
6.1.1. Experimental medium supplemented with FBS is required for N2A cell survival 58
6.1.2. The FBS concentration in the experimental medium influences senescence induction 61
6.1.3. LBH589 induces senescence in N2A cells 63

6.2. Temperature entrainment and Proteostasis 67
6.2.1. The insoluble protein content increases in senescent N2A cells 67
6.2.2. The insoluble protein content of N2A cells kept in temperature cycles increases less compared to N2A cells kept in constant condition 70
6.2.3. The insoluble protein content of U2OS cells kept in temperature cycles increases compared to U2OS cells kept in constant condition 75

6.3. Chaperone system of N2A and U2OS cells 79
6.3.1. Chaperones detected in N2A and U2OS cells in constant condition and after heat shock induction 79
6.3.2. Concentration of HSP90 does not change in N2A cells kept in temperature cycles in comparison with control cells kept in constant condition 83

7. DISCUSSION 84

7.1. Senescent N2A cells as an aging model 84
7.1.1. Senescent associated beta-galactosidase is a senescence marker 84
7.1.2. LBH589 is a trigger for senescence induction 85
7.1.3. FBS influences senescent induction negatively 87
7.1.4. Summary 88

7.2. Protein aggregation as a hallmark of aging 88
7.2.1. Insoluble protein extraction in N2A cells 89
7.2.2. Are protein aggregates beneficial or a burden for the cell? 91
7.2.3. Summary 92

7.3. Temperature cycles influence the Proteostasis network of N2A and U2OS cells 93
7.3.1. The insoluble protein content of N2A cells kept in temperature cycles decreases compared to N2A cells kept in constant temperature condition 95
7.3.2. The insoluble protein content of U2OS cells kept in temperature cycles increases compared to U2OS cells kept in constant temperature condition 98
7.3.3. Summary 99

7.4. Chaperones as mediators between the clock and Proteostasis 100
7.4.1. Chaperone expression differs in N2A and U2OS cells 100
7.4.2. No changes in HSP 90 expression in N2A cells kept in constant versus cycling temperature condition 101
7.4.3. Summary 103

8. BIBLIOGRAPHY 104

9. ABBREVIATIONS 145

10. ACKNOWLEDGMENTS 146

11. CURRICULUM VITAE FEHLER! TEXTMARKE NICHT DEFINIERT.

1. Summary

The elderly and patients suffering from age-associated neurodegenerative diseases (ND) often present with disrupted circadian timing systems such as alterations in their wake-sleep cycle. As aging and ND are associated with an increase of protein aggregates in the brain tissue, a correlation between protein aggregation and a disrupted circadian timing system seems likely.

This study aims to investigate the effect of the circadian clock on protein aggregation in aging neuronal cells. We established an aging neuronal cell model by inducing senescence with administration of a histone deacetylase inhibitor (LBH589) in mouse neuroblastoma cells (N2A cells). One hallmark of senescent cells in an increase of protein aggregates. Therefore, senescent neurons represent a low complexity *in vitro* model for aging cells in which to test discrete hypotheses. In order to investigate the effect of circadian entrainment on protein aggregation in senescent cells, we kept senescent N2A cells in 24 temperature cycles with 34 °C (12 hours) and 37 °C (12 hours). A comparison with control cells kept in 24h temperature cycles or constant temperature showed effects of a 24h zeitgeber cycle on aggregation.

Analyses of the protein content in N2A cells kept in temperature cycles revealed a decrease in insoluble protein content compared to cells kept in constant temperature condition. However, we observed no differences in the insoluble protein content of cells kept in 24h versus non-24h temperature cycles. Therefore, we suggest a direct effect of temperature on the insoluble protein content in N2A cells, without a significant influence of circadian clock oscillation. These findings support the hypothesis that the master pacemaker of the circadian timing system, the suprachiasmatic nucleus (SCN), influences protein aggregation through regulating temperature amplitudes and not as initially expected through circadian

temperature amplitudes. An impaired SCN and temperature alterations in elderly and ND patients, suggested by several studies, may influence the proteostasis network adverse.

Therefore, a better rhythmicity (like activity at day time, sleep during night) in elderly could lead to a better entrainment of the SCN and higher temperature amplitudes, which would protect the cell from protein aggregates and thus could be a therapy option for ND in future.

Furthermore, the effect of protein aggregates on cell survival could be a focus of future projects. If decreasing the insoluble protein content of a cell is beneficial, this could represent a target for new therapeutic approaches to treat or even prevent neurodegenerative diseases.

1. Zusammenfassung

Ältere Menschen und Patienten mit neurodegenerativen Erkrankungen (ND) leiden häufig unter Störungen ihrer zirkadianen Rhythmik, ein Beispiel hierfür ist die Veränderung ihres Schlafwachrhythmus. Da Alterungsprozesse und neurodegenerative Erkrankungen mit einer Erhöhung von Proteinaggregaten im Gehirn einhergehen, scheint ein Zusammenhang zwischen Proteinaggregaten und einem gestörten zirkadianen System naheliegend.

Diese Studie untersucht den Zusammenhang zwischen der zirkadianen Uhr und Proteinaggregaten in gealterten (seneszenten) neuronalen Zellen. Wir entwickelten ein Zellmodell mit gealterten neuronalen Zellen (Mausneuroblastom-Zellen, N2A Zellen), in denen wir Seneszenz mittels Histondeacetylase Inhibitor (LBH489) induzierten. Eine Eigenschaft von seneszenten Zellen ist der Anstieg von Proteinaggregaten, weshalb sie sich als vereinfachtes *in vitro* Modell zur Überprüfung unserer Hypothesen eigneten. Um den Effekt der zirkadianen Synchronisation auf den unlöslichen Proteinanteil in gealterte Zellen zu untersuchen, hielten wir seneszente N2A Zellen in 24h Temperaturzyklen mit 12 Stunden 34 °C und 12 Stunden 37 °C. Der Vergleich mit Kontrollzellen, die unter nicht-24h Temperaturzyklen oder konstanter Temperatur gehalten wurden, zeigten uns den direkten Einfluss von Temperatur auf den Proteingehalt auf.

Unsere Auswertungen ergaben, dass N2A Zellen, die unter Temperaturzyklen gehalten wurden, einen reduzierten Gehalt an unlöslichen Proteinen aufweisen. Der Gehalt an unlöslichen Proteinen in N2A Zellen, die unter 24h und nicht-24h Temperaturzyklen gehalten wurden, unterschied sich jedoch nicht. Daraus folgern wir einen direkten Temperatureinfluss auf den unlöslichen Proteingehalt in N2A Zellen, ohne einen signifikanten Einfluss von zirkadianer Oszillation. Diese Ergebnisse unterstützen die Hypothese, dass der zentrale zirkadiane Taktgeber (der Nucleus suprachiasmaticus, SCN) Proteinaggregation durch

Temperaturamplituden steuert und nicht wie zunächst angenommen über zirkadiane Temperaturoszillationen. Interessanterweise zeigten Studien, dass sich der SCN und Körpertemperaturen in Älteren und ND Patienten verändern. Folglich könnte ein Funktionsverlust des SCN mit einhergehenden Temperaturveränderungen einen negativen Effekt auf das Proteostasenetzwerk haben.

Eine ausgeprägter Rhythmus (wie Aktivität am Tag, Schlaf in der Nacht) in älteren Menschen könnte zu einem besseren Entrainment des SCN führen und somit zu höheren Temperaturamplituden im Körper. Dies wiederrum könnte einen protektiven Effekt auf Proteinaggregation haben und wäre ein interessanter Therapieansatz zur Bekämpfung von ND.

Ob Proteinaggregate einen Einfluss auf das Zellüberleben haben, könnte Schwerpunkt für zukünftige Projekte darstellen. Falls sich ein sinkender unlöslicher Proteinanteil vorteilig auf die Zelle auswirkt, stellt dies einen neuen Ansatz für therapeutische Verfahren dar, um neurodegenerative Erkrankungen zu behandeln oder sogar zu verhindern.

2. Introduction

2.1. Circadian Clock, Temperature entrainment and the link to Neurodegenerative diseases

2.1.1. System of the circadian clocks in mammals

The temporal coordination of internal biological processes among each other and the environment is crucial for the survival and health of organisms, from bacteria to human beings. Due to the importance of temporal coordination you can find circadian clocks, which are 24h-timing mechanisms composed of molecular oscillators, in nearly every mammal tissue (Bollinger and Schibler, 2014). Multiples of endogenous oscillators build a hierarchical timing system that functions by anticipating (stressful) environmental changes (Figure 2.1). The circadian clock in mammals is composed of a master pace maker, the suprachiasmatic nucleus (SCN), a part of the hypothalamus, and many subordinate endogenous peripheral clocks in most - if not all - cells (Ralph *et al.*, 1990; Bollinger and Schibler, 2014). The SCN processes light environment as it changes and transforms the signal into oscillation of hormones, temperature and other biochemical components (Balsalobre *et al.*, 2000; Stokkan *et al.*, 2001; Brown *et al.*, 2002a). The SCN is able to synchronize peripheral clocks via these messengers. The external and internal stimuli on the SCN and the peripheral clocks, which themselves oscillate and are able to synchronize /reset the circadian clock, are called zeitgebers (in German: time-giver) (Roenneberg, Daan and Merrow, 2003). One of the strongest zeitgeber on the SCN is light (day-night cycle) (Duffy and Wright, 2005; Roenneberg and Merrow, 2007).
One of the co-founders to define circadian clocks was Colin Pittendrigh, who listed different aspects of circadian clocks (Pittendrigh, 1960).

Some of his definitions are still commonly used to describe circadian clocks: they are (i) endogenous self-sustained rhythmic oscillators with a period of about 24 hours (Latin; *circa*: about, *diem*: a day) in constant condition, (ii) the period is temperature compensated and (iii) they are entrainable by zeitgebers to exactly 24 hours (Merrow *et al.*, 2015). Here, I will discuss these properties.

(i) *Self-sustained* in this context describes the continuity in rhythmicity of the clock even without zeitgebers. The Bunker experiment by Jürgen Aschoff, starting in 1963, are a good example for self-sustained rhythmicity in humans (Aschoff and Wever, 1962). Subjects were kept in a bunker and were shielded from any environmental impact; especially from daylight and other periodic input e.g. watches or phones. Even though the test persons had no connection to the outside world, they maintained a ca. 24h sleep-wake rhythm. Their day length, however, shifted from an average of about 24 hours to an average of about 25 hours, suggesting that there is an inner clock, which stays self-sustained and rhythmic without input of zeitgebers (Aschoff and Wever, 1962).

(ii) *Temperature compensation* means that the circadian oscillations, or rhythms, stay unaffected in their period of approximately 24 hours when kept within a physiological range of constant temperature. In other words, the period remains fairly constant, even without zeitgeber input. Not to be mistaken with synchronization: If cells get entrained by zeitgebers like 24h temperature cycles or light, the clock phase shifts and synchronizes to exactly 24 hours of these zeitgeber cycles (Bodenstein, Heiland and Schuster, 2012).

(iii) The ability of circadian clocks to entrain to zeitgeber cycles, implies that they are able to actively synchronize to cycling environmental signals (Roenneberg, Daan and Merrow, 2003). A good example is the wake-sleep cycle in humans which entrains to light (Duffy and Wright, 2005). Electricity made humans more and more independent of sunlight and enabled them to work indoors and especially after sunlight. Thus, electric lightening led to a change in light exposure, uncoupled from natural sunlight. It could be shown that this delays circadian clocks in humans, thus changing the timing of behavior relative to the natural day-night cycle (Wright *et al.*, 2013).

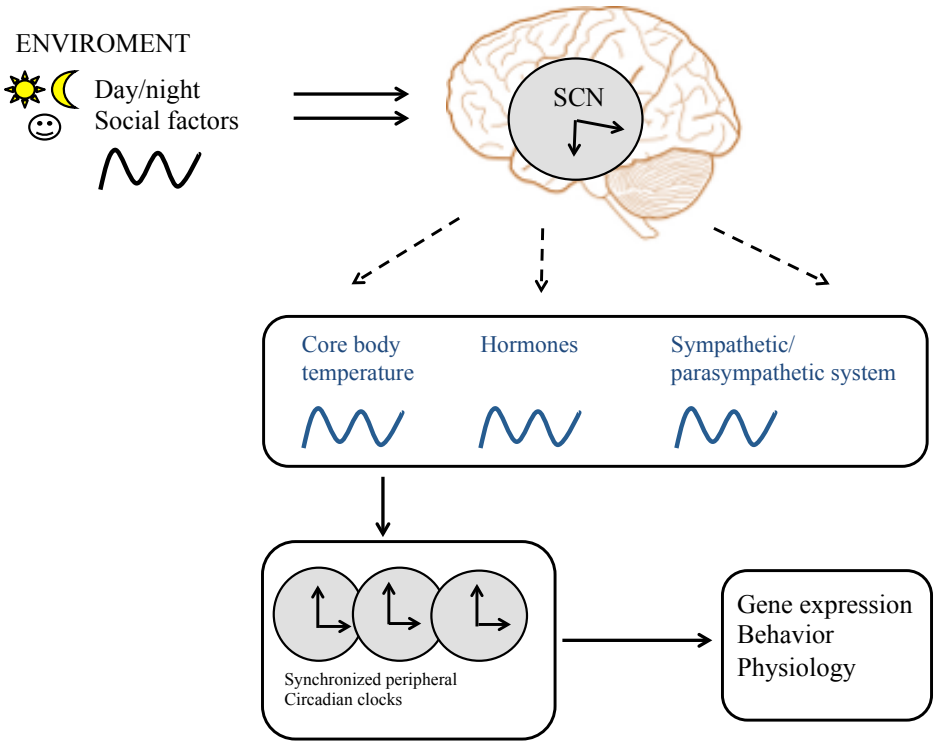


Figure 2.1 Scheme of the mammal circadian timing system. The master pacemaker (SCN = suprachiasmatic nucleus) detects changes in the environment, mainly light, to synchronize endogenous clocks in peripheral tissues via oscillation of e.g. temperature and hormones. The peripheral endogenous clocks control output pathways like gene expression, behavior and physiology over a 24-hour period.
Adapted from Scully et al., 2010.

Taking the hierarchical overview of figure 2.1, circadian clocks are present in the brain (= central clocks) and in peripheral organs or tissues (= peripheral clocks). They are entrainable and therefore linked to other systems. However, they also oscillate autonomously when decoupled from input pathways, e.g. shown in mouse and rat fibroblasts (Nagoshi et al. 2004; Welsh et al. 2004). Central clocks get entrained by zeitgebers from the external environment; the peripheral clocks entrain to endogenous oscillating messengers (= internal environment) (Albrecht, 2012).

In summary a circadian clock consists of an input pathway to allow entrainment, the core clock components and an output pathway to regulate downstream rhythms figure 2.2 (A).

On a cellular level, the circadian clock consists of core clock components, a system of genes whose products (proteins) are essential in building and regulating circadian oscillations (Takahashi, 2004). In figure 2.2 (B), a simplified model of circadian oscillation in a cell is illustrated. The model consists of interacting positive and negative transcriptional feedback loops. There are proteins which function as suppressors: if they accumulate to a certain level, they inhibit their own activation through interaction with activators. At the same time they influence the transcription/translation of activators directly by interacting with genes of activators. The activators on the other hand influence the transcription/translation of their suppressors. Examples of activating proteins in mammals are CLOCK (CLK) and brain and muscle Arnt-like protein 1 (BMAL 1). Examples of circadian core protein suppressors are period homologue proteins (PER) and cryptochomes (CRY) (Eckel-Mahan and Sassone-Corsi, 2013). These regulation mechanisms enable the clock to self-sustain in the absence of zeitgeber cues.

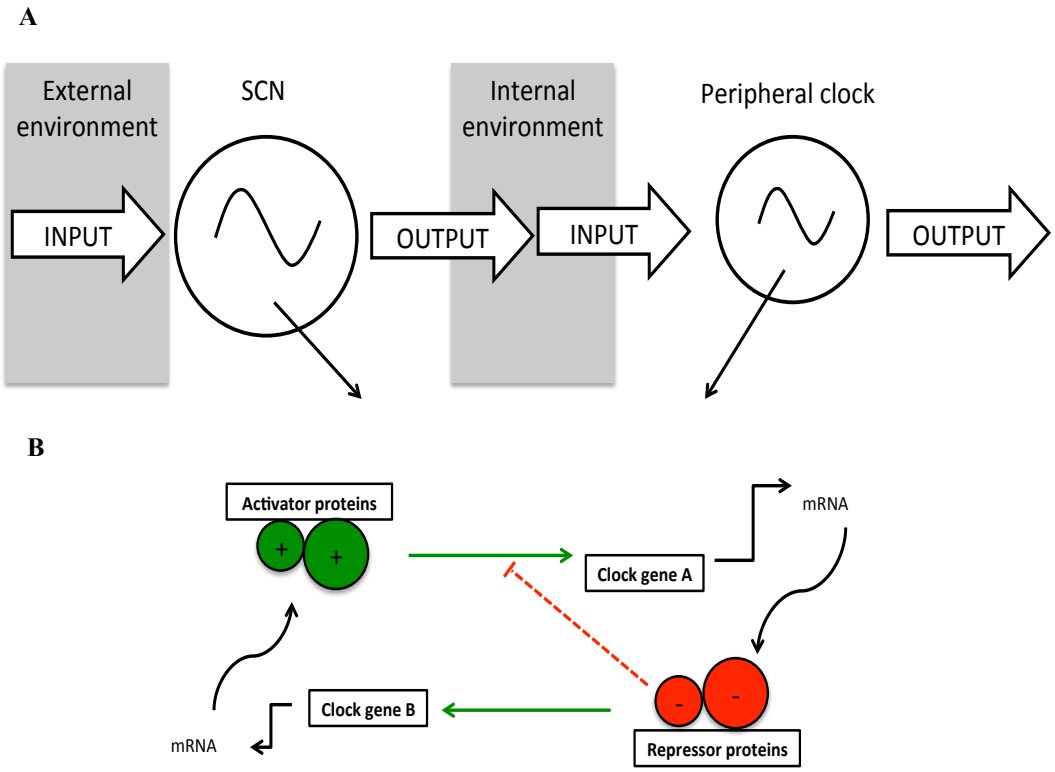


Figure 2.2 A Model of the circadian timing system with the key elements of the clock: input pathway, oscillation and output pathway of the circadian master pacemaker (suprachiasmatic nucleus = SCN) and peripheral clock. External environment factors, e.g. light, entrain the SCN; where as internal messengers regulated by the SCN entrain peripheral clocks in organs or tissues **B** Example of circadian oscillator model with 2 interacting feedback loops. Clock gene A is transcribed and then translated into a suppressor protein. The suppressor protein inhibits its own transcription via suppressing activator proteins. At the same time it activates the processing of activator proteins via clock gene B.
(Re-drawn after Eckel-Mahan & Sassone-Corsi 2013; Albrecht 2012; Lakin-Thomas 2006)

2.1.2. Link between circadian rhythm and neurodegenerative diseases in aging mammals

The circadian clock timing system connects the environment with the inner processes of mammals and influences biochemical, physiological and behavioral flows (Hu *et al.*, 2004; Morris, Yang and Scheer, 2012). Even though this connection is not yet understood in every system or tissue, rhythmicity is present in many physiological processes, e.g. the immune system (Scheiermann *et al.*, 2012), the endocrine system (Nicolaides *et al.*, 2014) and the body temperature (Tsujimoto *et al.*, 1990). But, what happens if such a complex and ubiquitous system fails or breaks down?

Looking at the hierarchical circadian timing system described in chapter 2.1.1, there are many possible levels or components that can fail and then lead to disruption in the circadian timing system. Therefore, in this chapter *I will* depict some of the key items of the circadian system and their interaction with my field of interest: neurodegenerative diseases in aging. Starting with describing behavioral output pathways, leading to the clocks in the brain and the SCN to the input pathways. Considering that neurodegenerative diseases (ND) like Parkinson disease (PD) and Alzheimer disease (AD, leading cause of dementia) are related to age (Reitz, Brayne and Mayeux, 2011; Reeve, Simcox and Turnbull, 2014b), studies with both elderly and elderly suffering from neurodegenerative diseases will be illustrated together for a clearer view.

Circadian output pathways of ND in aging mammals

A physiological rhythm that can be seen as an output pathway of the circadian clock system is the wake-sleep cycle. It is known that elderly or people suffering from ND, like dementia, have altered wake-sleep cycles. Their activity rhythm becomes irregular leading to fragmented sleep, more day time naps and later onset of sleep at night (Huang *et al.*, 2002; Hatfeld *et al.*, 2004; Merlino *et al.*, 2010; Weissová *et al.*, 2016).
The changing sleep pattern not only impacts the patients' quality of life (shown in Parkinson patients (Barone *et al.*, 2009)) but is also one of the main reasons why

elderly and those suffering from dementia get institutionalized (Pollak and Perlick, 1991; Bianchetti *et al.*, 1995). Considering the fact that elderly or patients suffering from ND have an irregular activity rhythm, one might ask if through entrainment with stronger zeitgeber signals the wake-sleep cycle would synchronize again.

Studies with elderly and patients suffering from dementia living in nursing houses show that increased exposure to light in the morning leads to a delayed peak of the activity phase and improves circadian rhythmicity for 10 days after light treatment (Ancoli-Israel *et al.*, 2002). Also, when exposed to bright light in the morning and evening, subjects had more consolidated sleep during the night (Ancoli-Israel *et al.*, 2003). When combining light with physical activity (walking) to entrain the circadian clock, subjects had less total wake time and better sleep efficiency (McCurry *et al.*, 2011). A study in the Netherlands with demented aged residents of care facilities suggests that bright light (a stronger zeitgeber) has a modest positive effect on mood and reduces cognitive decline (Riemersma-van der Lek *et al.*, 2008). Similar results could be shown in the United States, where a positive effect on depression scores and agitation, as well as sleep efficiency was assessed in AD patients (Figueiro *et al.*, 2014). In summary, even though the mentioned studies show an effect on the circadian clock, the beneficial consequence of bright light on neurodegenerative symptoms and especially long term effects are not yet assessed and need further investigations (Forbes *et al.*, 2014).

A good way to assess the effect of disrupted circadian clock timing systems on diseases is to study shift workers. Their wake-sleep cycle shifts and therefore their inner clocks desynchronize from the environmental natural light cycle. Studies show that long-term shifted wake-sleep cycles are associated with higher rates of serious morbidities like cancer and cardiovascular diseases (Kawachi et al. 1995; Knutsson et al. 1999; Suwazono et al. 2008).

Another output pathway of the circadian timing system that suggests a connection between the circadian rhythm and neurodegenerative diseases is food intake. In patients suffering from Alzheimer disease, there is a shift in circadian food intake with decreased input in the evening compared to healthy controls, contributing to a poor body mass index (Young and Greenwood, 2001). A change in the timing of food intake would shift the phase of the clock in the liver later, possibly creating a sub-optimal relationship with SCN regulated peripheral clocks.

Circadian central and peripheral oscillators of ND in aging mammals

On the level of central and peripheral oscillators, disruptions of the circadian clock mechanisms have been described in elderly and ND patients, but also in mammal models. Several studies suggest that oscillation mechanisms in the SCN change with age. Comparing to young rats, there are lower amplitudes of electrical activity in the SCN of older rats, implying that aging leads to SCN disruption and thus to a disregulated circadian system (E Satinoff *et al.*, 1993). Consistently, neurons in the SCN of PD mouse models are less excitable and phenotypes showed distracted wake-sleep patterns (Kudo *et al.*, 2011). Looking at the SCN of elderly and AD patients, studies suggest a decrease in certain neurons expressing vasoactive intestinal peptide (VIP) and a change in seasonal oscillation, compared to healthy or young controls (Swaab, Fliers and Partiman, 1985; Hofman and Swaab, 1995; Zhou, Hofman and Swaab, 1995). This loss of VIP expressing neurons correlates with alterations in the circadian rhythm amplitude of motor activity in elderly and AD patients (Wang *et al.*, 2015).

In AD mouse models, the impact of ß-amyloid plaques on circadian core clock components showed interesting results. In the SCN, ß-amyloid plaques induce core clock transcription factors' degradation, leading to alterations in clock gene expression (Song *et al.*, 2015). Another hint to an interaction between AD and the circadian clock is when looking at presenilin 2. Presenilin 2 gene mutations are on one hand associated with hereditary AD forms, on the other hand its gene expression is modulated by key clock components (Bélanger, Picard and Cermakian, 2006).

In contrast, when comparing mRNA and protein levels of core clock genes in the SCN, one could find no difference in core clock gene expression in young versus old rats (Asai *et al.*, 2001) or rhesus macaques (Eghlidi *et al.*, 2018).
As an example of peripheral clock changes in ND, there are findings that mRNA expressions of core clock genes in leucocytes differ among PD patients with lower levels in the evening. Lower expression of core clock genes, correlated positively with the severity of PD (assessed with the Parkinson Disease Rating Scale) (Cai *et al.*, 2010). In mouse models with circadian core clock gene deficiencies, e.g. Bmal1 or per1/2 knockout mice, mice show a reduced lifespan and suffered from conditions related to aging, like predisposed tumor incidences or osteoporosis (Lee, 2005; Kondratov *et al.*, 2006; Dubrovsky, Samsa and Kondratov, 2010).

To sum this up, there is evidence of alterations in central and peripheral clock components in aging and ND models. Nevertheless, the existing data does not allow us to draw general conclusions, because underlying mechanisms causing these alterations are not yet understood.

Circadian input pathways and ND in aging mammals

In the following, some aspects of input pathways that entrain circadian clocks in elderly and ND patients will be described. On the one hand zeitgebers from the 'external environment' synchronize cells in the SCN, on the other hand zeitgebers from the 'internal environment' synchronize peripheral clocks.

Beginning with external zeitgebers, one aspect could be that elderly or ND patients are exposed to a different external environment with e.g. a decline in zeitgeber strength. Fitting this hypothesis, studies show that elderly and severely demented patients in care house facilities are less exposed to light, possibly due to mental and physical decline (Campbell *et al.*, 1988; Ancoli-Israel *et al.*, 1997; Shochat *et al.*, 2000). When increasing light exposure of ND patients, there is an improvement of their wake-sleep cycle alteration (Ancoli-Israel *et al.*, 2003; McCurry *et al.*, 2011; Videnovic *et al.*, 2017). Another aspect might be an impairment of SCNs' neurons, which are responsible to transmit environmental circadian information to the SCN. One of the key neurons in transmitting light to

the SCN, in order to entrain the central clocks, are melanopsin-expressing retinal ganglion cells (mRGC). They transmit light information directly to the SCN (Hatori *et al.*, 2008; Do and Yau, 2010). Interestingly, AD Patients have fewer mRGCs, implicating that the main input pathways to the SCN is impaired (La Morgia *et al.*, 2016). In PD patients, data is also implicating a disruption in the environmental light input pathway. Shown in their retina with misfolded alpha-synuclein deposition (Bodis-Wollner *et al.*, 2014) and decreased dopamine levels (Harnois and Di Paolo, 1990). A further input pathway to the SCN comes from the cholinergic basal forebrain. In both elderly and AD patients, amyloid-ß accumulation was detected in this region (Baker-Nigh *et al.*, 2015), suggesting a decline in information flow to the SCN. Considering a study with AD model rats, where a decline in cholinergic paths from the basal forebrain led to circadian alterations in the SCN (Erhardt *et al.*, 2004), this might be another explanation for the disrupted circadian clock system in ND patients.

There are various internal zeitgebers influencing peripheral clocks that are altered in elderly or ND patients. Glucocorticoid is an example of hormone, which entrains peripheral clocks but not the SCN (showed in rat fibroblasts (Balsalobre *et al.*, 2000)). Studies suggest that nocturnal glucocorticoid levels increase in elderly (Ferrari *et al.*, 1995, 1996, 2001; Vgontzas *et al.*, 2003) and senile dementia patients (Ferrari *et al.*, 2001). In contrast, in PD patients higher cortisol levels were investigated (Hartmann *et al.*, 1997).

Another internal zeitgeber, mainly resetting peripheral clocks, is circadian body temperature (Brown *et al.*, 2002b; Saini *et al.*, 2012). Supported by studies, showing that the SCN is resistant to temperature entrainment (shown in Rat1 fibroblast and mouse cortical glia (Brown *et al.*, 2002c; Prolo, 2005; Buhr, Yoo and Takahashi, 2010)).

Thus, external temperature oscillation directly, or endogenous temperature oscillation regulated by the SCN, function as zeitgebers to entrain peripheral clocks. Compared to healthy young controls, both in elderly an AD patients a change in body temperature pattern can be noted: a decline in the endogenous circadian amplitude (highest to lowest temperature) and a desynchronisation of

temperature from activity rhythms (Satlin *et al.*, 1995; Harper *et al.*, 2005). In PD patients, there is a reduction of the core body temperature amplitude, too (Zhong *et al.*, 2013). The reason of both temperature and glucocorticoid changes in elderly and ND patients remain unclear.

In summary, *you* can see differences in several sub-items of the circadian clock between healthy (or young) mammals compared to ND (or old) mammals. If the disruption in the circadian timing system is the consequence of neurodegeneration, or if neurodegeneration is contributing to disruptions in the circadian system, however, is not yet understood (Videnovic *et al.*, 2014; Hood and Amir, 2017).

2.1.3. Features of neurodegenerative diseases in aging mammals

Neurodegenerative diseases (ND) are an umbrella term for diseases, which effect neuronal cells ("neuro") through loss of function and structure ("degeneration"). The main and most common NDs are Alzheimer disease (AD) and Parkinson disease (PD). AD, as the most common ND with approximately 60 - 80 % causative for dementia and PD, as the second most frequently ND with a prevalence of about 100 - 200 of 100.000 German citizens (von Campenhausen *et al.*, 2005; Alzheimer's Association, 2016).

In German clinical daily routine, the International Classification of Diseases (ICD-10, Deutsches Institut für Medizinische Dokumentation und Information) is used to classify somatic diseases, including neuronal diseases. ND is not classified as one group, since ICD-10 groups neurological diseases concerning the patients' clinical presentation and diagnostic results.
ND, however, summarizes neurological diseases with similar neuropathological findings (neurodegeneration and protein aggregates). This often involves post mortem findings and helps to define underlying pathologies. Thus, two main classifications can be used to describe neurological diseases: the clinical-anatomical classification and the neuropathological classification (Kovacs G.G. 2015). As my project focuses on the underlying pathological similarities of those neurological diseases, the term ND will be used.

The cause of ND is not yet understood, though a multifunctional genesis is assumed.

Many studies suggest a dysregulation in the protesostasis network as one causative aspect, leading to protein misfolding and protein aggregation (Klaips, Jayaraj and Hartl, 2018a). These protein aggregates, as an output of the proteostasis network, can be seen as hallmarks of both aging (Yang, Ang and Strong, 2005; Paz Gavilán *et al.*, 2006; David *et al.*, 2010b; Omata *et al.*, 2014) and ND (Klaips, Jayaraj and Hartl, 2018a). Even though the underlying proteins of aggregates differ in ND, there are many overlapping findings. Extracellular beta-Amyloid plaques for example, commonly associated with AD, has been identified in elderly, PD and Creutzfeldt-Jakob disease patients (Mann and Jones, 1990; Armstrong, 1995; Hainfellner *et al.*, 1998). Another example is alpha-synuclein aggregates, the hallmark of PD, which have been also identified in brains of AD patients (Uchikado *et al.*, 2006; Leverenz *et al.*, 2008). Although the etiology of ND is part of current research, there are many risk factors linked to ND. One of the most important risk factors is age, implying that in industrialized countries with increasing older population the proportion of ND will increase immensely in future (Reitz, Brayne and Mayeux, 2011; Reeve, Simcox and Turnbull, 2014a; Alzheimer's Association, 2016).

The clinical presentation of ND patients is very heterogenic, depending on the brain region affected (Alzheimer's Association, 2016). One important feature of all ND is the progression of symptoms, due to progress in neuronal degeneration.

The various ND diagnoses share predominant symptoms. In dementia, cognitive decline is in the foreground, whereas e.g. PD patients often show motoric symptoms in early stages of disease leading to cognitive decline in later stages (Alzheimer's Association, 2016).

The diagnosis is established based on clinical evaluation, adding typical diagnostic hints from laboratory results. Often, diagnoses can only be confirmed with post mortem autopsy results, proving typical pathological findings. ND cannot be cured and therapy is based on supportive care. Drugs used in ND, like acetyl cholinesterase inhibitor for AD and dopamine modulator for PD, improve symptoms, but do not address the cause of disease (Müller, 2012; Alzheimer's Association, 2016).

In summary, important features of ND are:

(1) Age as an important risk factor of most (not all) ND
(2) Involving multifactorial causes, including dysfunction in the proteostasis network
(3) Progress in neuronal degeneration, leading to progress in clinical presenting symptoms
(4) Complex diagnostic procedure, without having a single universal marker
(5) No curative therapy

(Klaips et al. 2018, Alzheimer's Association 2016; Reitz et al. 2011; Reeve et al. 2014a; Müller 2012)

2.2. Proteostasis in aging cells

2.2.1. Proteostasis network and chaperone system

Protein homeostasis is required to maintain a healthy cell (Chiti and Dobson, 2006). It implies a balance of protein synthesis, maintenance and degradation in order to have a functional system in a cell within an organism. To provide this balance a proteostasis network (PN) is needed, consisting of about 200.000 protein components (Klaips, Jayaraj and Hartl, 2018b).
Describing every detail of the PN would go beyond the scope of this work. Thus, main aspects and quality control strategies will be described, beginning with a short introduction of the chaperone network.

The chaperone system

The main regulating proteins in the PN are chaperones and co-chaperones. They assist, regulate and interfere in different steps of a protein's lifespan, without becoming part of the protein itself (Hartl, 1996; Hartl, Bracher and Hayer-Hartl, 2011). Because most of chaperone members are induced and up regulated under stress conditions, they are also known as stress proteins or heat shock proteins (HSPs) (Hartl, Bracher and Hayer-Hartl, 2011). Hence, the cell copes with stress-induced protein misfolding, through up regulating chaperones.

HSPs are classified according to their molecular weight, grouped in chaperone families. Popular reviewed systems are members of the HSP 70 (ca. 70 kDa) and HSP 90 (ca. 90 kDa) family (Hartl, Bracher and Hayer-Hartl, 2011; Fernández-Fernández and Valpuesta, 2018), especially in relation to aging and ND research (Klettner, 2004; Muchowski and Wacker, 2005; Brown, 2007).

The amount of chaperone members in every family varies among species, depending on the complexity of the PN. Hence, the number of chaperones increases from prokaryote to eukaryotes (Genevaux, Georgopoulos and Kelley, 2007; Vos *et al.*, 2008; Rizzolo *et al.*, 2017; Klaips, Jayaraj and Hartl, 2018a) with e.g. three HSP 70 members in Escherichia coli and 13 in humans (Kampinga *et al.*, 2008). Nearby, that also their interactions increase in complexity.

In eukaryotes e.g., with a more complex and finer tuned PN, HSP 70 depend on interactions with co-chaperones like HSP 40 (Nillegoda *et al.*, 2017).

The key functions of the chaperones are (i) assisting in de novo folding/refolding proteins (Young *et al.*, 2004) and (ii) trafficking unneeded proteins to the degradation machinery (Hartl, 1996; Majeski and Fred Dice, 2004), sharing one goal: to protect the cell from misfolded proteins. HSP 70 members and their co-chaperones play a role in nearly all processes to maintain proteostasis (Zuiderweg, Hightower and Gestwicki, 2017; Fernández-Fernández and Valpuesta, 2018). HSP 90 members are known to often work downstream of HSP 70 members. They play an important role in variant signaling pathways, including cell-cycle progression and apoptosis (McClellan *et al.*, 2007; Taipale, Jarosz and Lindquist, 2010). Another sub-group of chaperones, attracting growing attention, consists of small Heat shock proteins (smHSPs). Characterized by a molecular mass between 12-43 kDa and a special alpha-cystallin domain (Kappé *et al.*, 2003; Kriehuber *et al.*, 2010). Their function is - as well as in other chaperones - enclosed in the chaperone system. They interact with other HSPs in a broad manner and focus on assistance to avoid protein aggregation and amyloid formation (Lee and Vierling, 2000; Mogk *et al.*, 2003; Wilhelmus *et al.*, 2006; Peschek *et al.*, 2013).

The positive effect of the chaperone network to avoid protein aggregation and its toxicity is part of current research in aging and ND models. Studies show beneficial effects of e.g. HSP 70 in a drosophila PD model (et al. 2002), mouse AD model (Hoshino *et al.*, 2011) and in vitro (Chafekar *et al.*, 2012), decreasing protein aggregation.
Also, chaperones seam not only to assist in mechanism to avoid aggregation, but also decrease already built toxic aggregates, through transforming them into less-toxic aggregates (Muchowski *et al.*, 2000; Sittler *et al.*, 2001; Nagy *et al.*, 2016).

In summary, chaperones and co-chaperones are one of the key assistances in the PN. They consist of chaperone families, each with special characteristics and multiple members, working together as a system in order to stabilizes proteins and trying to stop unstable proteins from aggregation.

The proteostasis network

To understand the importance of the PN in maintaining a healthy cell, it is necessary to learn more about proteins in general. Seen in figure 2.3 (A), proteins get transcribed and then translated into a linear chain, composed of amino acids. In order to fulfill their function, they need to be folded in a three-dimensional structure, the so called "native state" (Balchin, Hayer-Hartl and Hartl, 2016; Clausen *et al.*, 2019). The information of their functional three-dimensional structure is recorded in their polypeptide chain (Anfinsen, 1973). Chaperones assist in protein synthesis and stabilize partially folded proteins, preparing them to fold correctly (Morán Luengo *et al.*, 2018). HSP 70 members are an example for key chaperones responsible for a smooth protein synthesis. They bind to client proteins, in cooperation with HSP 90 and co-chaperones like HSP 40 (Wegele, Müller and Buchner, 2004; Morán Luengo *et al.*, 2018).

Under physiological conditions, the protein's native state is the most favorable for its function. But if the protein's structure looses its stability as a consequence of stress or immense metabolic challenges, despite chaperone assistance, it might destabilize and misfold (Hartl and Hayer-Hartl, 2009). Partially folded or misfolded proteins expose hydrophobic structures, normally shielded in the native state, which may lead to protein aggregation (Hartl, Bracher and Hayer-Hartl, 2011; Klaips, Jayaraj and Hartl, 2018b).

The problem is, that protein aggregates loose their primordial function and may exhibit toxic effects on cell mechanisms. Therefore, protein aggregates need to be removed or excluded from the cell. They get either sequestered in well defined localizations in the nucleus and/or cytoplasm, building so cold aggresomes (Antón *et al.*, 1999; García-Mata *et al.,* 1999; Fabunmi *et al.*, 2000), or get degraded. There are two main degradation systems: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system (ALS). The UPS functions through conjugating misfolded or unneeded proteins with ubiquitin (Haas *et al.*, 1982; Hershko *et al.*, 1982, 1983). Ubiquitin marked proteins can then be recognized by proteasomes, which degrade them into their components (Hough, Pratt and Rechsteiner, 1986; Rock *et al.*, 1994). Proteins disposed through the ALS get

enzymatically digested in the lysosome. There are three main pathways, which traffic proteins to lysosomes: (i) via sequestration in an intermediate organelle, the "autophagosome" (= macroautophagy, (Itakura and Mizushima, 2010)), (ii) directly with invagination in the lysosome membrane (= microautophagy, (Ahlberg and Glaumann, 1985; Sahu *et al.*, 2011)) or (iii) chaperone mediated via e.g. HSP 70 and co-chaperones (Chiang *et al.*, 1989; Agarraberes and Dice, 2001). Other mechanisms to avoid aggregation, like down regulating of the translation machinery (Komar, Lesnik and Reiss, 1999; Siller *et al.*, 2010; Spencer *et al.*, 2012), are not closer specified in this text.
If errors in the sequestration or degradation processes occur, aggregates might transform into amyloid. Amyloid formations are aggregates, defined by beta-strands and often built via soluble oligomers. They are toxic for the cell and associated with diseases like ND (Chiti and Dobson, 2006; Klaips, Jayaraj and Hartl, 2018a).

The mechanisms of the PN, described above, have one big purpose in common: to uphold the cell's function. This is accomplished through maintaining a balance in protein synthesis, maintenance and degradation.

2.2.2. Changes of the proteostasis network in aging cells

Aging can be described as a time-dependent functional decline. From a cellular level (e.g. decline in metabolic processes (Lam *et al.*, 2009; Zhou *et al.*, 2009) to the entire human organism (e.g. cognitive decline (Harada, Natelson Love and Triebel, 2013)). One feature of aging and ND is a malfunctioning PN. It is described to decrease in function, leading to protein imbalances and accumulation of aggregates (Taylor and Dillin, 2011a; López-Otín *et al.*, 2013; Labbadia and Morimoto, 2015). Loss of function is described in several PN components, like the degradation machineries (UPS and autophagy (Cuervo and Dice, 2000; Komatsu *et al.*, 2006; Klaips, Jayaraj and Hartl, 2018b)) or the sequestration of aggregated proteins (Erjavec *et al.*, 2007). Thus, misfolded proteins and protein aggregates persist in the aging cell, worsening the PN functionality further (Bence, Sampat and Kopito, 2001; Bennett *et al.*, 2005). When down-regulating the PN, a negative effect on lifespan in flies and worms has been assessed (Tawo et al. 2017). Whereas, an up-regulated UPS system in vitro extends the cell's lifespan (Chondrogianni *et al.*, 2005; Hwang *et al.*, 2007). Those studies imply a strong relationship between a malfunctioning PN and aging processes.

Taking the decrease of functionality in PN components, it is not surprising that the interactions of the whole system become dysfunctional in an aging cell. Easily understood, when comparing stress coping mechanisms of a healthy cell with those of an aging or ND cell (Figure 2.3, B). When stress in an organism occurs, like metabolic changes or errors in protein synthesis, misfolded proteins and unstable intermediates increase in the cell. A healthy cell can cope with an increase of dysfunctional proteins, through stress response pathways. Coping strategies are to decrease protein synthesis (Harding *et al.*, 2000; Hollien *et al.*, 2009; Gardner and Walter, 2011; Shalgi *et al.*, 2013), up-regulating the chaperone system (Ananthan, Goldberg and Voellmy, 1986) or up-regulating degradation systems (Fornace *et al.*, 1989; Shang, Gong and Taylor, 1997).

In aging or ND cells, these stress response pathways malfunction. This leads to a dysbalance of proteostasis with an increase of misfolded proteins and protein aggregation (Poon *et al.*, 2006; Ben-Zvi *et al.*, 2009; David *et al.*, 2010a).
If the changes of the PN are consequences, or the cause of aging processes, and how these mechanisms can be delayed or stopped is part of current research.

There is growing evidence, however, that there is a strong relationship between an aging cell and neuronal degeneration in ND due to similar disruptions in the PN ((Feleciano *et al.*, 2019). For further details, please see reviews: (Saez and Vilchez, 2014; Labbadia and Morimoto, 2015; Klaips, Jayaraj and Hartl, 2018b))

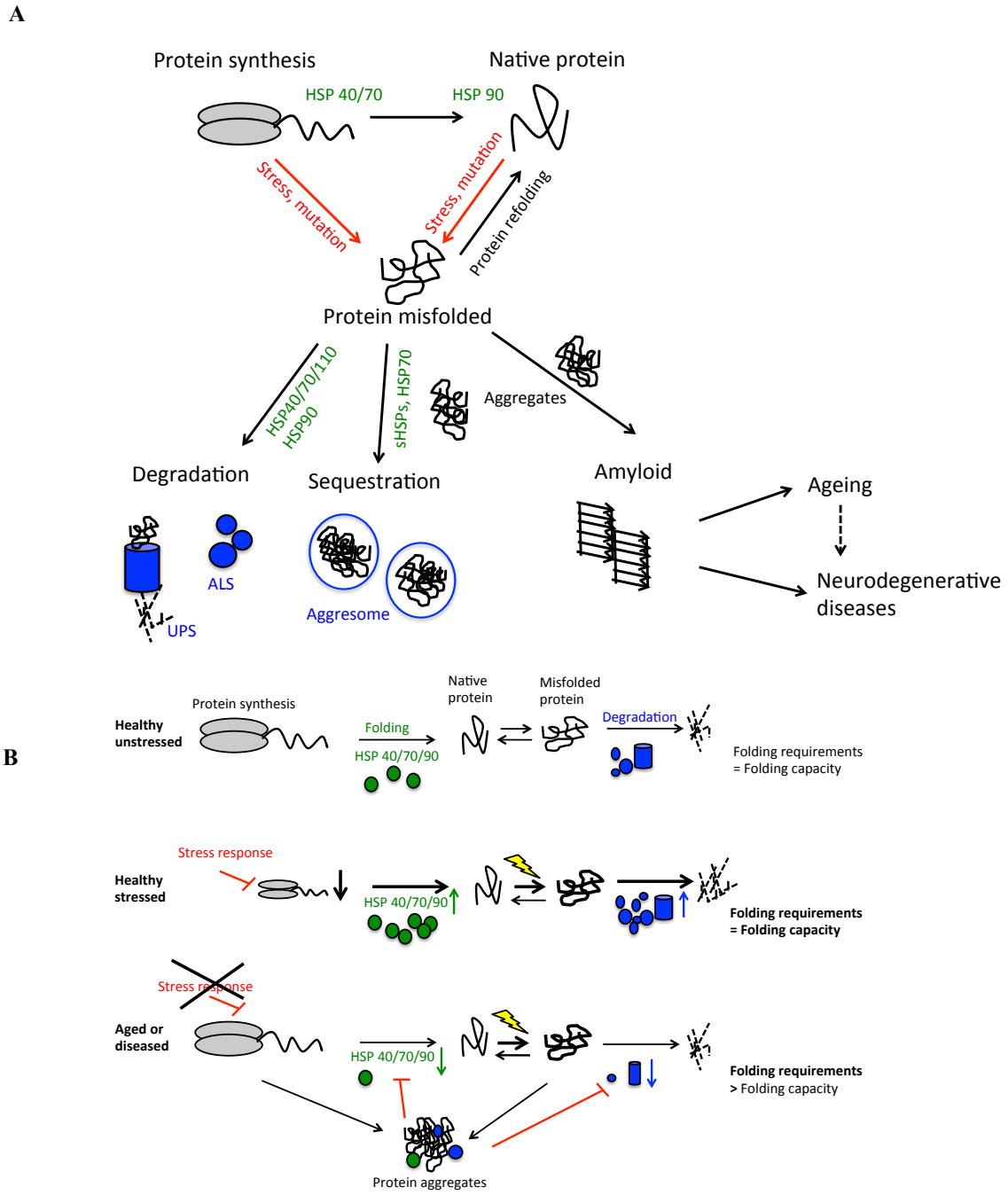


Figure 2.3 Main components of the PN (A). Chaperones and co-chaperones (green) are involved in many processes of the PN. Proteins get synthesized and folded to their native state. If errors occur, proteins might misfold. Misfolded proteins can either get refolded, or if not possible get degraded via the UPS/ALS. If clearing processes fail, misfolded proteins can aggregate and then either get sequestered or accumulate to amyloid, which is associated with ND or/and aging. Native unneeded proteins can also get degraded directly via the UPS or ALS (not shown). **PN in healthy and aged/diseased cells (B).** In healthy unstressed and stressed cells, protein synthesis and clearance is in balance. In healthy stressed cells, a stress response pathway restricts protein translation/transcription, up-regulates chaperones/co-chaperone and degradation systems. Therefore, the cell can cope with stress induced higher amounts of misfolded proteins. In aged or diseased cells, the PN is unbalanced due to an increase of protein aggregates, which influence chaperones/co-chaperones and the degradation systems negatively. The PN capacity is lower than its requirements. Hence, more aggregates accumulate.
Re-drawn after (Hipp, Park and Hartl, 2014; Klaips, Jayaraj and Hartl, 2018a).

2.3. Senescence, an aging cell

3.3.1. Definition of Senescence

Senescence is a special term to describe aging cells or organisms with distinct features. On a cellular level the term cellular senescence is used. An important feature of cellular senescence is irreversible proliferating arrest, due to either replicative telomerase shortening, or stressed induced cell cycle arrest (Hayflick and Moorhead, 1961; Bodnar *et al.*, 1998; Herbig *et al.*, 2004). On the one hand, senescence induction goes along with aging processes and a decline in the cell's functionality. On the other hand, these mechanisms prevent mutant cells from transforming into uncontrolled proliferation and dissemination (Braig *et al.*, 2005).

There are multiple stimuli, which induce senescence. They imply a potential threat for healthy organisms, like oxidative stress (Iwasa, Han and Ishikawa, 2003; Parrinello *et al.*, 2003), oncogene activation (Serrano *et al.*, 1997; Lin *et al.*, 1998) or an impaired autophagy system (Kang *et al.*, 2011). These stimuli mostly end in DNA damage and activate a DNA damage response pathway, which can induce senescence when overstrained (D'Adda di Fagagna *et al.*, 2003; Herbig *et al.*, 2004; Nakamura *et al.*, 2008; Rodier *et al.*, 2011). There are some exceptions, however, like drug-induced senescence with deacetylase inhibitors. They induce senescence through interacting with the histone complex (Ogryzko *et al.*, 1996; Munro *et al.*, 2004). Errors in biological processes and potential senescent triggers increase in an aging cell. Thus, it is nearby that senescent cells rise in aging tissue (e.g. shown in rat and human tissue (Dimri *et al.*, 1995a; Gruber *et al.*, 2007; Geng *et al.*, 2010). Hence, they protect ageing organisms from mutant cells, but also decrease cells' functionality in aging and aging associated diseases (Howcroft *et al.*, 2013).

Depending on tissues and cell types, different markers are used to identify senescent cells. One of the most widespread marker is the senescence associated ß-galactosidase (SA-ß-gal (Dimri *et al.*, 1995b; Lee *et al.*, 2006)). Other commonly used markers are kinase inhibitors, playing a role in cell cycle arrest (e.g. $p14^{INK4a}$ (Krishnamurthy *et al.*, 2004; Burd *et al.*, 2013), or lipofuscin, formations consisting of non-functional and non-degradable oxidized proteins, metals and lipids (Georgakopoulou *et al.*, 2012; Evangelou *et al.*, 2017). Each marker, however, has its errors. Detection of in vitro SA-ß-gal, for example, is described to occur also in some immortal cells, or cells influenced by environmental factors like H_2O_2 oxidation (Severino *et al.*, 2000). Also, the marker's detection depends on cell type and tissue. Lipofuscin can be detected in most of the biological materials, like fresh, frozen or archival cells (Evangelou *et al.*, 2017). SA-ß-gal staining, however, is only applicable in fresh tissue, because of its required enzymatic reaction (Debacq-Chainiaux *et al.*, 2009; Georgakopoulou *et al.*, 2012). A ubiquitous marker is still missing. Thus, it is important to choose each senescent marker carefully, considering cell type and processing.

3.3.2. Features of a senescent cell

The multiple triggers mentioned above induce irreversible growth arrest in cells, the foundation of senescence (Saretzki, 2010). Even though, the compounds of senescent features vary from cell to cell (Wiley *et al.*, 2017) depending on the trigger mechanism and cell type, there are some general similarities.

Beginning with their typical morphology: senescent cells appear bigger in size and are flattened (Saretzki, 2010), due to intracellular changes like cytoskeletal rearrangements (Druelle *et al.*, 2016). Also, senescent cells express different gene patterns than young cells (Linskens *et al.*, 1995; Shelton *et al.*, 1999), including senescent markers like SA-ß-gal (mentioned above (Dimri *et al.*, 1995b)). When a cell undergoes senescence, special stress induced pathways get activated.

They get mostly induced by stress or in response to DNA damage (Brenner, Stampfer and Aldaz, 1998; Robles and Adami, 1998; te Poele *et al.*, 2002). Two major players, assessed in senescent associated pathways, are p53 and p16/INK4a-RB (Harris & Hollstein 1993; Alcorta et al. 1996; Hara et al. 1996; Lin et al. 1998; Jin & Levine 2001; Sharpless et al. 2001). They are key tumor suppressors, carrying out the cell proliferation arrest in senescent cells. In human tissues p53 and p16/INK4a-RB can be detected through immune histochemical methods and has been investigated in aged human kidney samples (Melk *et al.*, 2004), peripheral blood t-cells of elderly (Liu *et al.*, 2009) or human lung epithelial cells (Parker *et al.*, 2008). Last but not least, studies suggest a paracrine and autocrine function of senescent cells. They secrete chemokines (Acosta *et al.*, 2008), matrix metalloproteinases (Coppé *et al.*, 2010) and cytokines (Kuilman *et al.*, 2008). Through secretion of mediators, they e.g. reinforce senescence and create an inflammatory milieu (Acosta *et al.*, 2008; Kuilman *et al.*, 2008), suggesting that they still play a regulating role within the human system.

It is important to note, that senescent cells are not equitable with quiescent or terminally differentiated cells. They undergo a terminal cell cycles arrest, are still metabolic active and express specific markers.

3. Aim of the project

Even though there is evidence that elderly and patients suffering from neurodegenerative-diseases often present with disrupted wake-sleep cycles and activity rhythms, little is known about the underlying causative mechanism.

In order to contribute to understanding the link between circadian rhythms and age-associated pathology, our aim was to assess the effect of temperature rhythms on protein aggregation in a senescent cell model.

1. Our goal was to establish a senescent cell model to assess protein aggregates, an age associated pathology.

2. In the senescent cell model we wanted to investigate the effect of 24h, non-24h temperature cycles and constant temperature condition on protein aggregates, to (i) see if temperature has an effect on protein aggregation and (ii) detect if (24h and non-24h) temperature cycles have a different effect on senescent cells than constant condition.

3. Also, our aim was to find chaperone candidates, which are present in the cells we used, for initial investigations if they are temperature cycle dependent. This will be interesting for future projects to draw a possible causative link between alterations in protein aggregation in cells kept in different temperature conditions.

4. Materials

4.1. Buffers and media for cell culture

Neuro-2A (N2A, ATCC® CCL-131™) and **U2OS** (ATCC® HTB-96™) cell lines were obtained from ATCC®.

4.1.1. Culture medium for routine

Dulbecco's Modified Eagle Medium (Thermo Scientific™ #31966-021), high glucose, and GlutaMAX™, supplemented with 10% fetal bovine serum (FBS, Gibco® Life Technologies) and 0.4% penicillin/streptomycin (10.000 Units/ml Penicillin, 10.000 µg/ml Streptomycin)

Trypsin :
TrypLE™ Express (Gibco® Life Technologies)

4.1.2. Experimental medium for senescence experiments

Dulbecco's Modified Eagle Medium/F12 (Thermo Scientific™ #11039-021) with 2.5 mM L-Glutamine and 15 mM HEPES supplemented with 0.4% penicillin/streptomycin (10.000 Units/ml Penicillin, 10.000 µg/ml Streptomycin)

Experimental medium N2A:
0.5% FBS was added (for U2OS no FBS)

For senescence induction:
15 µl of LBH589 (BioVision USA) per 10 ml of DMEM/F12 medium,
Stock solution: in 15 µM dimethylsulfoxid (DMSO), sterile filtered, kept at -20°C

For control cells:
15 µl DMSO per 10ml experimental medium was added instead of LBH589

4.1.3. Buffers

All buffers were prepared with ultra filtered water (ddH_2O), which was purified by an Astacus (membraPure) system.

Buffers in alphabetic order:

Blocking milk

TBS-T, 5 % Milk powder

Coomassie solution

0.1 % Coomassie brilliant blue R-250, 50 % Methanol, 10 % Glacial acetic acid

Loading buffer 5 x

313 mM Tris pH 6.8, 50 % Glycerol, 10 % Sodium dodecyl sulfate, 0.05 % Bromophenol-blue

Fixation buffer 10 x for b-galactosidase staining, according to The Senescence Cells Histochemical Staining Kit (SIGMA®, #CS0030)

20 % Formaldehyde, 2 % Glutaraldehyde, 70.4 mM Na_2HPO_4, 4.7 mM KH_2PO_4, 1.37 M NaCl, 26.8 mM KCl

PBS

0.01 M Phosphate buffer, 0.0027 M Potassium chloride, 0.137 M Sodium chloride, pH 7.4 (was prepared from Sigma PBS tablets - P4417)

Dulbecco's Phospate Buffered Saline

Without Calcium Chloride and Magnesium Chloride (Gibco™ Life Technologies #14190-144)

Ponceau solution

0.1 % Ponceau S, 5 % Acetic acid

Protease inhibitor

cOmplete™, EDTA-free Protease Inhibitor Cocktail (SIGMA-ALDRICH®, #11873580001) One tablet EDTA-free per 10 ml RIPA buffer or PBS

RIPA buffer

50 mM Tris-HCL (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 % NP-40, 0.5 % Sodium deoxycholat, 0.5 % Sodium dodecyl sulfate

Staining mixture 10 ml for b-galactosidase staining, according to The Senescence Cells Histochemical Staining Kit (SIGMA®, #CS0030)

1 ml of Staining solution 10 x, 125 µl of 400 mM Potassium ferricyanide, 125 µl of 400 mM, Potassium ferrocyanide, 8 mg X-gal solution, 8.5 ml ddH_2O

TBS-T

TBS buffer, 0.1 % Tween 20

Transfer buffer

25 mM Tris, 192 mM Glycine, 20 % Methanol

Tris buffered saline buffer (TBS buffer)

50 mM Tris, 137 mM NaCl, 2.7 mM KCl

Tris - Glycin - SDS running buffer 10 x

250 mM Tris pH 8.3, 1920 mM Glycine, 1 % Sodium dodecyl sulfate

Trypan blue solution

0.4 % Trypan blue solution, 0.81 % Sodium chloride, 0.06 % Potassium phosphate (SIGMA-ALDRICH®)

Urea buffer 2 x

8 M Urea, 2 % Sodium dodecyl sulfate, 50 mM Dithiothreitol, 50 mM Tris pH 8

Wetern Blot substrate

Pierce ^{TM} ECL Western Blotting Substrate

4.2. Gels

4.2.1. Hand cast 10 % Tris-glycine gel for protein aggregation assay (normalization)

Resolving solution (10 %) for two gels

6.6 ml of Acrylamide (AA) 30 %, 5 ml of 1.5 M Tris(hydroxymethyl)aminomethan (Tris) pH 8.8, 200 µl of Sodium dodecyl sulfate (SDS) 10 %, 100 µl of Ammonium persulfate (APS) 10 % , 10 µl of Tetramethylethylenediamine (TEMED), 8.1 ml ddH_2O

Stacking solution (4 %) for two gels

1.32 ml AA 30 %, 2.52 ml 0.5 M Tris pH 6.8, 100 µl SDS 10 %, 10 µl TEMED, 50 µl APS 10 %, 6 ml ddH_2O

4.2.2. Gel for protein aggregation assay (after normalization)

4-15 % Criterion™ TGX Stain-Free™ Precast gels
BioRad®

4.2.3. Hand cast 7.5 % Tris-glycine gel for Western Blot

Resolving solution (7.5 %) for two gels

5 ml of Acrylamide (AA) 30 %, 5 ml of 1.5 M Tris(hydroxymethyl)aminomethan (Tris) pH 8.8, 200 µl of Sodium dodecyl sulfate (SDS) 10 %, 100 µl of

Ammonium persulfate (APS) 10 %, 10 µl of Tetramethylethylenediamine (TEMED), 9.7 ml ddH_2O

Stacking solution (4 %) for two gels

1.32 ml AA 30 %, 2.52 ml 0.5 M Tris pH 6.8, 100 µl SDS 10 %, 10 µl TEMED, 50 µl APS 10 %, 6 ml ddH_2O

4.3. Antibodies

All primary and secondary antibodies (except anti-HSP70 at 4°C) were stored at -20°C. The aliquots of the secondary antibodies in use were stored at 4°C.

4.3.1. Primary antibodies

Alpha B Crystallin Antibody, 3A10.H4, StreeMarq Biosciences $_{INC}$ #SMC-165
Detects ~20 kDa, mouse anti-human Alpha B Crystallin Monoclonal IgG1

GAPDH Antibody, SPC-689, SIGMA-ALDRICH® #G8795
Detects ~36 kDa, rabbit anti-human GAPDH Polyclonal

GRP78 Antibody, StressMarq Biosciences $_{INC}$ #SPC107A
Detects ~ 78kDa, rabbit anti-rat GRP78 Antibody Polyclonal

HSP25/27 Antibody, 8A7, StressMarq Biosciences $_{INC}$ #SMC114
Detects ~25kDa or ~27kDa, mouse anti-human HDP25/27 monoclonal IgG1Kappa

HSP70 Antibody, Santa Cruz Biotechnology #E2313
Detects ~70kDa, mouse monoclonal IgG2a

HSP90 Antibody, 4F3.E8, StressMarq Biosciences $_{INC}$ #SMC149
Detects ~90kDa, mouse anti-human HSP90 (total) monoclonal IgG2a

Vinculin Antibody, V924, SIGMA-ALDRICH® #V9264
Detects ~116kDa, mouse anti-human Vinculin monoclonal IgG1

4.3.2. Secondary antibodies

Anti-mouse IgG (H+L)-HRP Conjugate, BIO RAD® #1706516
Goat anti-rabbit IgG (H+L)-HRP Conjugate, BIO RAD® #170651

5. Methods

5. 1. Cell culture

5.1.1. Cell lines and maintenance

All experiments were performed with Neuro-2A cells (N2A, ATCC® CCL-131™). For the protein aggregation assay U2OS cells (ATCC® HTB-96™) were used additionally. Both cell lines were provided by ATCC®. N2A cells were derived from a neuroblastoma tumor in a strain A albino mouse (Klebe RJ et al., 1969). Due to their tumor origin, they are immortal and are able to proliferate indefinitely. The fact that they are also able to differentiate into neurons within a couple of days, make them a great candidate to study senescent cells; an in vitro model reflecting elderly neurons in the human central nervous system. U2OS is a human osteosarcoma cell line. These cells are commonly used as a model for circadian clock experiments, because of their ability to synchronize to temperature cycles. Therefore, U2OS cells represent a good model to assess proteins in altering temperature conditions.

For routine purposes, cells were maintained in culture medium containing Dulbecco's Modified Eagle Medium (DMEM), high glucose and GlutaMAX^{TM}, supplemented with 10 % fetal bovine serum (FBS) and 0.4 % penicillin/streptomycin. Antibiotics were used to prevent bacterial growth in the cell culture flasks. Incubation took place in 5 % CO_2/ 95 % air atmosphere incubators at 37 °C. For both protein aggregation assay and beta-galactosidase staining, cells were maintained in experimental medium containing Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) with 2.5 mM L-Glutamine, 15 mM HEPES and 0.4 % penicillin/streptomycin.

N2A cells could not be cultured without FBS, therefore 0.5 % FBS was added. For U2OS medium no FBS was required. The incubators used for temperature cycle experiments generated no constant CO_2 levels. In order to maintain equal CO_2 levels in constant and temperature cycle incubators, flasks with closed lids were used for all conditions (T75, Sarstedt).

5.1.2. Subculturing cells

Subculturing cells is important for supporting further growth and passaging cells in order to render biological replicates. When cells become confluent, toxic metabolites accumulate and nutrient concentration is insufficient to maintain cell growth. Cells in an exponential growth phase are under best physiological conditions and in this phase cell death is at its minimum. Hence, it is important to maintain cells in the exponential growth phase (Cell culture basic handbook, gibco® life technologies™).

For maintenance of cell division, cells were split 1:10 every 3 days at 70-80 % confluence. Medium was removed with a Pasteur pipette, the adherent cell monolayer was washed once with Dulbecco's Phospate Buffered Saline (PBS) and incubated with 2 ml of TrypLE™ Express at 37 °C for 7-9 minutes. To make sure all cells detached from the bottom of the flask after incubation with trypsin, the flask was gently tapped and cells were checked under a light microscope. Detached cells appeared round and floated in suspension. To stop the enzyme activity of trypsin, 8 ml of medium was added. To avoid cell clumps and adherent cells, the suspension was pipetted over the bottom of the flask a couple of times. 9 ml cell suspension was removed and discarded. New medium was added to a volume of 10 ml in a T-75 flask.

Cells were transferred back into 5 % CO_2/95 % air atmosphere incubators at 37 °C, where they could attach to the surface of the flask and proliferate again.

5.1.3. Cell counting

To perform experiments under standardized conditions, using the same number of cells and their viability is important. For this aim, trypan blue staining was used to estimate the viable cell number.
A hemocytometer (Improved Neubauer, Baxter Scientific) is a glass slide with different chambers of a defined volume. The chamber was cleaned with 70 % ethanol before use and then a coverslip was attached. In order to make sure that the coverslip is properly attached to the hemocytometer, the presence of Newton's rings at the edges was checked. Adherent cells were first detached with trypsin (TrypLE™) for 7 minutes and suspended with culture medium. The cell suspension was diluted 1:1 with trypan blue and pipetted into one chamber of the hemocytometer. Cells were counted in the 4 corner squares of the chamber and the cell concentration was calculated (Cells/ml = counted cells/4 x dilution factor x 10^4 ml). Only vital cells, not colored by trypan blue, were counted. Dead cells appear dark blue because trypan blue traverses the broken cell wall.

5.2. Senescence induction

5.2.1. Senescence induction

For senescence induction, 15 µl of LBH589 per 10 ml of DMEM/F12 medium was used. LBH589 was provided by BioVision (San Francisco, USA) and was reconstituted in dimethyl sulfoxide (DMSO) to a final concentration of 15 µM. Long-term storage occurred in -70 °C, whereas aliquots for regular use were stored at -20 °C.
As a control, 15 µl of DMSO per 10 ml of DMEM/F12 medium was added instead of LBH589.

5.2.2. Adaption of FBS concentration

Senescent N2A cells were, unlike senescent U2OS cells, not able to survive without FBS. To evaluate the right FBS concentration for senescent N2A cells, $1x10^6$ cells per flask were seeded in closed lid (T75) flasks. At first lids were kept open and cells were incubated in culture medium with 10 % FBS at 37 °C for 2 days.

On day 3, culture medium was aspirated and cells were washed with PBS for 30 seconds before 10 ml experimental medium per flask was added. 15 µl of LBH589 per 10 ml medium for senescence induction and 15 µl DMSO per 10 ml medium for control cells were added. Different FBS concentrations were added to each flask: 0.1 %, 0.5 %, 1 % or 2 % FBS. Lids were closed and cells were incubated at 37 °C and medium (containing DMSO or LBH and the respective FBS concentrations) was replaced every two days. On day 2, 4 and 7 cells were investigated under the light microscope (LEICA ICC50) and 5 pictures per flask were taken with a LEICA ICC50 HD camera (magnification: 10 x). Pictures were analyzed with % Area calculation of ImageJ (an open platform for scientific image analyses), where cell confluence on the pictures was assessed. For every condition (LBH/DMSO and different FBS concentrations) the same HSB color adjustments in Image J were used to color the cells (Hue 0-255, saturation 14-255 and brightness 0-255). The colored cell areas were then calculated via Image J as the "area fraction" of the cells.

5.3. Beta-galactosidase staining

This method was used for its well-known ability to detect senescent cells in vitro and in vivo. Senescence associated beta-galactosidase expression (SA-b-gal) is one of the most utilized biomarker for senescence (Lee *et al.*, 2006). It is defined as the beta-galactosidase activity detected with X-gal staining at pH 6 and originates from the gene encoding lysosomal beta-D-galactosidase GLB1 (b-D-gal). The lysosomal b-D-gal has its maximal activity at pH 4.5 and therefore, only the SA-b-gal is detected with the staining at pH 6. This pH dependence is used in the assay to detect SA-b-gal activity at pH 6 in senescent cells, however not in immortal, quiescent or tumor cells that express b-D-gal (Dimri et al. 1995, Lee et al. 2006).

Staining was only performed with N2A cells. Riccardo Vanzo previously characterized senescence induction in U2OS cells in our lab.

5.3.1. Cell seeding and maintenance

50.000 N2A cells per well were seeded in six well plates in 2 ml standard medium. The number of cells was optimized to ensure at the same time non-confluent dishes and the maximum amount of cells per well. A high confluence would lead to positive results in SA-b-galactosidase staining, independent of the experimental senescence induction.

Cells were kept in culture medium at constant 37 °C condition with CO_2 for two days. After two days, when cells adhered to the flask surface, the culture medium was changed to the experimental medium. 3 µl of LBH589 was added to the experimental medium for inducing senescence. For the control cells 3 µl of DMSO, instead of LBH589, was added to the experimental medium.

FBS was added to both, the control experimental medium (with DMSO) and the senescence-inducted experimental medium (with LBH589). To evaluate the influence of FBS on senescence induction different FBS concentrations were tested. Either 0.5 % FBS or 1 % FBS were added to control and senescence-inducing experimental medium, respectively. For every condition (with LBH589 or DMSO and 0.5 % or 1 % FBS) three biological replicates were performed.

The plate was covered and closed airtight with parafilm to have comparable conditions to closed-lid flasks in the cycling temperature incubators. Cells were kept at 37 °C. Experimental medium (with LBH or DMSO, and different FBS concentrations) was changed every 2-3 days at the same time of the day, in order to minimize any influence on the endogenous circadian system.

5.3.2. Cell staining

On day 0, day 10 and day 15, cells were taken out of the incubator and stained under the fume hood.

The Senescence Cells Histochemical Staining Kit (SIGMA®, #CS0030) was used.

Experimental medium was aspirated and cells were washed twice with 1 ml of 1x PBS per well. The washing solution was carefully removed, to avoid cell detachment. 1.5 ml of 1x fixation buffer was added to each well, and cells were incubated for 6 minutes at room temperature. Fixed cells were washed three times with PBS. 1 ml of staining mixture was added, the plate was sealed with parafilm

to prevent it from drying out and incubated at 37 °C without CO_2 for 14 hours. The absence of CO_2 is important to provide a stable pH for the pH dependent staining process.

Senescent N2A cells appeared blue after the staining (senescent cells: figure 5.1, control cells: figure 5.2.). Dishes were analyzed using a phase contrast microscope at 40x magnification. 40 pictures per dish and in total 120 pictures per biological replicate were captured. The stained cells were counted blind and the percentage of senescent cells was calculated.

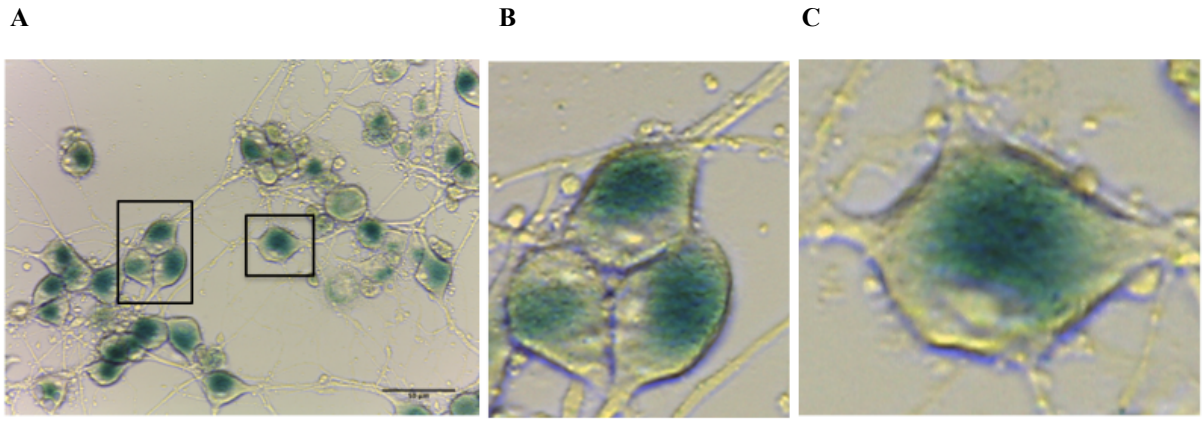


Figure 5.1. Beta-galactosidase staining of senescent N2A cells on day 15, LBH589 medium.
A Magnification: 40 x, Scale bar: 50 µm
B/C Zoom of stained cells of picture A

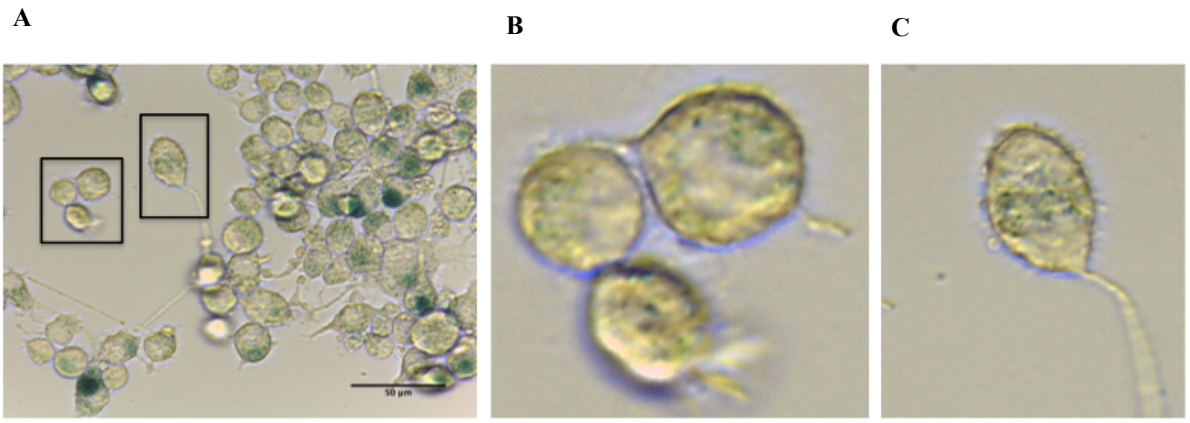


Figure 5.2. Beta-galactosidase staining of control N2A cells on day 15, DMSO medium.
A Magnification: 40 x, Scale bar: 50 µm
B/C Zoom of stained cells of picture A

5.4. Protein aggregation assay

The protocol used by Riccardo Vanzo (Institute of Medical Psychology Munich, now at The Center for Stem Cell Research, University of Copenhagen) for extracting insoluble protein content in U2OS cells (originally established by Della C. David, Department of Biochemistry and Biophysics, University of California, San Francisco, (David *et al.*, 2010a)) was adapted to N2A cells.
Accumulation of insoluble protein in senescent cells, kept at either constant or different cycling temperature conditions, was analyzed. Cells were harvested on day 0, 10 and 15 after senescence induction. Total, soluble and insoluble protein was extracted.
All experiments were performed with a minimum of 3 biological replicates.

5.4.1. Cell culture and senescence induction

Cell concentration was determined using trypan blue (see above). 1x 10^6 cells were seeded in tissue culture flasks (T75) and standard medium was added. U2OS cells were kept for 2 days in 5 % CO_2/95 % air atmosphere incubators at 37 °C. N2A cells were cultured for 3 days, because they needed one more day to attach to the bottom of the flask.
Senescence induction was performed after 2 days (U2OS cells) or 3 days (N2A cells) as described in chapter 5.2. Flasks with closed lids were used in order to maintain the same CO_2 concentration in all conditions. Medium was changed every 2-3 days; but not the day before harvesting to avoid any influence by addition of fresh medium.

5.4.2. Temperature cycles

The senescence-induced cells and control cells were kept in different temperature conditions: 37 °C constant condition and 34/37 °C temperature cycles. For cycling conditions, 24h temperature cycles (12:12 hours) and non-24h temperature cycles (9:9 hours) were used.

5.4.3. Cell harvesting

Cells were harvested on day 0, 4, 10 and 15 by scraping. Compared to trypsin, scraping leads to less membrane protein changes which would influence the total, soluble, and insoluble protein amount.

To stop cell metabolism, the following steps were performed on ice with ice-cold buffers and the centrifuge set to 4 °C. The medium was removed and cells were washed with 10 ml of PBS. After adding 3 ml of PBS with protease inhibitor (PBS+PI), cells were detached by scraping 5-7 times with a cell scraper (25cm, Sarstedt #3280400). Cells of one flask were collected in two 1.5 ml safe lock Eppendorf tubes and were centrifuged at 1000 g for 5 minutes at 4 °C. After removing the supernatant, the tubes containing the cell pellets were placed in liquid nitrogen and transferred to the -80 °C freezer for storage. One cell pellet was used for the protein extraction experiment (see below); the other one was kept for mass spectrometry (see mass spectrometry).

5.4.4. Protein extraction

One cell pellet (in 1.5 ml Eppendorf tube) per condition was transferred from -80 °C to ice for protein extraction. The following procedure was performed on ice with ice-cold buffers (except Urea buffer and the steps after addition of Urea buffer).

600 µl of RIPA buffer with protease inhibitor (RIPA+PI, one tablet of protease inhibitor “EDTA-free” per 10 ml of RIPA buffer) was added to each thawed cell pellet. Cell pellets were resuspended by pipetting 8-10 times per tube. 11.26 µl of 200 mM magnesium chloride per tube was added as a cofactor for DNaseI. For total cell lysis, suspension was pulled through a 22 G needle 10 times to disrupt DNA. 3 µl of DNaseI (recombinant, RNase free, Roche) was added and tubes were incubated on a shaker for 1 hour at 4 °C.

30 µl of each sample were transferred to a new Eppendorf tube, 30 µl of 2 x Urea buffer was added, samples were mixed and incubated at room temperature for 15 minutes. These samples were considered as the total protein content because both soluble and insoluble protein dissolved by adding Urea buffer. Total protein content tubes were frozen in liquid nitrogen and transferred to -80 °C. The residual cell lysate was centrifuged at 20.000 g, 4 °C for 15 minutes to pellet

insoluble protein content. The supernatant was transferred to new Eppendorf tubes (2 x 100µl), placed in liquid nitrogen and stored at -80 °C.

To obtain the insoluble protein content, the remaining supernatant was removed and the pellet was washed with 300 µl of RIPA+PI buffer followed by centrifugation (20.000 g, 15 minutes, 4 °C). This step was repeated two additional times. Then, the U2OS insoluble protein pellet was resuspended with 30 µl of 1x UREA buffer (1:1 RIPA+PI and 2 x UREA buffer, to obtain the same composition as in total protein content samples), whereas the N2A insoluble protein pellet was mixed with 55 µl of 1x Urea buffer. N2A cells yielded more insoluble protein content and a higher buffer volume was required to dissolve the pellet.

The insoluble protein content was mixed and kept at room temperature for 15 minutes, placed in liquid nitrogen and stored at -80 °C.

5.4.5. Gel electrophoresis

First total protein content was normalized across samples. For this, the samples of total protein content were transferred to ice and thawed. The sample buffer was prepared (5 x Protein loading buffer with 12.5 % ß-mercaptoethanol) and 7.5 µl of the sample buffer was added to each of the thawed total protein content tubes. The mixture was vortexed, shortly centrifuged and boiled at 95 °C for 5 minutes in a thermo block (Thermomixer comfort, Eppendorf). Boiled samples were stored at -20 °C and boiled again for 2 minutes when used.

For normalization, 10 % self-made Tris-glycine gels were used (figure 5.3.).

For each gel one spacer plate, one short plate and one comb were cleaned with tap water and 70 % ethanol and dried with Kimtech wipes. The short plate was layered on the spacer plate and arranged in a gel holder. First the solution for the 10 % resolving gel was prepared in a 50 ml tube as described in Materials. Once APS is added, the solution begins to polymerize. The solution was directly pipetted between the plates one-comb length away from the top. A 20 % ethanol layer was pipetted on the gel solution to avoid an uneven gel due to a dried surface. After 20-30 min the gel was polymerized and the ethanol layer was removed with a pipet. The 4 % stacking gel solution was prepared in a 10 ml tube and pipetted on top of the separating gel.

The comb (15 wells) was carefully placed in the solution. Once the stacking part polymerized, the gel was transferred to the gel electrophoresis tank, and the gel holder and tank were filled with 1 x Tris-glycine running buffer. The comb was removed and the wells were washed with running buffer before samples were loaded. 10 µl of total protein sample of each condition were loaded per well. 3 µl of PageRuler™ (prestained protein ladder, 10 to 180 kDa, ThermoScientific™) were used as a marker. Gels ran with 80 V until samples passed the stacking part and continued with 120 V until running front was close to the gel bottom.

After removing gels from the glass plates they were stained with Coomassie blue for 5 hours (on the shaker at room temperature) until gels were uniform blue. For destaining the background, gels were incubated with ddH_2O for 2 hours. DdH_2O was replaced every 15 minutes until bands appeared clear (Figure). Gels were photographed by a ChemiDocTM MP system (BioRad®).

The insoluble protein content samples were prepared after the normalization procedure with the total protein content (see below). Insoluble protein content samples were diluted with RIPA buffer according to the dilution ratio calculated during normalization and mixed with 5x sample buffer as described above. The processing of the insoluble samples was performed as described above.

5.4.6. Normalization

The quantification of the total protein bands was performed with Image Lab Version 4.1, a computer program from BioRad®. To quantify the amount of protein present in each lane, the whole signal of the lane was considered and determined with the Lane and Band Tool.

A dilution factor was calculated in order to load the same amount of total protein for each sample in the following normalized gels. The total and insoluble protein samples were diluted according to the ratio with RIPA buffer using the calculated dilution factor.

4-15 % Criterion™ TGX Stain-Free™ Precast gels were used to load 10 µl of the normalized total and 20 µl of the normalized insoluble protein samples. Gel electrophoresis was performed at 200 V. Compared to self-made gels, 4-15 % gradient Stain-Free™ Precast gels were faster in proceeding, more sensitive for protein quantification and higher reproducibility was secured. After photo activation with UV light, the fluorophores in the gel bind covalently to the protein

molecules and the now fluorescent bands can be detected immediately with a ChemiDoc MP without further staining. To confirm the quantification results Coomassie staining was performed as well.

As all samples were normalized to the total protein amount, relative differences in insoluble protein concentrations could be detected (figure 5.4.) and the insoluble:total protein ratio was calculated.

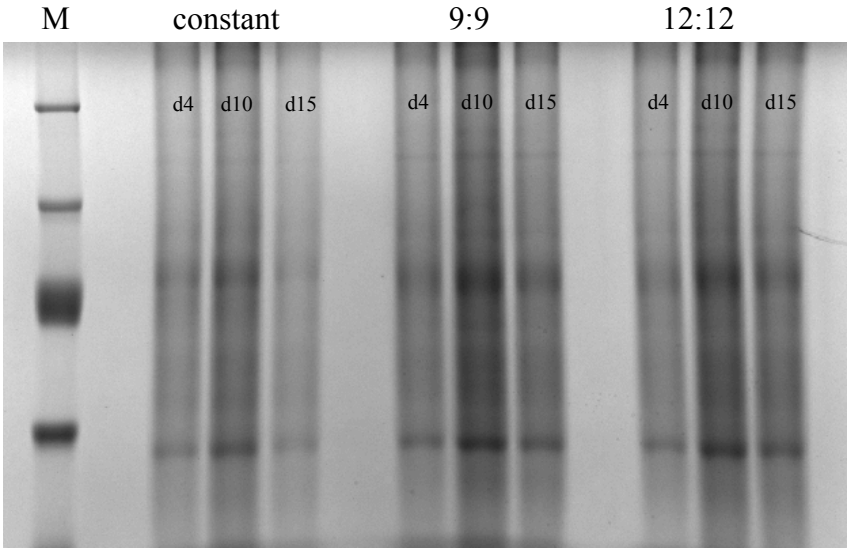


Figure 5.3. Total protein gel from N2A samples (10% hand cast Tris-gycine gel) stained with Coomassie blue for normalization. Cells were treated with LBH589 for 4, 10 and 15 days.
Conditions: constant 37 °C, non-24h temperature cycle 9:9 hours (34 °C/37 °C) and 24h temperature cycle 12:12hours (34 °C/37 °C).

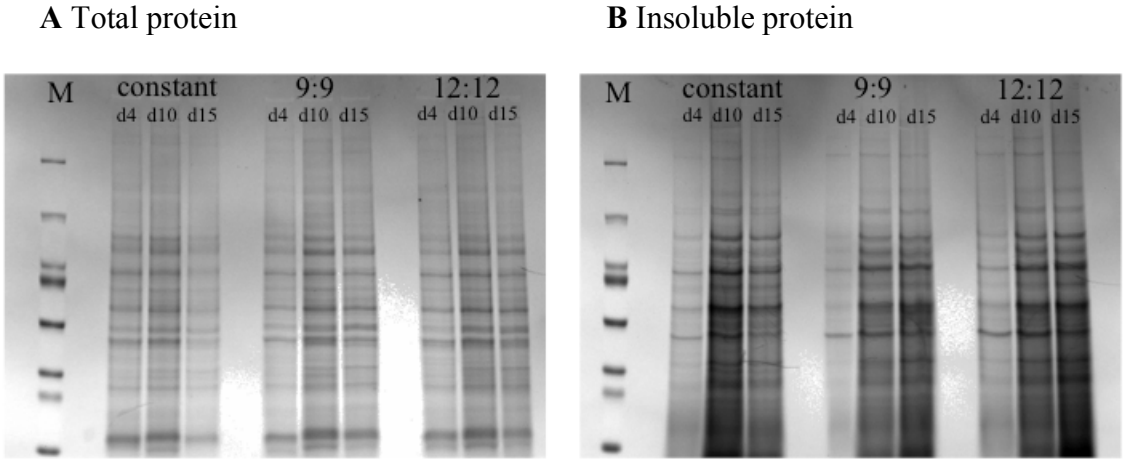


Figure 5.4. Protein gels from N2A samples (Criterion™ TGX Stain-Free™ Precast gels BioRad®) stained with Coomassie blue after normalization. Cells were treated with LBH589 for 4, 10 and 15 days. Conditions: constant 37 °C, non-24h temperature cycle 9:9 hours (34 °C/37 °C) and 24h temperature cycle 12:12 hours (34 °C/37 °C).
A Total protein gels of N2A cells treated with LBH589 after normalization.
B Insoluble protein gels of N2A cells treated with LBH589 after normalization.

5.5. Western Blot

Western Blot is a method to detect the relative amount of specific proteins indirectly through antibodies.

Cells were harvested following a time course protocol, which is used to evaluate the rhythms of different components in a cell during 24 hours. After harvesting cells, proteins were extracted and gel electrophoresis was performed. Proteins were blotted on a membrane, where specific protein bands were detected through antibodies.

5.5.1. Time course

1 x 10^6 N2A cells were seeded in T75 flasks with closed lids kept in culture medium and were entrained to a 12:12 hour (34/37 °C) temperature cycle for three days. The concept is to harvest cells every 4 hours for 24 hours (Figure 5.5). To avoid having to harvest through the night, a time course protocol was used where cells were seeded 12 hours apart and kept in two different incubators with temperature cycles shifted by 12 hours. Cells were then harvested every 4 hours for 12 hours (Figure 5.6).

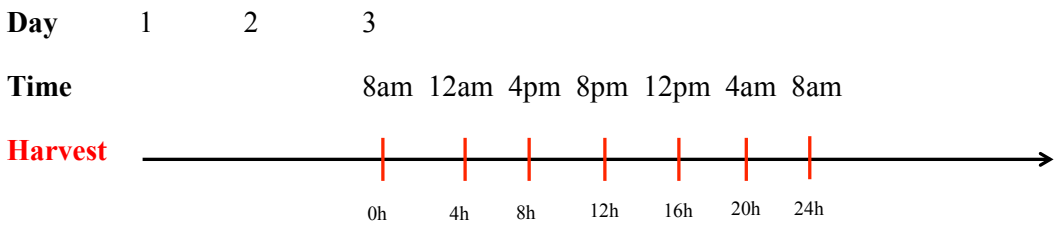
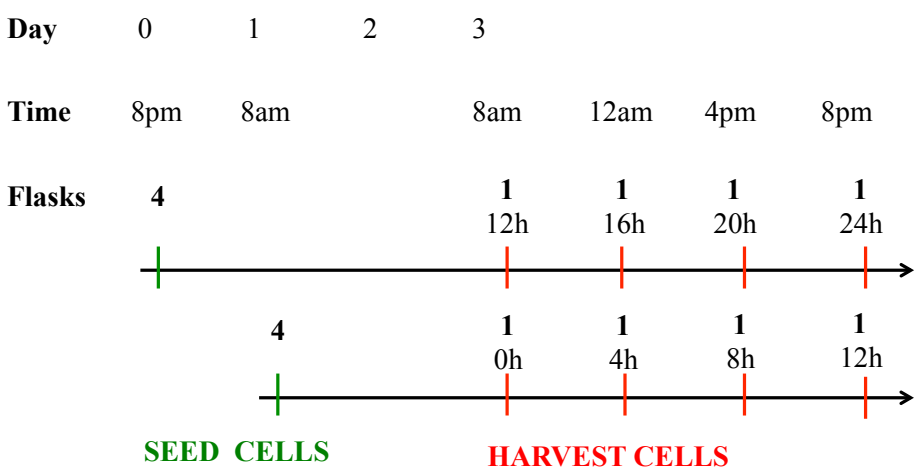


Figure 5.5 Concept of 24 hour time course: Harvest cells every 4 hours.

Figure 5.6. Concept of 24 hour time course, experimental realization with two temperature cycle



incubators (12 hours shifted) in order to avoid harvesting through the night. 1 x10^6 N2A cells per flask were seeded.

Day 0: cells were seeded in 4 flasks and put into incubator 1
Day 1: cells were seeded in 4 flasks and put into incubator 2.
Day 3: cells were harvested every four hours
Temperature cycles 12:12 hours (34 °C/37 °C). Same setting with constant condition 37 °C.

5.5.2. Protein extraction

All following steps were performed on ice. Cells were harvested as described before (chapter 5.4.3.) and cell pellets were kept at -80 °C.

After completing the time course, all cell pellets (in Eppendorf tubes) were processed together, in order to avoid processing differences. 600 µl of RIPA+PI and 11,26 µl of 200 mM magnesium chloride were added to each tube. Samples were pulled through a 22 G needle 10 times. Then, 3 µl of DNAseI were added to each tube (see protein aggregation assay). Because cells didn't undergo senescence, the amount of cells and DNA per tube was higher which leaded to viscous samples. In order to reduce viscosity, DNA was fragmented using sonication before samples were put on the shaker for one hour.

Sonication was performed with a diagenode Bioruptor®. 4 °C cold water was filled in the water tank, because the ultrasound waves produced by Bioruptor® generate heat. Therefore, 4 °C water guaranteed the preservation of the protein samples and prevented the damage of the instruments (Bioruptor® manual 2.1).

12 seconds short ultrasound wave pulses were used ("ON"). 48 seconds in between the pulses were necessary to re-establish a low temperature ("OFF"). After 10 minutes, 4 °C water was replaced and pulses were repeated for another 10 minutes. To complete the cell lysis, samples were transferred to a shaker for 1 hour at 4 °C. For Western Blot, only total protein was analyzed and samples were directly placed in liquid nitrogen and stored at -80 °C for further investigation.

5.5.3. Determination of protein concentration

Tubes with cell lysate were set on ice until thawed.

Protein concentrations were measured with the bicinchoninic acid assay (BCA, Pierce/Thermo Scientific), which quantifies the total protein amount. It works through the reduction of Cu^{+2} to Cu^{+1} by proteins. Cu^{+1} can then be detected colorimetrically at 562nm by a reagent containing bicinchoninic acid (Smith *et al.*, 1985).

Albumin (BSA) standards were prepared by dilution with RIPA buffer. The working reagent is an alkaline medium allowing the reduction of Cu^{+2} (biuret reaction) and contains bicinchoninic acid for the detection of Cu^{+1}. Before every BCA, working reagent was prepared containing 50 parts of Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tertrate in 0.1M sodium hydroxide) and one part of Reagent B (4% cupric sulfate).

In each well of a 96 well plate, 200 µl of working reagent were pipetted. 10 µl of Albumin standard or prepared sample were added. For standards and undiluted samples, three technical replicates were prepared to reduce variation. The plate was gently mixed on a shaker for 30 seconds before incubation at 37 °C for 30 minutes. For the colorimetric detection a Multiscan FC (Thermo Scientific^{TM}) with Multiscan software FC 3.1 was used. The concentrations were calculated relatively to the concentrations of the Albumin standards.

5.5.4. Tank Blotting

The sample preparation for gel electrophoresis was performed as described in the protein aggregation assay. 7.5 % self-made gels were used, 10 µl of protein samples were loaded per well and electrophoresed at 80 V through the stacking part, and at 120 V through the resolving part.

In the meantime, transfer buffer was prepared; 25 mM Tris and 192 mM glycine were diluted with ddH_2O and mixed with 20 % methanol. 4 heavy paper squares and 1 blotting membrane per gel were cut in 8 x 6 centimeter squares to fit the gel.

Gel, heavy paper squares, blotting membrane and two blotting pads were saturated in transfer buffer and piled into the blotting frame as follows: transparent side of blotting frame, blotting pad, two heavy paper squares, blotting membrane, gel, two heavy paper squares, blotting pad, black side of blotting frame. Air bubbles were squeezed out with a small role in order to avoid artifacts on the membrane.

The frame was inserted into a tank and filled with transfer buffer. A stir bar to maintain continuous mixture of transfer buffer and a cool pad to maintain stable 4 °C temperature from the beginning was added.

Tank blotting took place at 4 °C and was performed at 90 mA for 15 hours.

5.5.5. Ponceau staining

Ponceau staining was performed in order to verify transfer of proteins to the membrane. The membrane was rinsed in ddH_2O and stained in Ponceau for 1 minute at room temperature. Then, the membrane was rinsed 5 times in ddH_2O until the background was white and pictures were taken with a geldoc.

5.5.6. Immunolabeling

In order to remove SDS from the membrane, it was rinsed with TBS-T. The membrane was blocked with 5 % milk in TBS-T at room temperature for 1 hour. The primary antibody was added with its assessed dilution (see materials) to 10 ml of the milk/TBS-T mixture and incubated for 1 hour at room temperature. The membrane was washed with TBS-T 3 times for 10 minutes and the secondary antibody (1:5000) in milk/TBS-T was added.

Depending on the primary antibody, anti-mouse or anti-rabbit antibody was used as secondary antibody. It was incubated also for 1 hour at room temperature and washed with TBS-T 3 times for 10 minutes. Finally, the membrane was rinsed once with TBS without Tween and 1 ml of ECL Western Blotting Substrate Pierce ™ (500 µl reagent 1, 500 µl reagent 2) was pipetted on the membrane. The blot was kept in plastic foil to prevent it from drying out and then transferred to the Chemidoc (BioRad®).
Bands were detected and analyzed with Image Lab (BioRad®, see protein aggregation assay).

5.6. Heat shock experiment

A heat shock experiment was performed in order to evaluate if heat shock proteins can be detected in N2A cells and whether their protein levels increase when cells underwent stress through high temperature.
The protocol was adapted from K. Kaarniranta et al. (K. Kaarniranta *et al.*, 2002).
To induce heat shock, N2A cells and U2OS cells in sub-confluent flasks were stimulated at 43 °C for 15 minutes in an incubator. For cell recovery, flasks were transferred to a 37 °C incubator for 360 minutes. The control N2A cells were kept in constant 37 °C.
Cells were harvested, total protein was extracted and BCA, gel electrophoresis and western blot was performed as described above (see western blot). For gel electrophoresis a 10 % self-made gel was used. 20 µg of U2OS total protein sample and 45 µg of N2A total protein sample were loaded.

5.7. Statistical analyses

All statistics were performed using Prism version 7 (GraphPad Software, Inc.).
The primary outcomes were percentage of senescent cells (b-galactosidase staining) and the ratio of insoluble to total protein content in cells (protein aggregation assay). Triplicates were analyzed with 1-way or 2-way analysis of variance (ANOVA) and Tukey´s posthoc-test. P values less than $p < 0.05$ were considered as significant.

6. Results

6.1. Establishing a model for senescence-induced aggregation in neuroblastoma cells

Cellular senescence can be defined as an irreversible growth arrest of cells in the G1 cell cycle phase (Saretzki, 2010). There are several triggers to induce senescence in proliferating cells, including DNA damages via oxidative stress and cytotoxic drugs (Chang *et al.*, 2002). Through the DNA damage the cells loose their ability to proliferate and the aging process occurs.

I used the cytotoxic drug LBH589 (Panobinostat, Novartis Pharmaceuticals) to trigger senescence in cultured N2A cells. LBH589 functions as a histone deacetylase inhibitor, which supports acetylated histones (Manal *et al.*, 2016). Through acetylation, histones appear with less positive charge density. This leads to looser interaction between the negatively charged DNA and the now less positively charged histones. The DNA strains unfold, which leads to abnormal transcription of important cell cycle regulators (Manal *et al.*, 2016) and triggers the cell to undergo senescence (described in osteosarcoma cells, (Cain *et al.*, 2013)). The process of inducing senescence can be disrupted, however, by many factors. In cell culture one of these important influencing factors is the FBS concentration in the cell culture medium. FBS withdrawal can lead to cell death (Evangelopoulos, Weis and Krüttgen, 2005), where as a high FBS concentration in the medium can reduce the efficiency of senescence induction through proliferating pathways (Kalka and Hoyer, 1998).

To optimize the amount of senescent N2A cells per flask I therefore needed to adapt the FBS concentration in the media to allow for senescence induction on the one hand, without inducing too much cell death on the other hand. I assessed the influence of different FBS concentrations in cell medium on the cell survival and on the senescence induction.

For the cell survival I calculated the cell confluence and then used a b-galactosidase staining protocol to assess the amount of senescent cells.

6.1.1. Experimental medium supplemented with FBS is required for N2A cell survival

Senescent N2A cells, unlike senescent U2OS cells, survive no longer than four days with serum withdrawal (Evangelopoulos, Weis and Krüttgen, 2005). Because we required a senescent N2A cell model for more than four days for the following experiments, the right FBS concentration for the experimental medium had to be investigated: an FBS concentration (i) as low as possible in order to cause the least influence on the senescence induction and (ii) high enough for N2A survival. It is known that after seven days of inducing U2OS cells with serum withdrawal and addition of low levels of LBH589, cells undergo senescence (Cain *et al.*, 2013). Therefore, our aim was to obtain a sub-confluent cell culture flask on day 7. Senescence was induced with LBH589 and the confluence of N2A cells with different FBS concentrations was assessed on day 2, 4 and 7 after senescence induction.

Both the confluence of senescent N2A cells (LBH589) and of control N2A cells (DMSO) with each FBS condition (0.1 %, 0.5 %, 1 % and 2 % FBS) was analyzed with ImageJ, an open platform for scientific image analyses. The mean % area (area covered with cells) of all conditions was calculated from five representative pictures of one flask per condition. Only one biological replicate was done for this experiment, as it was just a preliminary test before assessing the effect of FBS on senescence in more detail.

The confluence both in LBH589-induced N2A cells and in DMSO control N2A cells significantly increased ($p < 0.0001$, two-way ANOVA, Tukey's post hoc multiple comparisons) in media containing FBS concentrations of 0.5 %, 1 % and 2 % from day 2 to day 7 (figure 6.1.). Cells incubated in medium with 0.1 % FBS, however, did not increase significantly in confluence: the mean % area remained below 15 % in both DMSO and LBH589 N2A cells (figure 6.1, A and B).

In addition, the confluence of LBH589- induced N2A cells declined on day 4 compared to day 2 in every FBS concentration. This reduction did not occur in control DMSO cells.

I found that a concentration of 0.5 % FBS and 1 % FBS is sufficient to have a significant and sufficient increase of N2A confluence from day 2 to day 7 ($p < 0.0001$, two-way ANOVA, Tukey's post hoc multiple comparisons). Therefore, I further assessed the influence of these two FBS concentrations on the senescence induction of N2A cells (chapter 6.1.2.).

Confluence of N2A cells during senescence induction

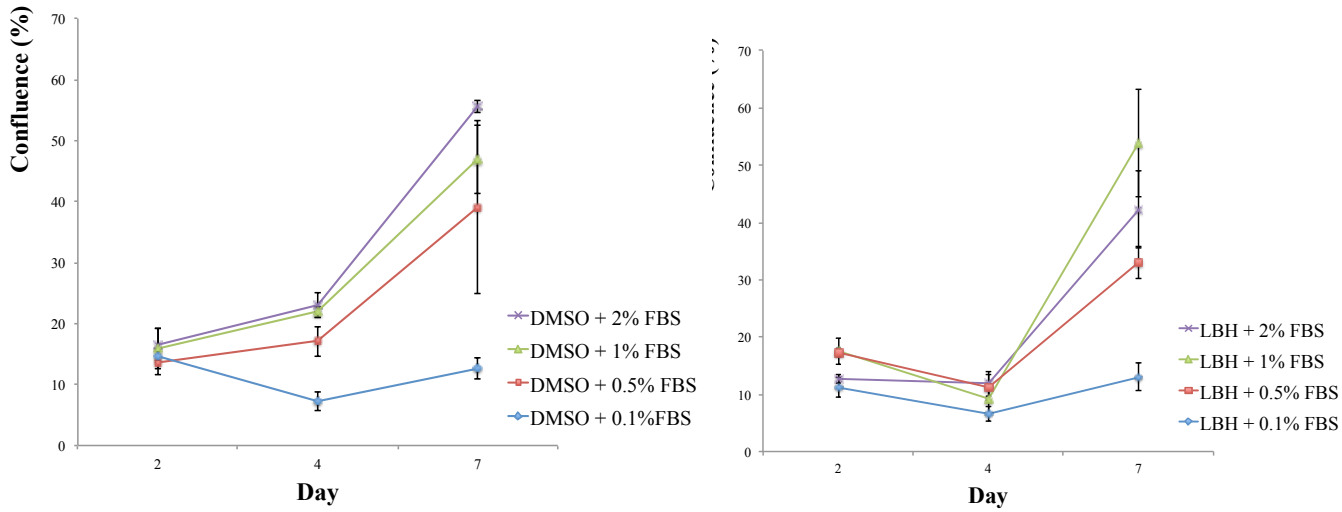


Figure 6.1. Confluence of N2A cells treated with DMSO (**A**) or LBH589 (**B**) and different FBS concentrations (0.1 %, 0.5 %, 1 %, 2 %) on days 2, 4 and 7 after senescence induction.
Five representative pictures of each flask were taken, the confluence (%) was assessed with ImageJ, data mean centered and expressed as mean ± SD.

Two-way ANOVA:
DMSO and LBH589, day 2, 4 and 7:
Interaction $p < 0.0001$(****), Time $p < 0.0001$(****), Condition $p < 0.0001$(****)

Tukey's post hoc multiple comparisons:

DMSO:	day 2 vs. day 7	day 4 vs. day 7
0.1 % FBS	ns	ns
0.5 % FBS	$p < 0.0001$(****)	$p < 0.0001$(****)
1 % FBS	$p < 0.0001$(****)	$p < 0.0001$(****)
2% FBS	$p < 0.0001$(****)	$p < 0.0001$(****)

LBH589	day 2 vs. day 4	day 2 vs. day 7	day 4 vs. day 7
0.1 % FBS	ns	ns	$p = 0.0210$(*)
0.5 % FBS	$p = 0.0337$(*)	$p < 0.0001$(****)	$p < 0.0001$(****)
1 % FBS	$p = 0.0027$(**)	$p < 0.0001$(****)	$p < 0.0001$(****)
2 % FBS	ns	$p < 0.0001$(****)	$p < 0.0001$(****)

6.1.2. The FBS concentration in the experimental medium influences senescence induction

It is already known that certain cell types differentiate in serum-free cultures, such as human embryonic stem cells into dopaminergic neurons (Schulz *et al.*, 2004) or neuronal stem cells and neuroblastoma cells into post mitotic neurons (Seeds *et al.*, 1970; Conti *et al.*, 2001). Some of the mechanisms behind this effect are understood: serum factors, such as high-density lipoproteins (HDL) block N2A differentiation and serum-free media induced N2A differentiation is associated with phosphorylation of epidermal growth factor receptor (EGFR) and other molecular signaling pathways (Evangelopoulos, Weis and Krüttgen, 2005). In this project, we combined reduction of serum with addition of the senescence-inducing histone-deacetylase inhibitor LBH. The influence of serum deprivation on senescence induction is not yet understood. Therefore, we assessed the influence of two different FBS concentrations (0.5 % and 1 %) and LBH589 on the senescence induction of N2A cells.

Senescence in N2A cells was analyzed with a b-galactosidase staining (see methods, chapter 5.3.). The senescence-associated b-galactosidase activity can be detected in cells undergoing senescence. Senescent cells are stained blue using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Senescence Cells Histochemical Staining Kit, SIGMA®) and are considered positive. The percentage of senescent cells was estimated, analyzing 120 pictures of each condition. On the pictures (magnification 40 x) positive-stained cells were counted blind and analyzed with two-way ANOVA (Prism version 7).
The morphology of senescent N2A cells changed clearly in comparison to control cells. They appeared bigger in size and developed more cell protuberances (figure 6.2.).

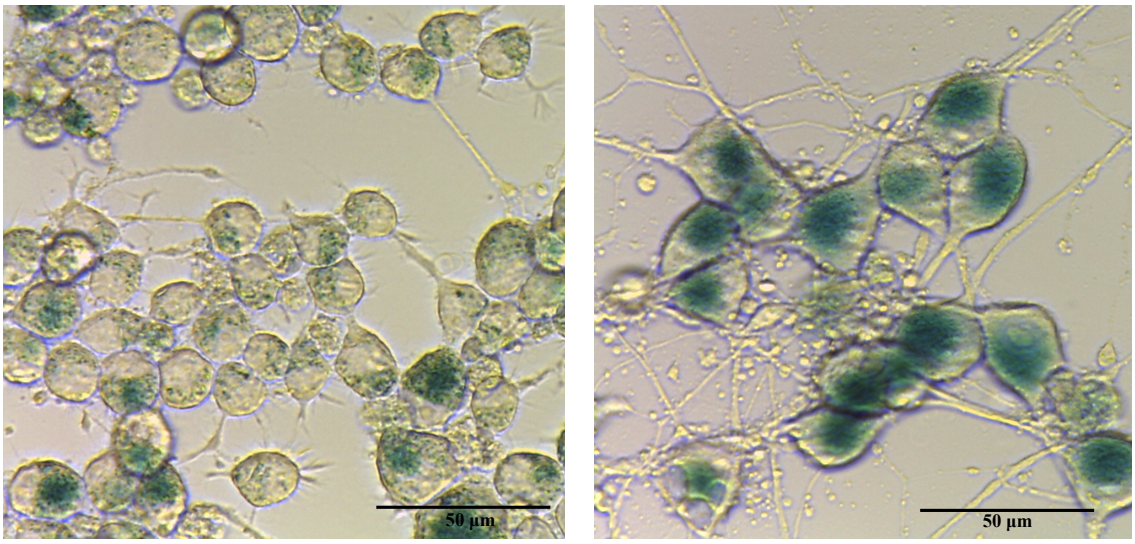


Figure 6.2. Beta-galactosidase staining of N2A cells. Picture of control N2A cells (A) and LBH589 induced N2A cells (**B**) stained with b-galactosidase staining on day 15. Media contained 0.5 % FBS. Magnification: 40 x, Scale bar: 50 µm.

We observed that more N2A cells underwent senescence with lower FBS concentrations (0.5 % FBS) than with a higher FBS concentration (1 % FBS) on days 4, 10 and 15 (all with addition of LBH589). In figure 6.3, the percentage of senescent N2A cells in 0.5 % or 1 % FBS media on day 0 and day 15 are depicted. On day 15, the percentage of senescent cell concentration was 83 % ± 7 % in the 0.5 % FBS medium and 73 % ± 8 % in the 1 % FBS medium compared to 4 % ± 1 % on day 0. On day 10, there were 70 % ± 20 % senescent cells in the 0.5 % FBS medium, in comparison with only 62 % ± 17 % senescent cells in the medium containing 1 % FBS (chapter 6.1.3.). Senescence also occurred earlier in some of the cells kept in the 0.5 % FBS medium (day 0 to day 4: 4 % ± 1 % to 45 % ± 29 % senescent cells, whereas the amount of senescent cells in 1 % FBS medium only increased from 4 % ± 1 % to 31 % ± 12 %, data see chapter 6.1.3.9).

Percentage of senescent N2A cells

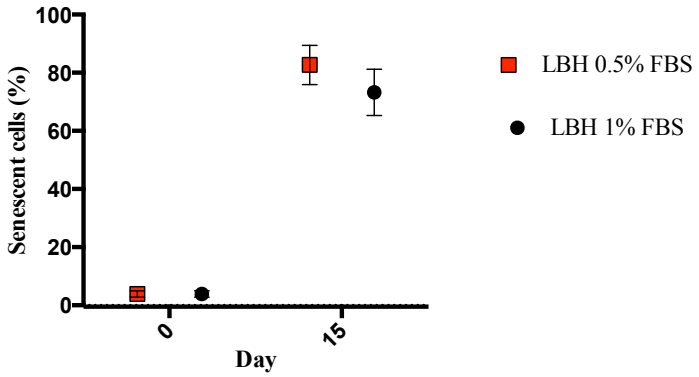


Figure 6.3. Comparison of senescent N2A cells (%) kept in medium with 0.5 % or 1 % FBS on day 0 and day 15. Senescence was induced with LBH589 and assessed with a b-galactosidase staining. Three biological replicates, each with two technical replicates.

6.1.3. LBH589 induces senescence in N2A cells

To estimate the effect of LBH589 on inducing senescence in N2A cells obtained in different FBS media, we used DMSO medium as a control and assessed the percentage of cells undergoing senescence with the b-galactosidase staining on day 0, 4, 10 and 15 (3 biological replicates, each with two technical replicates).
We observed a significant increase in senescent N2A cells from day 0 to day 15 in every condition, 0.5 % or 1 % FBS and DMSO/LBH589-induced cells (figure 6.4/B, 6.5/B). Interestingly, not only cells incubated in LBH589 but also control cells partially underwent senescence. This emphasizes once more (chapter 6.1.2.) that FBS deprivation plays a role in senescence induction. Nevertheless, more LBH589-induced cells underwent senescence (when looking at both 0.5 % FBS and 1 % FBS media) compared to control cells.

When looking at the 0.5 % FBS condition (figure 6.4.) there was a significant increase in senescent N2A cells induced with LBH589 from day 0 to day 15. In DMSO control cells, however, there was only a trend in the increase of senescent cells.

Senescent cells already occurred on day 4; 28 % of the cells were senescent in the DMSO medium, whereas 45 % were senescent in the LBH589 medium. On day 10, there was an average of 43 % senescent cells in the DMSO medium and 70 % in the LBH589 medium. On the last day of the experiment (day 15), 59 % of control cells and 83 % of LBH589-induced cells underwent senescence.

In the 1 % FBS condition (figure 6.5.), the difference in senescence between control cells and LBH589-induced cells was not as striking as in the 0.5 % FBS condition. Nevertheless, in each independent experiment, there was an increase in senescent cells kept in LBH589 medium compared to control cells (figure 6.5/C, one outliner with a decrease in senescent cells on day 15). A different initial cell number in each biological replicate may have contributed to this inconsistency of senescent cells throughout the replicates. In the medium containing DMSO, the average of senescent cells increased from 17 % ± 10 % on day 4 to 49 % ± 21 % on day 10 and 66 % ± 15 % on day 15. Whereas senescent cells incubated in LBH589 increased from 31 % ± 12 % senescent cells on day 4 to 62 % ± 17 % on day 10 and 73 % ± 8 % on day 15.

We concluded that the FBS concentration influences the senescence induction via LBH589 in N2A cells. The 1 % FBS containing medium decreased the effect of LBH589 on senescence induction. More control cells underwent senescence in the 1 % FBS medium than in the 0.5 % FBS medium. Due to higher FBS levels the proliferation of N2A cells might increase in DMSO medium, leading to cell overgrowth and cellular stress. Higher cellular stress induces senescence (Lloyd, 2002). Our goal, however, was to reduce N2A proliferation and its effect of senescence induction. Therefore, we used the lower FBS concentration (0.5 % FBS) in the experimental medium.

LBH-treatment increases the percentage of senescent N2A cells in 0.5 % FBS medium

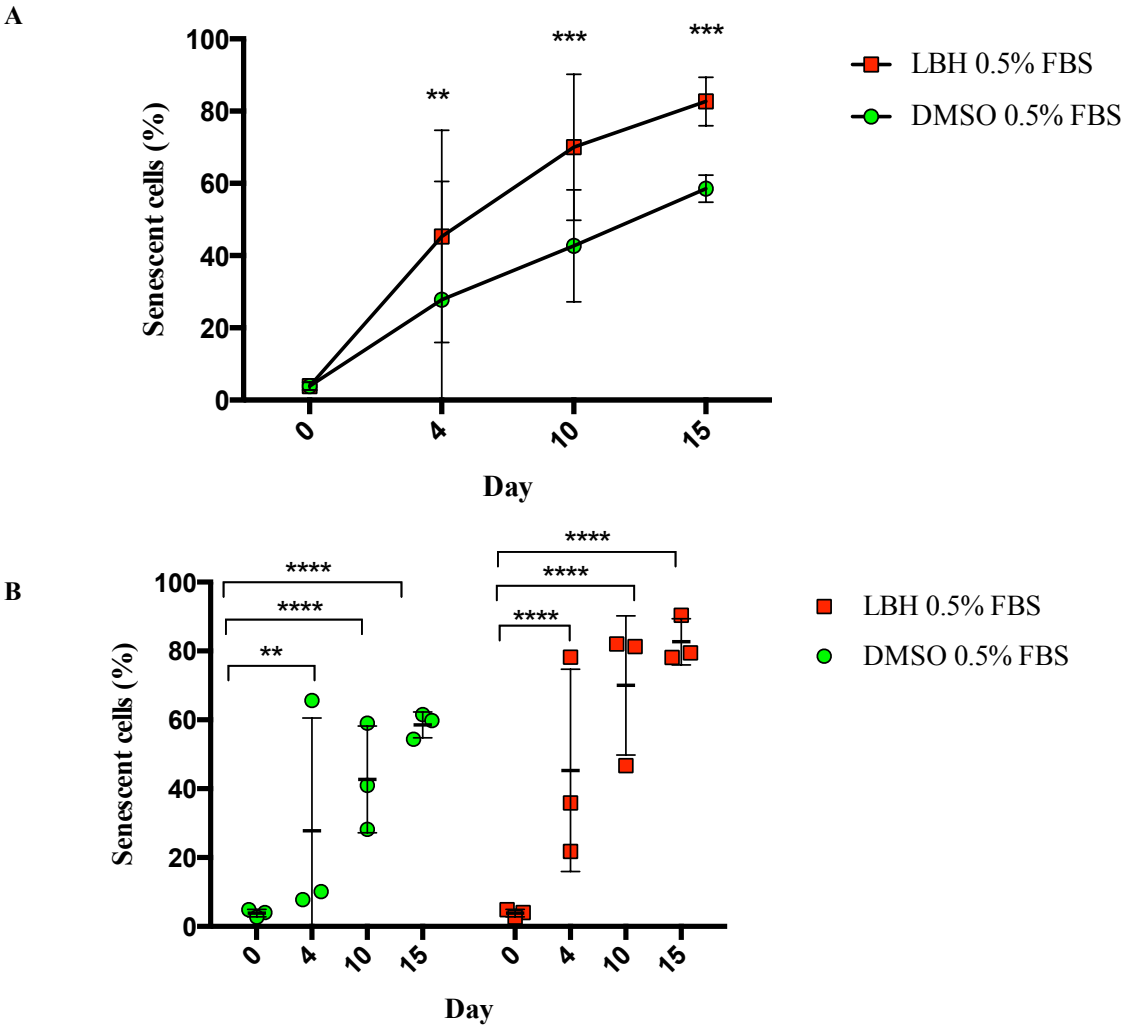


Figure 6.4. Beta-galactosidase staining of N2A cells treated with LBH589 versus cells treated with DMSO, on days 0, 4, 10 and 15. Cells were kept in constant condition (37 °C). Medium contained 0.5 % FBS. 3 biological replicates, each with 2 technical replicates

A : Differences between conditions on distinct day.
B : Differences per condition over days.

Two-way ANOVA:
Time p = 0.0099(**), Condition p = 0.356(*)

Sidak's post hoc multiple comparisons:

B	**DMSO 0.5 % FBS**	**LBH 0.5 % FBS**
Day 0 vs. day 4	p = 0.0012(**)	p < 0.0001(****)
Day 0 vs. day 10	p < 0.0001(****)	p < 0.0001(****)
Day 0 vs. day 15	p < 0.0001(****),	p < 0.0001(****)
Day 4 vs. day 10	p = 0.0143(*)	p = 0.001(***)
Day 4 vs. day 15	p = 0.0003(***)	p < 0.0001(****)
Day 10 vs. day 15	p = 0.0105(*)	p = 0.031(*)

1 % FBS medium reduces senescence induction of N2A cells

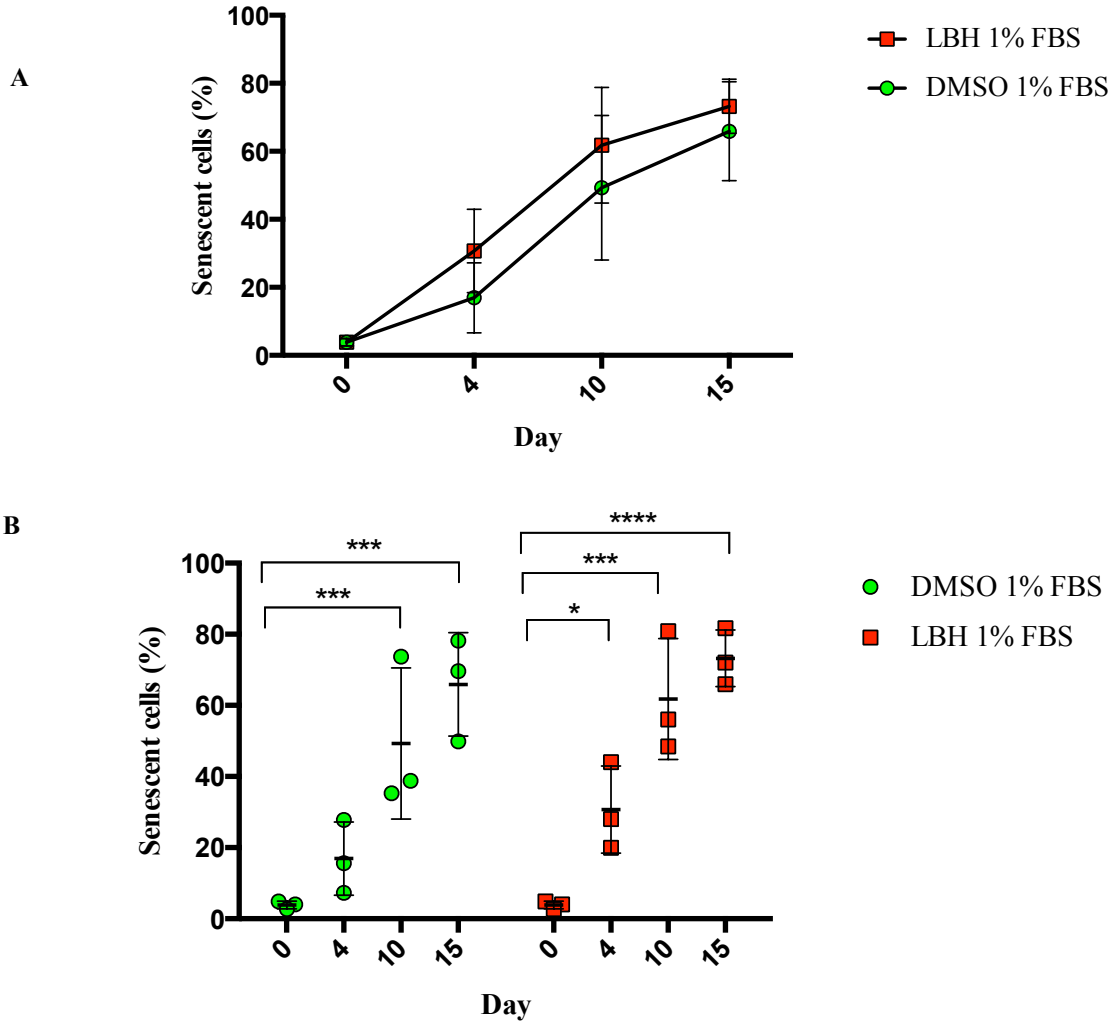


Figure 6.5. Beta-galactosidase staining of N2A cells treated with LBH589 versus cells treated with DMSO, on days 0, 4, 10 and 15. Cells were kept in constant condition (37 °C). Medium contained 1% FBS. Three biological replicates, each with two technical replicates

A : Differences between conditions on distinct day.
B : Differences per condition over days.

Two-way ANOVA:
Time p = 0.0017(**)

Sidak's post hoc multiple comparisons:

B	**DMSO 1 % FBS**	**LBH 1 % FBS**
Day 0 vs. day 4	ns	p = 0.0156(*)
Day 0 vs. day 10	p = 0.001(***)	p = 0.0002(***)
Day 0 vs. day 15	p = 0.0002	p < 0.0001(****)
Day 4 vs. day 10	p = 0.006(**)	p = 0.0074(**)
Day 4 vs. day 15	p = 0.0006(***)	p = 0.0014(**)
Day 10 vs. day 15	ns	ns

6.2. Temperature entrainment and Proteostasis

6.2.1. The insoluble protein content increases in senescent N2A cells

Many studies suggest that the decline of protein homeostasis (proteostasis) plays an important role in aging (Ben-Zvi *et al.*, 2009; David *et al.*, 2010a). To understand the influence of aging on the insoluble protein content, one "output" of the proteostasis machinery, we extracted proteins from senescent-induced N2A cells. First we needed to assess if there is an increase in insoluble protein content in N2A cells undergoing senescence. Second cells were harvested every 2-3 days in order to define the days with the highest increase of insoluble protein content. These days were later used to harvest cells kept in different temperature conditions and evaluate the influence of the circadian clock on insoluble protein content (chapter 6.1.3.).

Senescence was induced with LBH589 for 15 days and cells were harvested and lysed every two to three days. Insoluble and soluble protein was extracted, and the amount measured by gel electrophoresis and Coomassie staining. The ratio of insoluble - to total - protein content was calculated as described in methods (chapter 5.4.6.). This ratio does not correspond to the actual ratio of insoluble to total protein but is an arbitrary ratio (as described in chapter 5.4.6 Normalization, methods). To measure the actual ratio the exact amount of protein would have to be determined, a method that was not available to us. Senescent N2A cells contained a clearly visible pellet of insoluble protein after the last centrifugation step (chapter 5.4.4. protein extraction, methods)) that could easily be visualized by Coomassie staining after gel electrophoresis.

I found that the insoluble protein content of N2A cells incubated in LBH589 increased from day 0 to day 12 (figure 6.6.), starting from the insoluble/total protein ratio of 0.54 ± 0.22 on day 0 to an insoluble/total protein ratio of 1.67 ± 0.05 on day 12. On day 15 the insoluble/total protein ratio decreased to 1.27 ± 0.17.

Results show that the insoluble protein content increases in senescent N2A cells when compared to proliferating cells. In summary, senescence has an influence on the proteostasis machinery and insoluble protein content can be used to assess this effect.

We concluded that the insoluble protein content could be used in the following experiments as an output of the proteostasis machinery, to evaluate the effect of temperature cycles on the proteostasis system.

Insoluble/total protein ratio of LBH589-induced N2A cells day 0 to day 15, mean centered

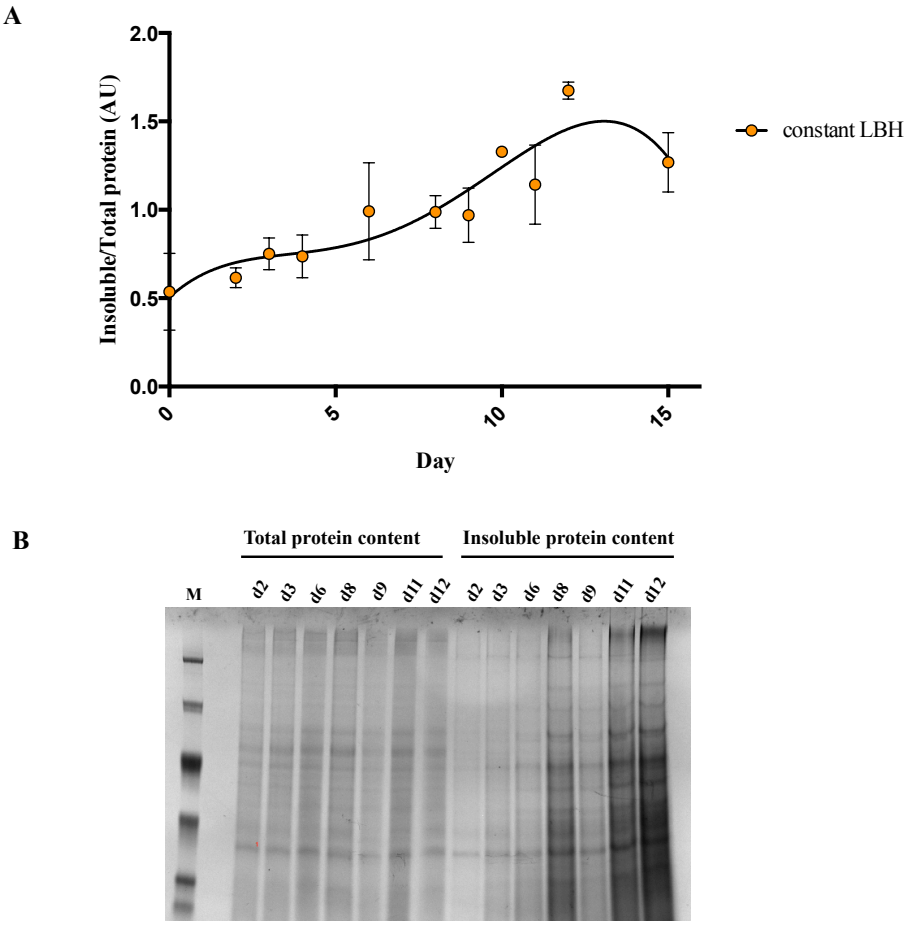


Figure 6.6 A. Insoluble protein content (insoluble/total protein ratio) of senescent-induced N2A cells. Senescence was induced on day 0 by LBH589. Cells were kept in constant conditions (37 °C) and a sample was harvested every two to three days. Mean ± SD of three experiments, three biological replicates.
B. Protein gel of LBH589-induced N2A cells kept at constant condition (37 °C). Days 2, 3, 6, 8, 9, 11 and 12 (constant condition days 4, 10 and 15, see figure 6.7/D) of total and insoluble protein content after normalization. Coomassie stained gradient TGX stain-free precast gel.

Ordinary one-way ANOVA:
$P < 0.0001$(****)

Tukey's post hoc multiple comparisons

Insoluble protein content of senescent-induced N2A cells	
Day 0 vs. day 6	p = 0.0458(*)
Day 0 vs. day 8	p = 0.0491(*)
Day 0 vs. day 10	p = 0.0001(***)
Day 0 vs. day 11	P = 0.0032(**)
Day 0 vs. day 12	p < 0.0001(****)
Day 0 vs. day 15	p = 0.0003(***)
Day 4 vs. day 10	p = 0.0042(**)
Day 4 vs. day 12	p < 0.0001(****)
Day 4 vs. day 15	p = 0.0122(*)
Day 6 vs. day 12	p = 0.0008(***)
Day 8 vs. day 12	p = 0.0007(***)
Day 9 vs. day 12	p = 0.0005(***)
Day 11 vs. day 12	p = 0.0122(*)

6.2.2. The insoluble protein content of N2A cells kept in temperature cycles increases less compared to N2A cells kept in constant condition

To investigate the influence of temperature and the circadian rhythm on the insoluble protein content in N2A cells, cells were kept in different temperature conditions: constant 37 °C, 24h (12:12 hours, 34/37 °C) or non-24h (9:9 hours, 34/37 °C) temperature cycles. We chose 9:9 hours temperature cycles because former luminescent temperature entrainment experiments done in our laboratory showed that cell-autonomous circadian oscillators could not synchronize to non-24h (9:9 hours) cycles, but to 24h (12:12 hours) temperature cycles. Proteins were extracted on days 4, 10 and 15 and the insoluble protein content of both LBH589-induced N2A cells and control cells (DMSO) were analyzed.

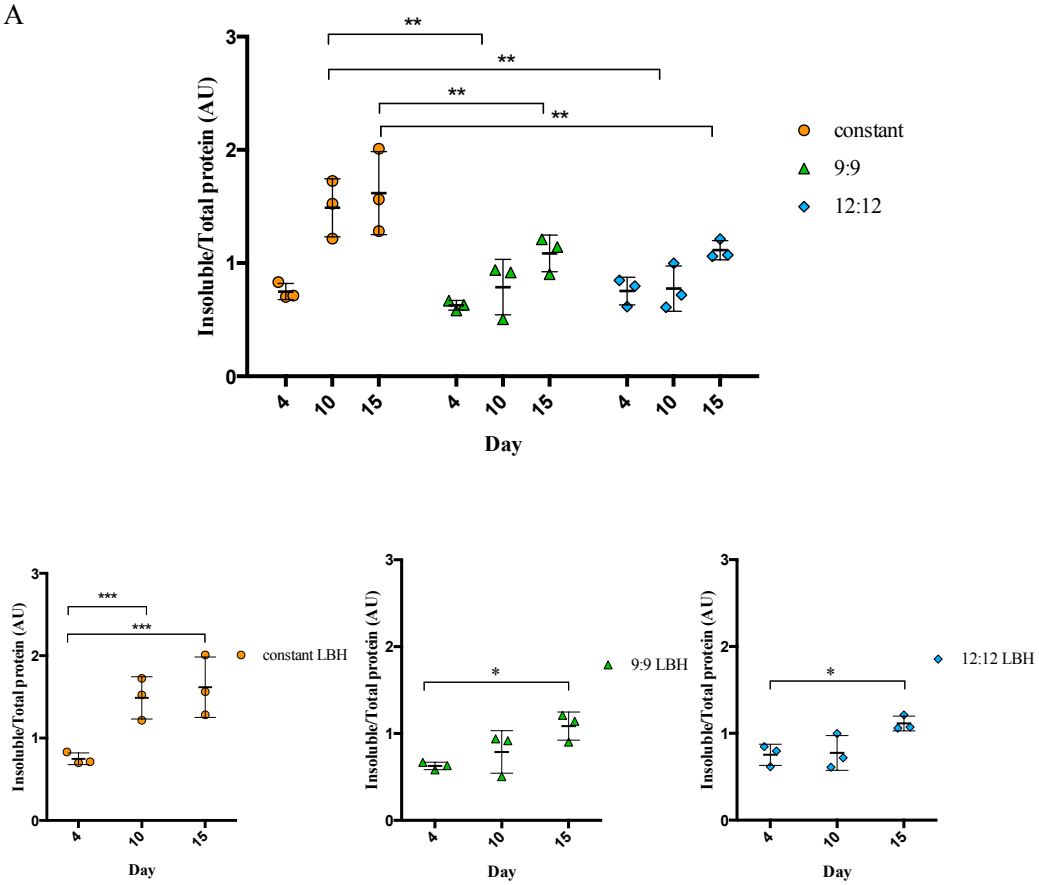
When looking at each condition separately, there is an increase in insoluble protein content both in LBH-induced (figure 6.7/A) and in DMSO control cells (figure 6.8/A) kept in different temperature conditions (constant, 9:9 and 12:12). In senescent N2A cells (LBH589), however, a significant increase in insoluble protein content was found from day 4 (9:9 ratio = 0.63 ± 0.04, 12:12 ratio = 0.75 ± 0.12) to day 15 (9:9 ratio = 1.09 ± 0.16, 12:12 ratio = 1.11 ± 0.08, $p < 0.01$, Two-way ANOVA, Tukey's post hoc multiple comparisons) in cells kept in 9:9 or 12:12 hour temperature cycles (figure 6.7/A). Senescent cells kept under constant temperature conditions even showed a significant increase of insoluble protein from day 4 (ratio = 0.75 ± 0.07) to day 10 (ratio = 1.49 ± 0.26), as well as from day 4 (ratio = 0.75 ± 0.07) to day 15 (ratio = 1.62 ± 0.37, $p < 0.001$, Two-way ANOVA, Tukey's post hoc multiple comparisons). In DMSO control cells only a trend was observed in each temperature condition (figure 6.8/A). This confirmed our findings (chapter 6.2.1.) that the insoluble protein content in N2A cells increases when cells undergo senescence. On top, there is a sharper increase in insoluble protein content in senescent-induced cells kept in constant versus temperature cycle conditions. Whether cells were incubated in 24h or in non-24h temperature cycles had no effect on the increase of insoluble protein content in senescent cells (figure 6.7/A).

In DMSO control cells, there is no significant difference in the insoluble protein content on day 10 or day 15 between different temperature conditions (figure 6.8/A).

Because there are lower insoluble protein ratios in senescent N2A cells in both temperature cycles compared to constant condition, we conclude that the temperature fluctuations and not a circadian mechanisms has an influence on the insoluble protein content in N2A cells.

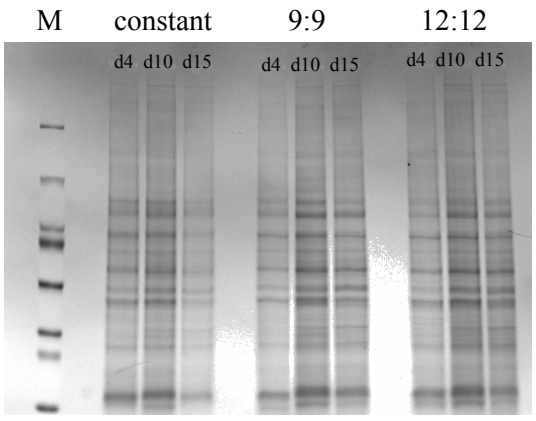
Figure 6.7.

A



B

Total protein content of senescent N2A cells



Insoluble protein content of senescent N2A cells

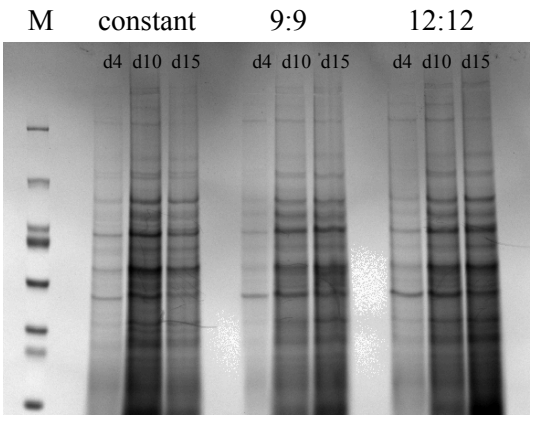
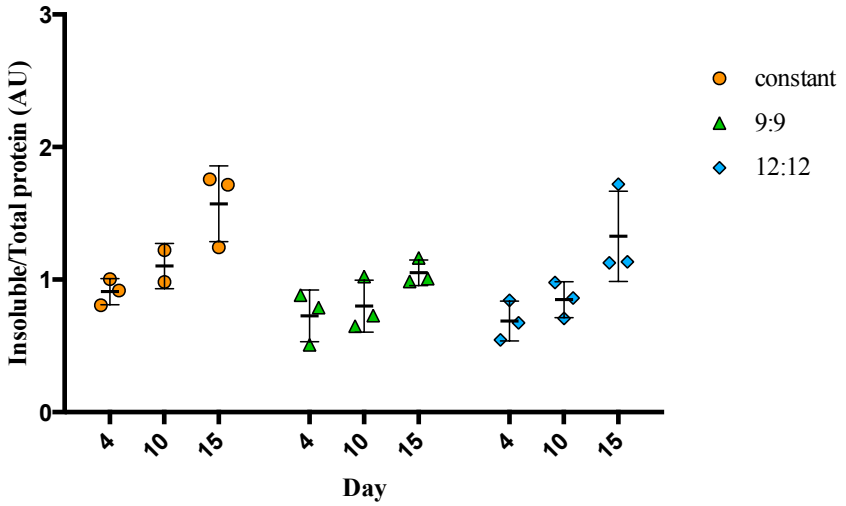


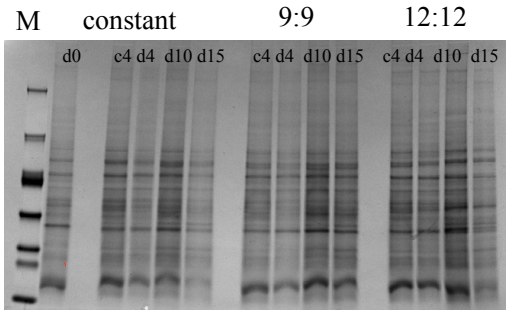
Figure 6.8.

A



B

Total protein content of control N2A cells



Insoluble protein content of control N2A cells

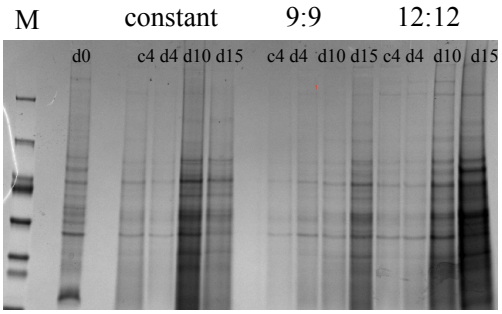


Figure 6.7/6.8. Insoluble protein content (insoluble/total protein ratio) of senescent (LBH589) and control (DMSO) N2A cells on days 4, 10 and 15. N2A cells were incubated in constant conditions (37 °C), 24h temperature cycles 12:12 hours (34 °C/37 °C) or non-24h temperature cycles 9:9 hours (34 °C/37 °C).

Figure 6.7. Senescent (LBH589-induced) N2A cells
A Insoluble protein content of senescent N2A cells, mean centered (as described in methods 5.4.). Differences between conditions on distinct day (upper graph) and differences per condition over days (lower graphs) are shown.
B Example of total and insoluble protein gel of senescent N2A cells. 4-15 % gradient gel stained with Coomassie.
M = marker, d = day, c = control.

LBH589, Two-way ANOVA:
Time p = 0.0004(***)

Tukey's post hoc multiple comparisons:

	constant vs. 9:9	constant vs. 12:12
Day 10	p = 0.0011(**)	p = 0.0010(**)
Day 15	p = 0.0061(**)	p = 0.0084(**)

	Constant	9:9 hours	12:12 hours
Day 4 vs. day 10	p = 00008(***)	ns	ns
Day 4 vs. day 15	p = 0.0003(***)	p = 0.014(*)	p = 0.0436(*)

Figure 6.8. Control (DMSO) N2A cells
A Insoluble protein content of control N2A cells (DMSO), mean centered.
Day 10, constant condition: only two replicates are illustrated.
B Example of total and insoluble protein gel of control N2A cells. 4-15 % gradient gel stained with Coomassie.
M = marker, d = day, c = control.

DMSO, Two-way ANOVA and Tukey's post hoc multiple comparisons:
Not significant

6.2.3. The insoluble protein content of U2OS cells kept in temperature cycles increases compared to U2OS cells kept in constant condition

We also investigated the influence of different temperature conditions (constant 37°, temperature cycles 9:9 and 12:12 (34 °C/37 °C)) on the insoluble protein content of U2OS cells. Our aim was to explore if there is a difference in insoluble protein changes in other cell lines. Besides the lack of FBS in the experimental medium, the experimental design was performed as described for N2A cells.

Looking at each condition separately (figure 6.9/A), one can observe a trend; the insoluble protein content in U2OS cells induced with LBH589 increased from day 4 to day 15 in every condition. In the non-24h temperature cycle we examined a significant increase in insoluble protein from day 4 (ratio = 1.01 ± 0.03) to day 15 (ratio = 1.69 ± 0.40, day 4 vs. day 15 p=0.0812(*), Two-way ANOVA, Tukey's post hoc multiple comparisons). In control cells the increase of insoluble protein content from day 4 to day 15 seems not as striking (figure 6.10/A). These findings show, as also seen in N2A cells (chapter 6.2.2.), that there is an increase of insoluble protein content in cells undergoing senescence.
When comparing the different conditions in senescent (figure 6.9/B, C) and control (figure 6.10) U2OS cells, we observed an increase in insoluble protein content in LBH589-induced U2OS cells on days 4, 10 and 15 in constant condition versus temperature conditions in two out of three biological replicates. However, there was no significant difference in the insoluble protein content when comparing the means of all replicates in the different conditions (figure 6.9/A, 6.10/A). It is conspicuously that one of three replicates is an outliner with a lower insoluble protein content in U2OS cells kept in temperature cycles than in constant conditions (except when comparing constant condition vs. 12:12 hours temperature cycle on day 15). Therefore a technical error seems likely. Possible could be an error in the protein extraction process, with loss of insoluble protein content during resuspension of the viscous protein pellet. Also a problem in normalizing the total protein content is reasonable. To investigate if technical problems were the case, more replicates would have been needed.

In summary, temperature cycles have a different effect on the insoluble protein content in U2OS cells than in N2A cells. In U2OS cells there is a rather increased insoluble protein content in cells kept in temperature cycles compared to cells kept in constant condition.

In N2A cells, however, there is a lower insoluble protein content in cells kept in temperature cycles compared to cells kept in constant conditions.

Figure 6.9.

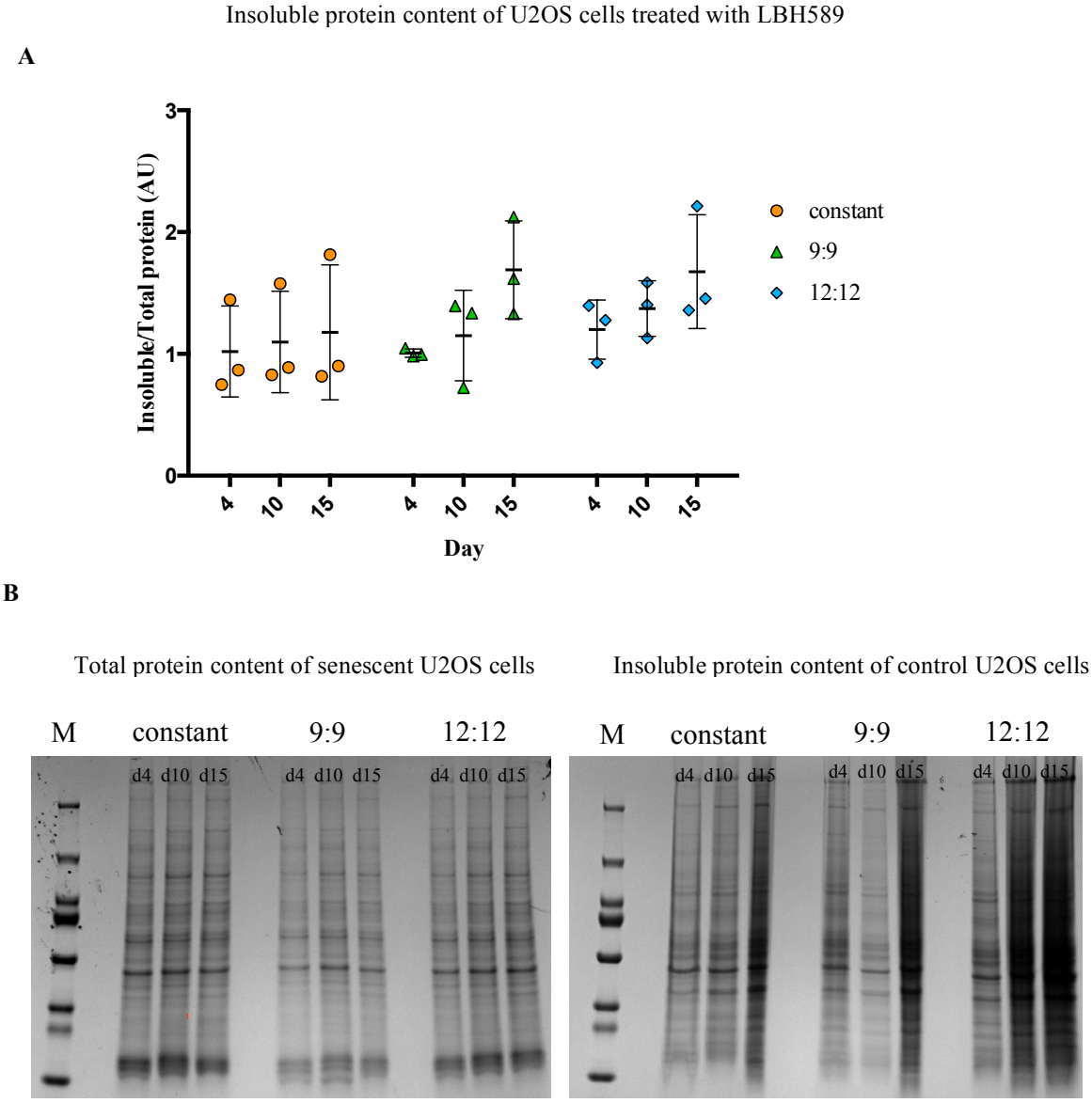
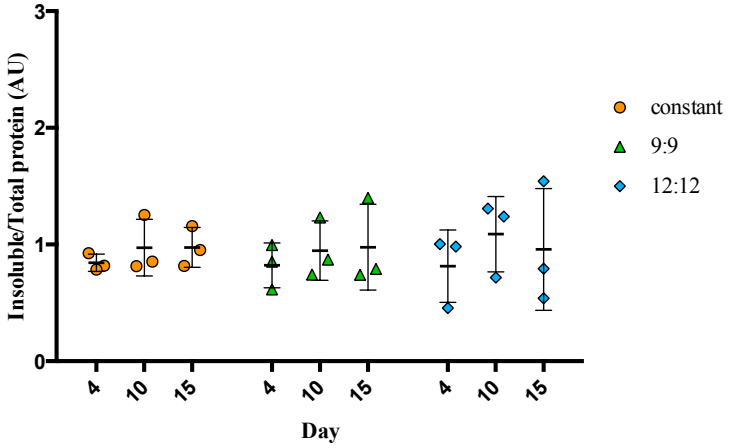
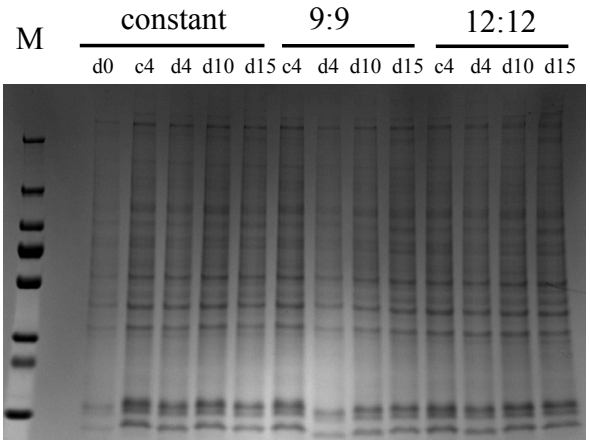


Figure 6.10.

Insoluble protein content of U2OS cells treated with DMSO



Total protein content of control U2OS cells



Insoluble protein content of control U2OS cells

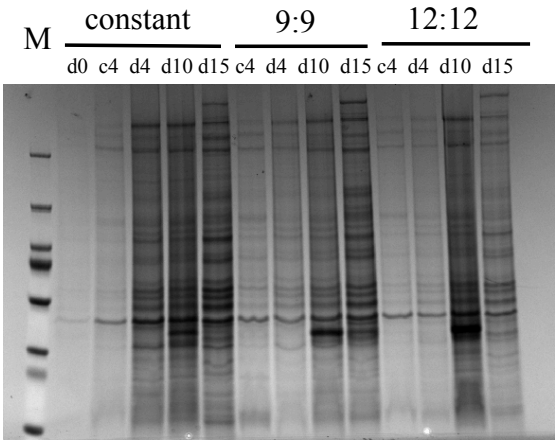


Figure 6.9/6.10. Insoluble protein content (insoluble/total protein ratio) of senescent (LBH589) and control (DMSO) U2OS cells on days 4, 10 and 15. U2OS cells were incubated in constant conditions (37 °C), 24h temperature cycles 12:12 hours (34 °C/37 °C) or non-24h temperature cycles 9:9 hours (34 °C/37 °C).

Figure 6.9. Senescent (LBH589-induced) U2OS cells
A Insoluble protein content of senescent U2OS cells, mean centered. Differences between conditions on distinct day and differences per condition over days are shown.
B Example of total and insoluble protein gel of senescent U2OS cells. 4-15 % gradient gel stained with Coomassie.
M = marker, d = day, c = control.

LBH589, Two-way ANOVA:
Time p = 0.216(*)

Tukey's post hoc multiple comparisons:

	Constant	9:9 hours	12:12 hours
Day 4 vs. day 10	ns	ns	ns
Day 4 vs. day 15	ns	p = 0.0812(*)	ns

Figure 6.10. Control (DMSO) U2OS cells
A Insoluble protein content of control U2OS cells (DMSO), mean centered.
B Example of total and insoluble protein gel of control U2OS cells. 4-15 % gradient gel stained with Coomassie.
M = marker, d = day, c = control.

DMSO, Two-way ANOVA and Tukey's post hoc multiple comparisons:
Not significant

6.3. Chaperone system of N2A and U2OS cells

Proteostasis (protein homeostasis) describes a network of proteins and systems that are responsible to stabilize and regulate protein procedures in cells. Because proteins are involved in nearly every process in a cell, proteostasis is essential for cell survival, and therefore for healthy organisms (Klaips, Jayaraj and Hartl, 2018a). Chaperones and Co-chaperones are one of the key proteins in proteostasis. They assist in folding de novo proteins, refolding misfolded proteins and help to degrade useless proteins (Jiang *et al.*, 2012; Rivera *et al.*, 2018). One of the best studied chaperones are heat shock proteins. These proteins are able to protect cells against stress when induced by stress factors, e.g. high temperature (Amin, Cumming and Latchman, 1996). Therefore, we assessed if heat shock protein levels are detectable or change when N2A or U2OS cells are induced with heat.

In the following experiments our aim was to investigate the link between heat shock proteins and the insoluble protein content in N2A or U2OS cells. Our hypothesis was that some of the selected heat shock proteins are up- or down regulated in the different temperature conditions used for our experiments. This would explain the change in insoluble protein content in N2A (chapter 6.2.2.) or U2OS cells (chapter 6.2.3.) kept in different temperature conditions and would bring us closer in understanding the connection of proteostasis with temperature entrainment.

6.3.1. Chaperones detected in N2A and U2OS cells in constant condition and after heat shock induction

Because the gene expression pattern of heat shock proteins differs from cell to cell (K. Kaarniranta *et al.*, 2002), we first needed to investigate if the selected heat shock proteins are detectable in N2A and U2OS cells. We selected well known heat shock proteins that cover different protein control systems: Two candidates from the HSP70 superfamily (GRP78, HSP70), two candidates from the small heat shock protein HSPB family (HSP25/27, Alpha B Crystallin) and one candidate from the HSP90 family (HSP90). For further background explanations of chaperone candidates please see chapter 2.2.1. in the introduction.

The heat shock protein candidates were detected via western blot in N2A and U2OS total protein samples. First, I tested which chaperone candidates can be detected in N2A and U2OS cells, before I secondly set up time line experiments (chapter 6.3.2.). Cells were either kept in a culture medium without treatment at 37 °C or heat shock (43 °C for 15 minutes) was induced. After heat shock induction cells were incubated in 37 °C for 6 hours to recover. Cells then were harvested and lysed as described in methods (chapter 5.6. heat shock experiment). 20 µg of U2OS and 45 µg of N2A total protein samples were loaded on a self-made 10 % gel. After preceding the gel, immunolabeling with selected heat shock protein-antibodies was performed. HSP90 had the highest molecular weight with 90 kDa, followed by GRP78 (78 kDa), HSP25/27 (25 or 27 kDa) and alpha B Crystallin with 21 kDa. ß-Actin was used as a loading control with a molecular weight of approximately 42 kDa.

The detection of chaperones differed in N2A and U2OS cell samples. The small heat shock protein HSP25/27 and the HSP70 were only detectable in U2OS cells, both in cells kept in constant condition and cells induced with heat. The small heat shock protein Alpha B Crystallin could not be detected in both N2A and U2OS cells, regardless of heat induction or cells kept in constant condition. HSP90 and GRP78, however, were expressed in both cell lines, kept in constant temperature condition (figure 6.11.) and after heat shock.
Because there have been problems in the experiment setting, e.g. some of the N2A protein samples were to viscous for loading and running in the gel, no further conclusions could be drawn and further investigations need to follow.

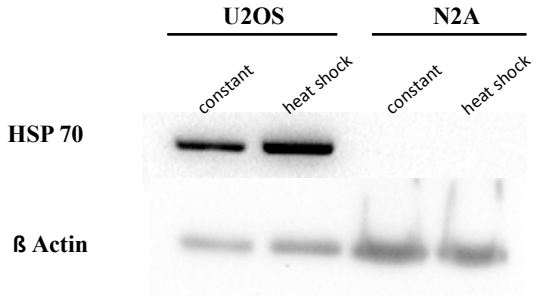
	N2A cells	**U2OS cells**
HSP 70 (~70 kDa)	-	+
HSP 90 (~90 kDa)	+	+
GRP 78 (~78 kDa)	+	+
HSP 25/27 (~25 kDa or ~27 kDa)	(-) band with wrong size	+
Alpha B Crystallin (~21 kDa)	-	-

Figure 6.11. Western blot detection (+ detected, - not detected) of 5 chaperone candidates in N2A and U2OS total protein samples. Samples of cells kept in constant condition (37 °C) and after heat shock induction were analyzed.

HSP70 was detected in U2OS total protein samples in both the constant condition (37 °C) and after heat shock induction. There was an increase in the HSP70 concentration (HSP70/ß-Actin ratio) in U2OS cells induced with heat shock (figure 6.12/B). In the N2A total protein samples, HSP70 could not be detected in cells with western blot in both constant condition samples and heat shock induced samples.

HSP70 detection in U2O and N2A cells

A



B

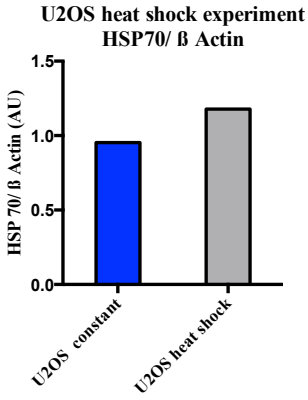


Figure 6.12. A Western Blot of HSP70 and ß-Actin. U2OS and N2A samples kept in constant condition at 37 °C and induced with heat shock (43 °C for 15 minutes, 37 °C recovery for 6 hours).
B HSP70/ß-Actin Ratio of U2OS cells kept in constant condition and after heat shock induction. One replicate was performed.

In both the N2A and U2OS samples, HSP90 was detected in the total protein of cells kept in constant condition and after heat shock induction. In the U2OS samples, a decrease in HSP90/ ß-Actin in heat shock versus constant condition was assessed, whereas in N2A samples a slight increase was detected. To draw a conclusion, however, more than one replicate would have been necessary.

HSP90 detection in U2OS and N2A cells

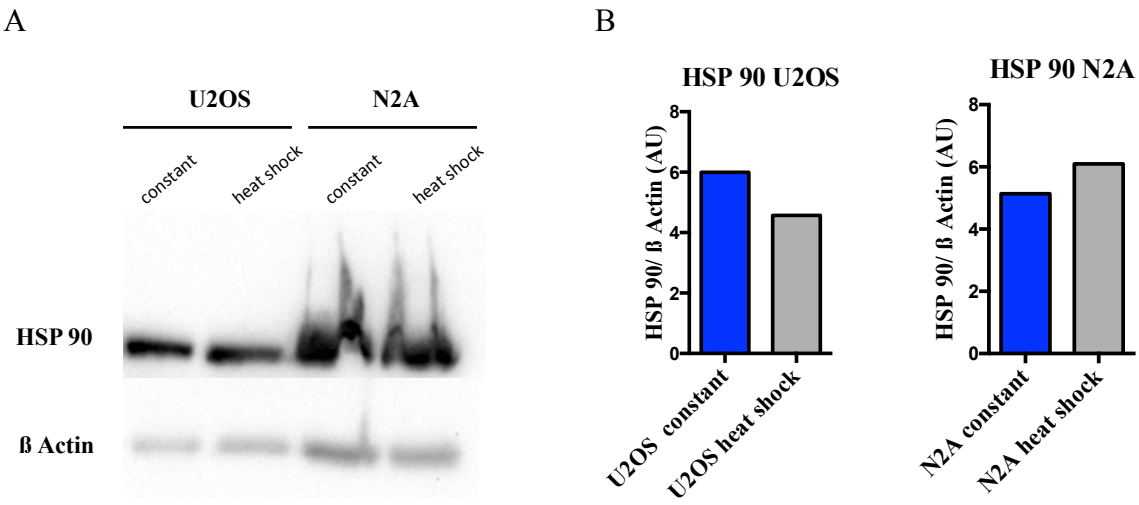


Figure 6.13. A Western Blot of HSP90 and ß actin. U2OS and N2A samples kept in constant condition at 37 °C and induced with heat shock (43 °C for 15 minutes, 37 °C recovery for 6 hours) **B** HSP90/ß Actin Ratio of U2OS and N2A cells kept in constant condition and after heat shock induction. One replicate was performed.

In summary, heat shock protein expression differs from N2A to U2OS cells. Further investigations need to be done, however, in order to assess the effect of heat shock on heat shock protein levels in N2A and U2OS cells. We investigated that HSP90 was well detectable in both cell lines. Therefore we chose HSP90 as an candidate to investigate heat shock protein levels in N2A cells kept in constant versus cells kept in 24h temperature cycles (see next experiment).

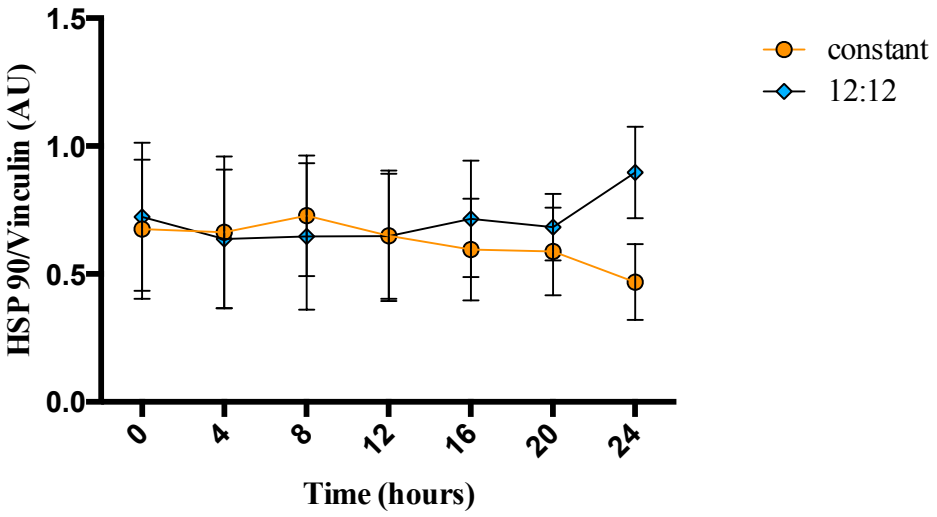
6.3.2. Concentration of HSP90 does not change in N2A cells kept in temperature cycles in comparison with control cells kept in constant condition

HSP90 expression might be circadian or might be altered by the temperature change in the entrainment protocol used here. This might affect the proteostasis network and lead to the changes in insoluble protein content observed here. To asses HSP90 expression I performed a HSP90 time course with N2A cells kept in constant 37 °C versus cells kept in 24h (12:12) temperature cycle (34/37 °C). N2A cells were not senescent (not LBH589-induced). Seven time points were analyzed during the 24-hour time course. Vinculin was used as housekeeping protein because it was better detectable than ß-Actin in N2A cells.

The HSP90/Vinculin ratio slightly changes when looking at the different time points (figure 6.14/A). However, a no significant difference between the constant and 12:12 cycling condition nor any kind of rhythmic expression was found.

HSP90/Vinculin time course

A



B

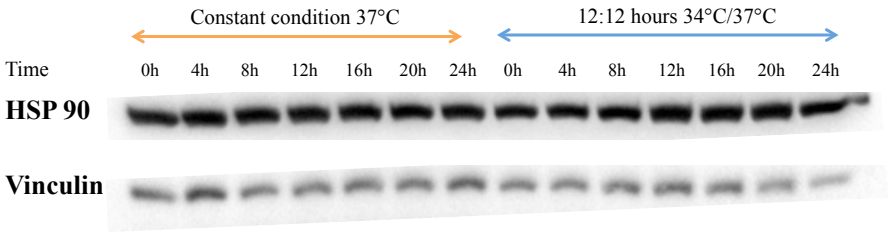


Figure 6.14. Western Blot analysis of HSP90/Vinculin Ratio **(A).** N2A cells kept in constant condition (37 °C) versus cells kept in 24h temperature cycle 12:12 hours (34 °C/37 °C). Cells were harvested every 4 hours on day 3.
Three biological replicates were performed.
B One out of three western blots, HSP-90 and Vinculin labeled.

7. Discussion

7.1. Senescent N2A cells as an aging model

A senescent N2A cell model has been used to assess the effect of the circadian clock on aging neuronal cells. As Ian P. Johnson discusses in "Age-related neurodegenerative disease research needs aging models", research addressing age-related neurodegenerative diseases (ND) concentrates on disease models rather than on aging models. ND, however, like Alzheimer's disease (AD) and Parkinson's disease (PD), are usually age associated. His view matches our idea of using a senescent cell model: Understanding general mechanisms of aging could help to lay open the role of aging processes in the development of ND (Johnson, 2015). As described in the introduction, senescent cells can be seen as a hallmark of aging, increasing in various aging tissues and organs (Dimri *et al.*, 1995b; Herbig *et al.*, 2006; Gruber *et al.*, 2007; Geng *et al.*, 2010).

7.1.1. Senescent associated beta-galactosidase is a senescence marker

Senescent and terminally differentiated cells have to be distinguished from quiescent cells. Terminally differentiated and senescent cells are in irreversible growth arrest and fulfill explicit functions depending on their cell type (Winden *et al.*, 2009; Baser *et al.*, 2019). Quiescent cells, in contrast, are in reversible growth arrest and can be brought back to proliferation (Coller, Sang and Roberts, 2006). One way to verify the cell's state is through defined markers, e.g. senescence markers (Hernandez-Segura, Nehme and Demaria, 2018).

I used a senescence-associated-b-galactosidase (SA-b-gal) staining to detect LBH589 (LBH) induced senescent cells (chapter 5.3.). It is a widely used method and well probed in cell culture (Dimri *et al.*, 1995c; Lee *et al.*, 2006), most

importantly also in neuronal cells such as rat hippocampal neurons (Geng *et al.*, 2010) and mouse brain tissue (Tominaga *et al.*, 2019).

False positive results may occur if cells are held under high confluence, due to an increase of stress induced SA-b-gal. Therefore, it was important to avoid N2A overgrowth and high cell confluence. I achieved a balanced N2A growth by adapting the FBS concentration (high concentration ends in cellular overgrowth and senescence inhibition (Seeds *et al.*, 1970; Evangelopoulos Maria, Wüller Stefan, 2009)) and LBH concentration (high concentration is toxic ending in cellular death (Cain *et al.*, 2013)) in the medium. The average N2A cell confluence - with the medium chosen for all experiments (supplemented with 0.5 % FBS) - was kept below 70 % (figure 6.1.). The confluence was measured with ImageJ and % area was calculated up until day 7. It is a simple method for obtaining the approximate confluence, without involving the vertical growing cells. On day 10 and 15 confluence was only approximately estimated under the light microscope (examples see figure 6.2.) during b-gal-staining experiments, without reaching a confluence of over 70 %. In future works it would be interesting to measure the influence of the exact N2A confluence on stress induced SA-b-gal expression in order to optimize senescence detection.

The use of several markers could be a good alternative way to avoid false positive results, this also being proposed in studies with e.g. senescent fibroblasts (Wiley *et al.*, 2017) and discussed in reviews (Sharpless and Sherr, 2015; Hernandez-Segura, Nehme and Demaria, 2018). A highly sensitive and specific combination of senescent markers, however, is still a subject of current research.

7.1.2. LBH589 is a trigger for senescence induction

We used LBH as an agent to induce senescence in N2A cells and SA-b-gal staining to detect senescent cells. LBH functions as an deacetylase-inhibitor, inducing senescence through inhibition of the histone complex and activation of stress response pathways (Ogryzko *et al.*, 1996; Munro *et al.*, 2004). LBH is also known as Panobinostat, a drug for the first-line treatment of multiple myeloma (Van Veggel, Westerman and Hamberg, 2018).

In cancer treatment, the toxic effect of LBH is used to induce cell cycle arrest of tumor cells and apoptosis (Ungerstedt *et al.*, 2005). Also, senescence induction seems to play a key role in cancer treatment with histone deacetylase, assessed in gynecologic cancer cells (Terao *et al.*, 2001) and murine lymphomas (Schmitt *et al.*, 2002). Our goal was to use the senescence inducing effect of LBH, but avoid apoptosis induction. To achieve this, we used a low dose of LBH, known to induce senescence in osteosarcoma cells (Cain *et al.*, 2013), and added different FBS concentrations (discussion, chapter 6.1.3.). In so doing so, we wanted to investigate the right FBS concentration with LBH to achieve a sub-confluent N2A cell flask. As LBH is dissolved in DMSO, control groups were treated with DMSO only to exclude toxicity-related effects of DMSO. Although using well-established doses of LBH (Cain *et al.*, 2013), N2A cells showed a small decrease in cell confluence when LBH was added from day 2 to day 4 (figure 6.1, B). Despite using low LBH doses, this implies a slight toxic effect of LBH. In the DMSO control medium, growth was also reduced, but to a lesser extent (figure 6.1, A). Nevertheless, after day 4 an increase of N2A cell confluence could be recorded. Protein analysis was therefore performed after day 4 when cells recovered from the initial toxic effects.

To induce senescence via LBH *in vitro* is a relatively fast method to accomplish an aging model, compared to the aging process in living species or *in vivo* studies. On average, there were 45 % senescent cells on day 4, increasing up to 83 % on day 15 (figure 6.4.) assessed with SA-b-gal staining in medium containing LBH and 0.5 % FBS. These findings match studies with LBH induced senescent osteosarcoma and neuroblastoma cells, in which growth arrest markers were detected after approximately 7 days (Cain *et al.*, 2013; Waldeck *et al.*, 2016). On the one hand, a fast senescence induction is practical for implementing experiments, on the other hand, some cellular mechanism may possibly differ compared to the slower neuronal degeneration in elderly or ND patients. Because so little is known about the details of aging processes it seems reasonable to start with basic senescent *in vitro* research and to then confirm results *in vivo*.

There is evidence that LBH also induces cell differentiation (Cain *et al.*, 2013). Studies identified differentiated cells with certain markers and staining, such as S100 as a marker in neuroblastoma cells (human and mouse cell line (Waldeck *et al.*, 2016)) or Alizarin staining in osteosarcoma cells (Cain *et al.*, 2013). Cain et al. demonstrated both, the effects of LBH on senescence and differentiation in osteosarcoma cells. However, it is not distinguishable why some cells undergo senescence and others differentiate when induced with LBH.
For the assessment of further factors influencing this 'fate of a cell' (apoptosis, senescence or differentiation) when induced with LBH, could be interesting for future works. It would optimize the senescent model, excluding other possible confounders from non-senescent cells.

7.1.3. FBS influences senescent induction negatively

FBS also plays a role in senescence induction and/or SA-b-gal detection. In control N2A cells, without LBH and low FBS concentrations, I still detected senescent cells with SA-b-gal staining. Indeed, fewer cells underwent senescence and senescence occurred later in most cells (day 4: 28 % senescent cells, up to 59 % senescent cells on day 15 (figure 6.4.)). FBS withdrawal might induce stress in N2A cells, activating cell cycle arrest thus leading to senescence induction. This would support my findings that lower FBS concentrations led to lower N2A confluences (figure 6.1.) and a trend of higher senescent cell levels (when comparing N2A cells in medium supplemented with 0.5 % FBS, versus 1 % FBS (figure 6.3.). In conclusion higher FBS concentration activates cell proliferation and inhibits senescent induction. When comparing 0.1 % FBS with 0.5 % FBS, the positive effect of FBS on cell confluence seems striking (figure 6.1.). With 0.5 % and higher FBS concentrations, the effect on confluence eases, leading to less difference when comparing 0.5 % with 2 % FBS concentration (figure 6.1.). Studies with FBS withdrawal only assessed the activation of N2A differentiation and differentiation pathways (Seeds *et al.*, 1970; Evangelopoulos, Weis and Krüttgen, 2005), not senescent induction, and hence leaving open whether senescent pathways are activated in N2A cells with FBS withdrawal as well. Another explanation could be a false positive detection of SA-b-gal in differentiated N2A cells. This, however, has only been described in a few cell types, such as melanocytes (Dimri *et al.*, 1995b).

In neuronal cells senescence was well detected, this being confirmed with other features of senescent cells, such as negative proliferation markers (Geng *et al.*, 2010), typical morphology and cytokine expression (Yan *et al.*, 2017), and other senescent markers e.g. p16 (Tominaga *et al.*, 2019).

7.1.4. Summary

The use of a senescent N2A cell model is a good way to investigate aging processes on a cellular level and assess the influence of the circadian clock on aging neuronal cells. It is relatively easy to accomplish and (compared with *in vivo* aging models) experiments are quickly repeatable. It also helps to initially understand basic mechanisms in the aging process. It may then enable us to understand the mechanisms leading to aging and aging associated neuronal diseases in future. Mechanisms suggested by our *in vitro* aging model can then subsequently be confirmed *in vivo*. As Johnson declares, treatments for age associated ND are still in the early stages. He concludes that ND research needs more aging models in order to understand underlying causes of ND and forward ND treatment (Johnson, 2015).

7.2. Protein aggregation as a hallmark of aging

Our goal was to assess the effect of the circadian clock on the PN of an aging cell model. Due to the PN' complexity in eukaryotes, consisting of about 1000 - 1400 components interacting with another (Balch *et al.*, 2008; Kim *et al.*, 2013; Hipp, Park and Hartl, 2014; Klaips, Jayaraj and Hartl, 2018b), we concentrated on one part of the PN: protein aggregates. The effect of the circadian clock on protein aggregates caught our interest because aggregates play a key role in both aging processes (Yang, Ang and Strong, 2005; Paz Gavilán *et al.*, 2006; David *et al.*, 2010c; Omata *et al.*, 2014) and age associated ND (Masters *et al.*, 1985; Baba *et al.*, 1998). Although it is known that most ND occur with age, the causative link remains unclear. Studies propose that protein aggregates, present in both aged and ND organisms, might explain this link between aging and ND. This is shown, for instance in aged Caenorhabditis elegans, where aged associated protein aggregates initiate amyloid beta aggregation (Groh *et al.*, 2017), a hallmark of AD. Or as assessed in AD transgenic mouse models, where tau-containing

neurofibrillary tangle accumulation has been associated with cellular senescence (Musi *et al.*, 2018), implicating a strong relationship between senescence and ND on a cellular level.

7.2.1. Insoluble protein extraction in N2A cells

We extracted proteins with RIPA buffer, Urea buffer, DNaseI, using syringes and sonication (chapter 5.4.4). Insoluble proteins were extracted by way of centrifugation (3 times at 20.000 g, 4°C for 15 minutes), forming a pellet on the tube's bottom. Extracting the insoluble protein content did not exclude the eventuality that cell membranes, lysosomes or mitochondria components might still be present in protein samples. This should not influence our experimental setting, however, because our control probes from day 0 also did not exclude this eventuality. We used electrophoresis to detect bands of protein samples. In this process, the components mentioned above are not retained in the gel, but run straight through it due to their size and charge. Moreover, gels were processed and stained with Coomassie blue, a method that only detects proteins.
We therefore expected no detection of cell components other than proteins in the gels and were able to compare the protein content of different conditions with the protein content of day 0 control protein samples.

Because we could not exclude proteins associated to but not part of the aggregates and we only quantified the protein content, we spoke rather of the insoluble protein content than of specific protein aggregates. On top proteins and therefore properties of aggregates vary depending on cell type and disease models. In order to eliminate the effect that absolute protein amounts vary among samples depending on the exact cell number, we calculated the relative insoluble protein amount: the ratio of insoluble protein amount divided by total protein amount (detected on protein gel, chapter 5.4.6.). To be able to compare different replicates, the ratio had been normalized. We could show that the average insoluble protein content increased in senescent N2A cells from day 0 to day 12 (figure 6.6, A). Our findings matched the general consensus, that protein aggregates can be seen as markers of aging. On day 15, however, the average insoluble protein content dropped slightly.

This might occur due to senescent cell degeneration and death, leading to a relatively higher amount of proliferating cells after a certain time.

In future experiments we not only want to quantify the protein amount but also investigate the aggregates' morphology in detail. This would help us to classify the aggregates occurring in senescent N2A cells. Most of the aggregates described in studies are amorphous, not showing any structural hallmarks. Whereas some proteins, often seen in age-dependent proteinopathies, form ordered fibrillar aggregates with mostly beta sheet formation (= amyloid) (Benzinger *et al.*, 1998; Der-Sarkissian *et al.*, 2003; Ross and Poirier, 2004). Interestingly, some aggregates are known to occur both in aging and ND. For example in human brains, beta amyloid was present in the elderly and patients suffering from AD (Armstrong, 1995). This emphasizes once more, that protein aggregates might play an important role in the link between aging and ND.

7.2.2. Are protein aggregates beneficial or a burden for the cell?

But what does it mean for a cell to have higher amounts of protein aggregates, is it beneficial or a burden? The answer to this question is still a subject of current discussion. Some studies suggest negative effects of protein aggregates on the healthy cell's well functioning PN, like inhibition and impairment of cellular degradation machinery components (Bence, Sampat and Kopito, 2001; Bennett *et al.*, 2005), and protein aggregation as an unregulated chaotic process due to PN collapse. Examples of PN collapse were assessed in senescent fibroblasts with alteration in protein degradation (Dice, 1982). Furthermore, in aging mouse hepatocytes a decrease of autophagic vacuoles has been investigated (Terman, 1995), implicating an impaired PN and an increase in protein aggregation. This is supported by findings in mice, where autophagy mechanisms of the PN have been suppressed. Here an accumulation of protein aggregates in neuronal cells occurred, implicating an important role of autophagy in preventing the cell from age associated ND (Hara *et al.*, 2006; Komatsu *et al.*, 2006).

Other publications describe protein aggregation as a rather well controlled mechanism. These see aggregates as a coping mechanism to sequester misfolded proteins into defined cellular assemblies due to an overload in proteosome capacity (Johnston, Ward and Kopito, 1998). This is regulated - among other factors - depending on their protein aggregation state and ubiquitination status (Kaganovich, Kopito and Frydman, 2008). Hence, sequestration might function as a control mechanism in the cell in order to clear the cell from potential toxic effects or stress. An enhancement of cellular fitness in cells and yeast (Escusa-Toret, Vonk and Frydman, 2013; Saarikangas and Barral, 2015) has been reported, supporting the hypothesis that controlled aggregation might be beneficial. In future projects we will carry out a cell survival assay in order to get an idea of the relation between protein aggregation and its effect on senescent N2A survival. In so doing, we will be able to predict the influence of aggregates on the life span *in vitro*.

7.2.3. Summary

Our senescent N2A model is an aging model that shows an increase in insoluble protein content when compared with proliferating cells. This increase in aggregation matches the findings that components of the PN decline with aging, leading to an increase in protein misfolding and aggregation (Cuervo and Dice, 2000; Komatsu *et al.*, 2006; Erjavec *et al.*, 2007; Klaips, Jayaraj and Hartl, 2018a). Whether this is a beneficial coping mechanism of the N2A cell (sequestration), or whether aggregation leads to a decline of the healthy cell will be the subject matter of future research work. For our project, the insoluble content in senescent neuronal cells was a good way to measure the effect of the circadian clock on the PN in terms of one output component.

7.3. Temperature cycles influence the Proteostasis network of N2A and U2OS cells

Most of elderly and ND patients suffer from a disrupted wake-sleep cycle with fragmented sleep during the night and napping during the day (Ancoli-Israel *et al.*, 1997; Huang *et al.*, 2002; Hatfeld *et al.*, 2004; Merlino *et al.*, 2010; Weissová *et al.*, 2016). In influencing their wake-sleep cycle through stronger light stimuli (= stronger external zeitgeber cues), studies suggest benefits for elderly and ND patients including adjusted sleep patterns (Ancoli-Israel *et al.*, 2003; McCurry *et al.*, 2011; Figueiro *et al.*, 2014), cognitive improvement (Riemersma-van der Lek *et al.*, 2008) and positive effects on circadian rhythmicity (Ancoli-Israel *et al.*, 2002, 2003; Figueiro *et al.*, 2014). Drawing the link to protein aggregates, (chapter 2.2.2.) an output of the PN and hallmarks for aging and ND, we wanted to assess the possible influence of the circadian clock on protein aggregation. Our aim was to understand the connection between the disrupted circadian clock in aging or aging associated diseases and its pathology, protein aggregates. Does circadian rhythmicity influence the insoluble content in neuronal cells?

To approach this question, we used a neuronal cell line in our experiments, which has been isolated from a strain A albino mouse tumor (N2A cells) by R.J. Klebe and F.H. Ruddle in 1969 (ATCC® CCL-131™). I induced senescence to achieve a model similar to aging in the brain of elderly and ND patients. Taking the hierarchical circadian clock system described in the introduction (chapter 2.1.1.), senescent N2A neurons with their endogenous clock can be seen as peripheral clocks. Therefore, we needed an oscillating endogenous zeitgeber, which entrains our senescent N2A cells *in vitro*. Many studies suggest that there is not one exclusive internal zeitgeber but rather many oscillating mediators such as hormones (Balsalobre *et al.*, 2000; McNamara *et al.*, 2001) or temperature (Brown *et al.*, 2002a), which connect the information of the SCN with peripheral clocks. Multiple pathways that influence the cellular clock were also detected *in vitro*, which has been investigated in mammal fibroblasts (Balsalobre, Marcacci and Schibler, 2000). We used temperature cycles in our experiments as an internal zeitgeber in order to entrain senescent N2A cells. This decision was carefully made. First, we knew from previous luciferase experiments with clock genes of

U2OS cells, performed in our lab, that cells could be entrained by temperature cycles (data not published).
Experiments were performed with a cycle amplitude of 3° between 34° and 37° degrees (12 hours 34°, 12 hours 37°). Cells revealed no entrainment when kept in constant temperature condition or non-24h temperature cycles (9 hours 34°, 9 hours 37°). Thus, we used 24h (12:12 hours) and non-24h (9:9 hours) temperature cycles with a temperature range of 34° to 37° (chapter 5.4.2.). A similar setting was used in a study with rat fibroblasts *in vitro*. Here, the oscillation in gene expression had been clock dependent when entrained with temperature cycles (assessed with 33° - 37°, 4° amplitude (Brown *et al.*, 2002a)). Our temperature amplitude of 3° in temperature cycles matches *in vivo* mouse studies, where the brain temperature amplitude was also measured 3° from 35° during night/non-active phase to 38° during day/active phase (Baud, Magistretti and Petit, 2013). Secondly, temperature cycles are widely used for entrainment, for instance in neurospora (Liu *et al.*, 1998; Merrow, Brunner and Roenneberg, 1999), drosophila (Krishnan *et al.*, 2001) and mammal fibroblasts (Brown *et al.*, 2002a). And thirdly, temperature is known to become regulated by the SCN through activity patterns and sleep-wake cycles, established in mammals ((Decoursey *et al.*, 1998; Kramer *et al.*, 2001). Therefore, temperature is known to be clock regulated and correlates with activity and wake-sleep cycles. It thus draws the link to alterations in wake-sleep cycles of elderly and ND patients. Studies estimated a change in the endogenous circadian temperature amplitude and phase in elderly (Czeisler *et al.*, 1992) and a phase delay of circadian temperature and activity rhythm in dementia patients (Satlin *et al.*, 1995; Ancoli-Israel *et al.*, 1997).

We concluded that changes in physiological temperature oscillations correlate with a disrupted circadian timing system, with aging/age-associated diseases respectively, and are a key junction between a disrupted circadian timing system and aging associated pathologies. Hence, temperature is an interesting zeitgeber to assess protein aggregates in cells. In order to minimize confounding effects, the time when medium had to be changed was kept as short as possible, ensuring that all cells were handled the same way. In addition, the same air atmosphere was used for cells kept in different incubators at different temperature conditions by using closed lids (incubators for temperature entrainment had no CO_2 regulator).

Also, N2A cells were counted and the quantity per flask was adapted to reduce differences between cell flasks and conditions. One has to note, however, that our model is simplified from reality in order to acquire basic knowledge about the underlying mechanisms.

In humans the body temperature varies depending on the localization of measurement and gender (Sund-Levander, Forsberg and Wahren, 2002). The temperature range between day/active and night/not-active in men is approximately 36.7° - 37.5° (rectal) and in women about 36.8° - 37.1° (rectal), (Sund-Levander, Forsberg and Wahren, 2002). Therefore, the amplitude of the temperature is much smaller.

7.3.1. The insoluble protein content of N2A cells kept in temperature cycles decreases compared to N2A cells kept in constant temperature condition

Interestingly, I found a decrease in insoluble protein content of N2A cells kept in temperature cycles versus cells kept in constant condition (figure 6.7.). The insoluble content in cells kept in 24h and non-24h temperature cycles did not differ, however (figure 6.7, A). This suggests that (i) temperature has an impact on the insoluble protein content in senescent N2A cells and (ii) that temperature influences the PN directly rather than through circadian clock mechanisms.

In primary experiments, I assessed that the insoluble protein content increases in senescent N2A cells (figure 6.6.). Therefore, the decrease in insoluble protein content in N2A cells, kept in temperature cycles as opposed to constant temperature conditions, may occur due to either fewer senescent cells or senescent cells with less insoluble protein content. This would imply that temperature amplitudes (range from the lowest to the highest temperature) protect the cell from senescent induction or protein aggregation. The potential benefit of temperature cycles is consistent with the fact that temperature cycles - not constant temperature conditions - are physiological in mammals with lower temperatures at night and higher temperatures during the day (Sund-Levander, Forsberg and Wahren, 2002; Baud, Magistretti and Petit, 2013). Interestingly, in the elderly the temperature amplitude declines (Czeisler *et al.*, 1992), suggesting that the SCN output (temperature) alters in aging. Furthermore, the decrease in temperature amplitude could be one explanation why protein pathology increase

in tissues of elderly and ND patients (Taylor and Dillin, 2011b). As already described in the introduction, studies of the SCN in aging mammals support that the SCN is impaired in older versus young mammals.

It could be shown that mRNA levels of some clock genes are less expressed in the SCN of aged rats (Asai *et al.*, 2001) and hamsters (Kolker *et al.*, 2003) when induced with light compared to young controls. Also, there is evidence that neuronal firing patterns in the SCN of old rats are aberrant, implying a disruption in the SCN and its output pathways in aging (E. Satinoff *et al.*, 1993). Additionally, the amount of neurons which modulate rhythmicity in the SCN decrease (assessed in male humans (Zhou, Hofman and Swaab, 1995)) and their polypeptide expression becomes arrhythmic (assessed in female rats (Krajnak *et al.*, 1998)). In summary, it is possible that through an impairment of the SCN in aged mammals the temperature amplitude declines leading to an increase in protein aggregation. To confirm this hypothesis the relationship between the impairment of the SCN, temperature amplitude and aggregation in aging must be further investigated.

Another aspect could be that constant temperature condition, being less physiological, induces more stress in N2A cells or leads to an impaired stress response, which ends in an increase of misfolded proteins and aggregation. Normally, a healthy cell copes with stress induced increase of misfolding proteins through stress response mechanisms such as reducing protein synthesis (Harding *et al.*, 2000; Hollien *et al.*, 2009; Gardner and Walter, 2011; Shalgi *et al.*, 2013), up-regulation of the chaperone system (Ananthan, Goldberg and Voellmy, 1986) and degradation machineries (Fornace *et al.*, 1989; Shang, Gong and Taylor, 1997). When talking about stress response pathways there are three main ones described: the heat shock response pathway (HSR) (Lindquist, 1986), the unfolded response pathway (UPR) (Hetz, Chevet and Oakes, 2015) and the oxidative stress response pathway (OxR) (Sykiotis and Bohmann, 2010). These mechanisms are beneficial for the cell with positive effects on lifespan (assessed in C.elegans (Hansen *et al.*, 2007)) and negative effects on lifespan if not functioning (Ben-Zvi *et al.*, 2009). In aging, these stress response pathways decline, suggesting that a cells' capacity to cope with stress decreases with aging

(Sala, Bott and Morimoto, 2017). This coincides with our results that the insoluble protein content in senescent cells is higher when compared to control cells. The activation of these coping mechanisms may be disturbed when cells are kept in a constant temperature condition.

But what role does temperature play in activating stress response pathways? Well assessed is the effect of temperature on the HSR. It is regulated by heat shock factor 1 (HSF 1) which is systemically controlled by thermo-sensory neurons (assessed in *C.elegans* (Prahlad, Cornelius and Morimoto, 2008; Tatum *et al.*, 2015) and serotonergic stimuli (Tatum *et al.*, 2015).

HSF 1 stays inactive in its monomeric state and is associated to chaperones such as HSP 90 (Zou *et al.*, 1998a) and HSP 70 (Zheng *et al.*, 2016). When stress in a cell occurs, chaperones are disconnecting from HSF1. Free HSF 1 then forms trimers and induces transcription of heat shock proteins such as HSP 70 and HSP 90 through heat shock elements (Baler, Dahl and Voellmy, 1993; Shi, Mosser and Morimoto, 1998; Zou *et al.*, 1998a). Heat shock proteins protect the cell from protein aggregation. The temperature level to induce stress depends on the cell type or organism, which adds to the complexity of HSR. In *C.elegans* stress induction has been assessed with a temperature of 30 - 34° (Prahlad, Cornelius and Morimoto, 2008), whereas in N2A cell culture (K Kaarniranta *et al.*, 2002) or other cell types temperatures between 41° and 43° were used to induce a HSR (Zou *et al.*, 1998a; K Kaarniranta *et al.*, 2002). Also, the mechanisms following activation vary among cells. As an example, it could be shown that HSR in N2A cells including HSP 70 induction is regulated on a translational level, whereas the HSR of IMR-32 neuroblastoma cells are regulated on the transcriptional level (K Kaarniranta *et al.*, 2002). In hippocampal neurons there even seems to be a lack of HSF 1 (K Kaarniranta *et al.*, 2002). In summary, the HSR is complex and differs among cell types. Therefore, we wanted to look at the HSR of N2A cells for follow up experiments through assessing chaperone expressions with and without heat shock. Furthermore, our aim was to investigate whether stress response is induced by constant temperature or temperature cycles on senescent N2A cells, by investigating HSP 90 expression (discussion, chapter 6.4.).

7.3.2. The insoluble protein content of U2OS cells kept in temperature cycles increases compared to U2OS cells kept in constant temperature condition

In contrast to N2A experiments, in U2OS cells there was an increase in insoluble protein content of two out of three biological replicates in cells kept in temperature cycles versus cells kept in constant temperature condition (figure 6.9, B/C). This increase occurred in both 24h and non-24h temperature cycles, implicating once more that temperature has a direct impact on the PN rather than via circadian clock mechanisms.

This data is not significant, however, possibly due to technical errors in one of the replicates. When comparing the insoluble protein content within one condition, one can only see a significant increase in cells kept in non-24h temperature condition (figure 6.9, A).

This may be due to later senescent occurrence in U2OS cells than in N2A cells and differences in the expression of PN components depending on the cell type (discussion below). Therefore, a longer observation period could be necessary to see an effect. In contrast, Cain et al and Riccardo Vanzo (Institute of Medical Psychology Munich, now at The Center for Stem Cell Research, University of Copenhagen) assessed that LBH induces senescence in U2OS cells after approximately seven days. They detected senescent cells also with SA-b-gal-staining (Cain *et al.*, 2013). In future experiments, SA-b-gal staining will be repeated with LBH induced senescence cells at constant temperature and in temperature cycles. Hence, we will obtain information about senescent induction and cell survival in senescent cells kept in different temperature conditions.

Assuming that the insoluble protein content of U2OS cells does not decrease in temperature cycles, other than in N2A cells, it is suggested that temperature has a different effect on the PN depending on the cell type. This is consistent with findings that protein expression patterns vary in human tissue including PN components (Uhlen *et al.*, 2015; Sala, Bott and Morimoto, 2017). Therefore, the PN in U2OS cells (origin in bone tissue) may react differently on stimuli such as temperature.

Another factor that may have an impact on the different insoluble protein results in N2A and U2OS cells is FBS. The medium used for U2OS cells contained no FBS because U2OS cells were more robust to nutrition withdrawal.

In N2A cells however, FBS was necessary for cell survival. Therefore, in U2OS medium without FBS cell proliferation has been rather inhibited, leading to less cell confluence. Furthermore, as investigated in other institutes, FBS induces cell differentiation (Seeds *et al.*, 1970; Evangelopoulos, Weis and Krüttgen, 2005). Therefore, U2OS cells without FBS may differentiate rather than undergoing senescence, especially when comparing to senescent induced N2A cells (medium contained 0.5 % FBS). In addition to this, we used a slightly different protein extraction procedure in U2OS cells than in N2A cells. In N2A cells we used sonication in order to handle the viscous protein samples. In U2OS cells no sonication was needed because protein samples were less viscous. Nevertheless, sonication is described to increase protein aggregation (Stathopulos *et al.*, 2004), not explaining our results with generally lower insoluble protein content in U2OS cells.

7.3.3. Summary

A decrease in the insoluble protein content in N2A cells kept in temperature cycling versus constant temperature condition has been investigated. There was no significant difference in insoluble protein content of cells kept in 24h or non-24h temperature cycles, suggesting an influence of temperature directly on the cell than via circadian clock mechanisms. In contrast, the insoluble protein content of U2OS cells seems to increase in temperature cycles. Also, no differences in the insoluble protein content between 24h and non-24h temperature condition was detected. We conclude that temperature has a direct effect on the PN of both N2A and U2OS cells. In addition, this data suggests that the PN of different cell types react individually on temperature stimuli, leading to higher or lower insoluble protein contents if kept in temperature cycles.

7.4. Chaperones as mediators between the clock and Proteostasis

Chaperones play a key role in maintaining protein balance in a cell. They protect the cell from protein misfolding and protein aggregation (Hartl, 1996; Hartl, Bracher and Hayer-Hartl, 2011). If errors in the chaperone system occur, misfolded proteins and protein aggregates increase. Because the insoluble protein ratio of N2A cells kept in temperature cycles is lower than in constant temperature condition (figure 6.7.), we analyzed different chaperone expressions depending on the temperature condition. Chaperones may be higher expressed (or more active) in temperature cycles or lower expressed (or inhibited) in constant temperature condition. Therefore, we wanted to first assess whether key members of chaperone families are expressed in U2OS and N2A cells under constant temperature conditions and after heat shock induction. And secondly, we wanted to investigate the influence of the circadian clock and temperature on its expression.

7.4.1. Chaperone expression differs in N2A and U2OS cells

Different expression patterns of chaperones in U2OS and N2A cells were found (figure 6.11.). This is consistent with finding that even among neuronal cells different HSP 70 expressions occur (K. Kaarniranta *et al.*, 2002), implying that chaperone expression patterns differ from cell to cell. Also, when a cell ages or degenerates the chaperone expression changes (Brehme *et al.*, 2014), suggesting that chaperones are involved in aging processes and protein aggregation pathology.

HSP 90 and GRP 78 were expressed in both N2A and U2OS cells, whereas HSP 70 and HSP 25/27 were only detected in U2OS cells. Considering that each chaperone has its distinct function, one assumes a different output of the PN in U2OS and N2A cells including protein aggregation. Therefore, the different chaperone expression patterns might be one cause of the differences in insoluble protein content between U2OS and N2A cells (chapter 6.2.2 and 6.2.3.).

Especially HSP 70 seem to play an important role in protecting the cell or organism from protein aggregation, as assessed in drosophila (Auluck *et al.*, 2002), mouse models (Hoshino *et al.*, 2011) and *in vitro* (Chafekar *et al.*, 2012). Thus, the PN of U2OS cells may cope with a temperature trigger differently through expressing certain chaperones such as HSP 70 and having better coping mechanisms to minimize insoluble proteins. Because chaperones were assessed with western blot, which cannot detect low protein concentrations, one has to consider that chaperones not detected in N2A cell protein samples might still be expressed in low concentrations. Kaarniranta et al. were able to detect HSP 70 with western blot after heat shock induction (43 °, 15 minutes) and a recovery time of 180 minutes (K Kaarniranta *et al.*, 2002), possibly due to higher protein concentrations. The HSP 70 response is regulated on a translational level and needs time to up regulated (K. Kaarniranta *et al.*, 2002). Therefore, we also used a recovery time after heat shock induction. In our experiment a six hours recovery time was used, because prior experiments show a with Western Blot well detectable HSP70 level in N2A cells after a recovery time of six hours (K Kaarniranta *et al.*, 2002). Nevertheless, we did not detect HSP 70 in N2A cells even after heat shock (figure 6.12). Reasons for this could be that (i) the recovery time was too long or (ii) protein samples with lower protein concentrations had been used. In U2OS cells, however, HSP 70 expression increased after heat shock (figure 6.12, B). In N2A cells, an up-regulation of HSP 90 after heat shock was investigated (figure 6.13.). To draw further conclusions, more than one replicate will be needed in future experiments.

7.4.2. No changes in HSP 90 expression in N2A cells kept in constant versus cycling temperature condition

It is known that protein aggregates increase in elderly (Yang, Ang and Strong, 2005) and ND patients (Masters *et al.*, 1985; Baba *et al.*, 1998), suggesting an impairment of the chaperone system. Based on studies that show correlations between age or age-associated ND and disrupted circadian timing systems (Huang *et al.*, 2002; Hatfeld *et al.*, 2004; Merlino *et al.*, 2010; Weissová *et al.*, 2016), we wanted to investigate the link between the circadian clock and chaperones in N2A cells. Thus we chose HSP 90 as a chaperone candidate to assess the influence of temperature entrainment on its expression. HSP 90 was well detectable in N2A

cells and is known to play a key role in interacting with proteins like steroid hormone receptors and protein kinases (Taipale, Jarosz and Lindquist, 2010) as well as with ND associated proteins (Dou *et al.*, 2003). Also, HSP 90 is strongly linked to HSF 1. HSP 90 is induced by HSF 1 and inhibits HSF 1 under non-stress condition (Zou *et al.*, 1998b). HSF 1 is a heat shock transcription factor, which regulates heat shock response.

If stress occurs and misfolded proteins increase, HSF-1 binds to specified DNA sides, the heat shock elements (Parker and Topol, 1984; Wu, 1984) and induces - among others - transcription of chaperones, co-chaperones and mitotic regulators (Trinklein *et al.*, 2004; Vihervaara and Sistonen, 2014). Interestingly, it also induces transcription of key circadian clock genes and plays an important role in synchronizing the cellular clock (Tamaru *et al.*, 2011). It binds to the DNA in a oscillatory manner which is influenced by temperature (Reinke *et al.*, 2008). In summary, it is a key element in translating entraining input of temperature oscillation in cellular clock processes. Furthermore, it is associated with aging processes, which has been assessed in *C. elegans* (Hsu, Murphy and Kenyon, 2003). Here, down-regulating HSF 1 activity led to a reduced life span and an increase in tissue aging.

In my experimental setting, I wanted to generally assess the influence of 24h temperature cycles on HSP 90 expression and compare it to the constant temperature condition. Surprisingly, no significant differences were found between HSP 90 expression in the constant versus cycling temperature condition (figure 6.14). Nor was there any kind of rhythmic expression found. However, N2A cells were not senescent. This would need a more complex experimental setting, with first senescent induction and secondly temperature entrainment. Because HSF 1 seems to have an impact on aging processes (assessed in *C. elegans* (Hsu, Murphy and Kenyon, 2003)) and chaperones are protective to aging associated ND pathology, the effect of temperature entrainment of senescent cells will be an interesting part of future experiments however.

7.4.3. Summary

We showed that chaperone expression differs in N2A and U2OS cells. HSP 90 and GRP 78 were expressed in both cells, whereas HSP70 and HSO 25/27 were only detected in U2OS cells. Furthermore, we assessed the influence of constant condition and temperature cycle on HSP 90 expression in N2A cells. HSP 90 it is strongly linked to HSF 1, which is an important transcription factor and regulator in the heat shock response. Even though HSF 1 is known to play a role in the process of entraining circadian clocks by temperature, we could not identify an effect of temperature between 34° and 37 °C on HSP 90 expression. There were no differences between HSP 90 expressions in constant versus cycling temperature condition, nor an effect on rhythmicity. In future works it will be interesting to investigate the effect of 24h and constant temperature condition on senescence induced cells with more chaperone candidates. Rhythmic chaperone candidates that differ in cycling temperature versus constant temperature condition might bring us closer in understanding the causative link between temperature and insoluble protein alterations in senescent N2A and U2OS cells. If the underlying mechanisms are better understood in future, chaperones might function as a therapy option to fight neurodegenerative diseases.

8. Bibliography

Acosta, J. C. *et al.* (2008) 'Chemokine signaling via the CXCR2 receptor reinforces senescence.', *Cell*, 133(6), pp. 1006–18. doi:10.1016/j.cell.2008.03.038.

Agarraberes, F. A. and Dice, J. F. (2001) 'A molecular chaperone complex at the lysosomal membrane is required for protein translocation', *Journal of Cell Science*, 114(13), pp. 2491–2499. Available at: https://jcs.biologists.org/content/114/13/2491.long.

Ahlberg, J. and Glaumann, H. (1985) 'Uptake--microautophagy--and degradation of exogenous proteins by isolated rat liver lysosomes. Effects of pH, ATP, and inhibitors of proteolysis.', *Experimental and molecular pathology*, 42(1), pp. 78–88. doi: 10.1016/0014-4800(85)90020-6.

Albrecht, U. (2012) 'Timing to Perfection: The Biology of Central and Peripheral Circadian Clocks', *Neuron*, 74(2), pp. 246–260. doi: 10.1016/j.neuron.2012.04.006.

Alcorta, D. A. *et al.* (1996) 'Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts.', *Proceedings of the National Academy of Sciences of the United States of America*, 93(24), pp. 13742–7. doi: 10.1073/pnas.93.24.13742.

Alzheimer's Association (2016) '2016 Alzheimer's disease facts and figures.', *Alzheimer's & dementia : the journal of the Alzheimer's Association*, 12(4), pp. 459–509. doi: 10.1016/j.jalz.2016.03.001.

Amin, V., Cumming, D. V. E. and Latchman, D. S. (1996) 'Over-expression of heat shock protein 70 protects neuronal cells against both thermal and ischaemic stress but with different efficiencies', *Neuroscience Letters*, 206(1), pp. 45–48. doi 10.1016/0304-3940(96)12421-6.

Ananthan, J., Goldberg, A. L. and Voellmy, R. (1986) 'Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes.', *Science (New York, N.Y.)*, 232(4749), pp. 522–4. doi: 10.1126/science.3083508.

Ancoli-Israel, S. *et al.* (1997) 'Variations in circadian rhythms of activity, sleep, and light exposure related to dementia in nursing-home patients.', *Sleep*, 20(1), pp. 18–23. Available at: https://pubmed.ncbi.nlm.nih.gov/9130329/.

Ancoli-Israel, S. *et al.* (2002) 'Effect of light treatment on sleep and circadian rhythms in demented nursing home patients.', *Journal of the American Geriatrics Society*. NIH Public Access, 50(2), pp. 282–9. doi: 10.1046/j.1532-5415.2002.50060.x.

Ancoli-Israel, S. *et al.* (2003) 'Increased Light Exposure Consolidates Sleep and Strengthens Circadian Rhythms in Severe Alzheimer's Disease Patients', *Behavioral Sleep Medicine*. Lawrence Erlbaum Associates, Inc. , 1(1), pp. 22–36. doi: 10.1207/S15402010BSM0101_4.

Anfinsen, C. B. (1973) 'Principles that govern the folding of protein chains.', *Science (New York, N.Y.)*, 181(4096), pp. 223–30. doi: 10.1126/science.181.4096.223.

Antón, L. C. *et al.* (1999) 'Intracellular localization of proteasomal degradation of a viral antigen.', *The Journal of cell biology*, 146(1), pp. 113–24. doi: 10.1083/jcb.146.1.113.

Armstrong, R. A. (1995) 'Beta-amyloid deposition in the medial temporal lobe in elderly non-demented brains and in Alzheimer's disease.', *Dementia (Basel, Switzerland)*, 6(3), pp. 121–5. doi: 10.1159/000106933.

Asai, M. *et al.* (2001) 'Circadian profile ofPer gene mRNA expression in the suprachiasmatic nucleus, paraventricular nucleus, and pineal body of aged rats', *Journal of Neuroscience Research*. John Wiley & Sons, Ltd, 66(6), pp. 1133–1139. doi: 10.1002/jnr.10010.

Aschoff, J. and Wever, R. (1962) 'Spontanperiodik des Menschen bei Ausschluß aller Zeitgeber', *Naturwissenschaften*. Springer, 49(15), pp. 337–342. doi: 10.1007/BF01185109.

Auluck, P. K. *et al.* (2002) 'Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease.', *Science (New York, N.Y.)*, 295(5556), pp. 865–8. doi: 10.1126/science.1067389.

Baba, M. *et al.* (1998) 'Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies.', *The American journal of pathology*, 152(4), pp. 879–84. Available at:http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1858234.

Baker-Nigh, A. *et al.* (2015) 'Neuronal amyloid-β accumulation within cholinergic basal forebrain in ageing and Alzheimer's disease', *Brain*, 138(6), pp. 1722–1737. doi: 10.1093/brain/awv024.

Balch, W. E. *et al.* (2008) 'Adapting Proteostasis for Disease Intervention', *Science*, 319(5865), pp. 916–919. doi: 10.1126/science.1141448.

Balchin, D., Hayer-Hartl, M. and Hartl, F. U. (2016) 'In vivo aspects of protein folding and quality control.', *Science (New York, N.Y.)*, 353(6294),p. aac4354. doi: 10.1126/science.aac4354.

Baler, R., Dahl, G. and Voellmy, R. (1993) 'Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1.', *Molecular and Cellular Biology*. American Society for Microbiology Journals, 13(4), pp. 2486–2496. doi: 10.1128/MCB.13.4.2486.

Balsalobre, A. *et al.* (2000) 'Resetting of circadian time in peripheral tissues by glucocorticoid signaling.', *Science (New York, N.Y.)*, 289(5488), pp. 2344–7. doi: 10.1126/science.289.5488.2344.

Balsalobre, A., Marcacci, L. and Schibler, U. (2000) 'Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts.', *Current biology : CB*, 10(20), pp. 1291–4. doi: 10.1016/s0960-9822(00)00758-2.

Barone, P. *et al.* (2009) 'The PRIAMO study: A multicenter assessment of nonmotor symptoms and their impact on quality of life in Parkinson's disease.', *Movement disorders : official journal of the Movement Disorder Society*, 24(11), pp. 1641–9. doi: 10.1002/mds.22643.

Baser, A. *et al.* (2019) 'Onset of differentiation is post-transcriptionally controlled in adult neural stem cells', *Nature*. Nature Publishing Group, 566(7742), pp. 100–104. doi: 10.1038/s41586-019-0888-x.

Baud, M. O., Magistretti, P. and Petit, J.-M. (2013) 'Sustained sleep fragmentation affects brain temperature, food intake and glucose tolerance in mice', *Journal of Sleep Research*. John Wiley & Sons, Ltd (10.1111), 22(1), pp. 3–12. doi: 10.1111/J.1365-2869.2012.01029.X.

Bélanger, V., Picard, N. and Cermakian, N. (2006) 'The Circadian Regulation of *Presenilin-2* Gene Expression', *Chronobiology International*. Taylor & Francis, 23(4), pp. 747-766. doi: 10.1080/07420520600827087.

Ben-Zvi, A. *et al.* (2009) 'Collapse of proteostasis represents an early molecular event in Caenorhabditis elegans aging', *Pnas*, 106(35), pp. 14914–14919. doi: 10.1073/pnas.0902882106.

Bence, N. F., Sampat, R. M. and Kopito, R. R. (2001) 'Impairment of the Ubiquitin-Proteasome System by Protein Aggregation', *Science*, 292(5521), pp. 1552–1555. doi:10.1126/science.292.5521.1552.

Bennett, E. J. *et al.* (2005) 'Global Impairment of the Ubiquitin-Proteasome System by Nuclear or Cytoplasmic Protein Aggregates Precedes Inclusion Body Formation', *Molecular Cell*, 17(3), pp. 351–365. doi: 10.1016/j.molcel.2004.12.021.

Benzinger, T. L. *et al.* (1998) 'Propagating structure of Alzheimer's beta-amyloid(10-35) is parallel beta-sheet with residues in exact register.', *Proceedings of the National Academy of Sciences of the United States of America*, 95(23), pp. 13407–12. doi: 10.1073/pnas.95.23.13407.

Bianchetti, A. *et al.* (1995) 'Predictors of mortality and institutionalization in Alzheimer disease patients 1 year after discharge from an Alzheimer dementia unit.', *Dementia (Basel, Switzerland)*, 6(2), pp. 108–12. doi: 10.1159/000106930.

Bodenstein, C., Heiland, I. and Schuster, S. (2012) 'Temperature compensation and entrainment in circadian rhythms', *Physical Biology*. IOP Publishing, 9(3), p. 036011. doi: 10.1088/1478-3975/9/3/036011.

Bodis-Wollner, I. *et al.* (2014) 'α-synuclein in the inner retina in parkinson disease', *Annals of Neurology*, 75(6), pp. 964–966. doi: 10.1002/ana.24182.

Bodnar, A. G. *et al.* (1998) 'Extension of life-span by introduction of telomerase into normal human cells.', *Science (New York, N.Y.)*, 279(5349), pp. 349–52. doi: 10.1126/science.279.5349.349.

Bollinger, T. and Schibler, U. (2014) 'Circadian rhythms - from genes to physiology and disease.', *Swiss medical weekly*, 144(July), p. w13984. doi: 10.4414/smw.2014.13984.

Braig, M. *et al.* (2005) 'Oncogene-induced senescence as an initial barrier in lymphoma development.', *Nature*, 436(7051), pp. 660–5. doi: 10.1038/nature03841.

Brehme, M. *et al.* (2014) 'A Chaperome Subnetwork Safeguards Proteostasis in Aging and Neurodegenerative Disease', *Cell Reports*, 9(3), pp. 1135–1150. doi: 10.1016/j.celrep.2014.09.042.

Brenner, A. J., Stampfer, M. R. and Aldaz, C. M. (1998) 'Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation.', *Oncogene*, 17(2), pp. 199–205. doi: 10.1038/sj.onc.1201919.

Brown, I. R. (2007) 'Heat Shock Proteins and Protection of the Nervous System', *Annals of the New York Academy of Sciences*. John Wiley & Sons, Ltd (10.1111), 1113(1), pp. 147–158. doi: 10.1196/annals.1391.032.

Brown, S. A. *et al.* (2002) 'Rhythms of mammalian body temperature can sustain peripheral circadian clocks.', *Current Biology*. Elsevier, 12(18), pp. 1574–83. doi: 10.1016/S0960-9822(02)01145-4.

Buhr, E. D., Yoo, S.-H. and Takahashi, J. S. (2010) 'Temperature as a universal resetting cue for mammalian circadian oscillators.', *Science (New York, N.Y.)*, 330(6002), pp. 379–85. doi: 10.1126/science.1195262.

Burd, C. E. *et al.* (2013) 'Monitoring Tumorigenesis and Senescence In Vivo with a p16INK4a-Luciferase Model', *Cell*, 152(1–2), pp. 340–351. doi: 10.1016/j.cell.2012.12.010.

Cai, Y. *et al.* (2010) 'Expression of clock genes Per1 and Bmal1 in total leukocytes in health and Parkinson's disease.', *European journal of neurology*, 17(4), pp. 550–4. doi: 10.1111/j.1468-1331.2009.02848.x.

Cain, J. E. *et al.* (2013) 'Sustained Low-Dose Treatment with the Histone Deacetylase Inhibitor LBH589 Induces Terminal Differentiation of Osteosarcoma Cells', *Sarcoma*. Hindawi Publishing Corporation Sarcoma, 608964(11), p. 11. doi: 10.1155/2013/608964.

Campbell, S. S. *et al.* (1988) 'Exposure to light in healthy elderly subjects and Alzheimer's patients.', *Physiology & behavior*, 42(2), pp. 141–4. doi: 10.1016/0031-9384(88)90289-2.

von Campenhausen, S. *et al.* (2005) 'Prevalence and incidence of Parkinson's disease in Europe', *European Neuropsychopharmacology*. Elsevier, 15(4), pp. 473–490. doi: 10.1016/J.EURONEURO.2005.04.007.

Chafekar, S. M. *et al.* (2012) 'Pharmacological tuning of heat shock protein 70 modulates polyglutamine toxicity and aggregation.', *ACS chemical biology*, 7(9), pp. 1556–64. doi: 10.1021/cb300166p.

Chang, B.-D. *et al.* (2002) 'Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent', *Pnas*, 99. doi: 10.1073_pnas.012602599.

Chiang, H. L. *et al.* (1989) 'A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins.', *Science (New York, N.Y.)*, 246(4928), pp. 382-5. doi: 10.1126/science.2799391.

Chiti, F. and Dobson, C. M. (2006) 'Protein Misfolding, Functional Amyloid, and Human Disease', *Annual Review of Biochemistry*. Annual Reviews , 75(1), pp. 333–366. doi: 10.1146/annurev.biochem.75.101304.123901.

Chondrogianni, N. *et al.* (2005) 'Overexpression of proteasome beta5 assembled subunit increases the amount of proteasome and confers ameliorated response to oxidative stress and higher survival rates.', *The Journal of biological chemistry*, 280(12), pp. 11840–50. doi: 10.1074/jbc.M413007200.

Clausen, L. *et al.* (2019) 'Protein stability and degradation in health and disease', *Advances in Protein Chemistry and Structural Biology*. Academic Press, 114, pp. 61–83. doi: 10.1016/BS.APCSB.2018.09.002.

Coller, H. A., Sang, L. and Roberts, J. M. (2006) 'A new description of cellular quiescence.', *PLoS biology*. Public Library of Science, 4(3), p. e83. doi: 10.1371/journal.pbio.0040083.

Conti, L. *et al.* (2001) 'Shc signaling in differentiating neural progenitor cells', *Nature neuroscience*, 4(6), pp. 579–586. doi: 10.1038/88395.

Coppé, J.-P. *et al.* (2010) 'A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen.', *PloS one*. Edited by M. V. Blagosklonny, 5(2), p. e9188. doi: 10.1371/journal.pone.0009188.

Cuervo, A. M. and Dice, J. F. (2000) 'Age-related Decline in Chaperone-mediated Autophagy*', *The Journal of biological chemistry*. in Press. doi: 10.1074/jbc.M002102200.

Czeisler, C. A. *et al.* (1992) 'Association of sleep-wake habits in older people with changes in output of circadian pacemaker.', *Lancet (London, England)*, 340(8825), pp. 933–6. doi: 10.1016/0140-6736(92)92817-y.

D'Adda di Fagagna, F. *et al.* (2003) 'A DNA damage checkpoint response in telomere-initiated senescence', *Nature*, 426(6963), pp. 194–198. doi: 10.1038/nature02118.

David, D. C. *et al.* (2010) 'Widespread Protein Aggregation as an Inherent Part of Aging in C. elegans', *PLoS Biology*. Edited by J. Ahringer. Public Library of Science, 8(8), p. e1000450. doi: 10.1371/journal.pbio.1000450.

Debacq-Chainiaux, F. *et al.* (2009) 'Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and in vivo', *Nature Protocols*, 4(12), pp. 1798–1806. doi: 10.1038/nprot.2009.191.

Decoursey, P. J. *et al.* (1998) 'Relationship of Circadian Temperature and Activity Rhythms in Two Rodent Species', *Physiology & Behavior*. Elsevier, 65(3), pp. 457–463. doi: 10.1016/S0031-9384(98)00187-5.

Der-Sarkissian, A. *et al.* (2003) 'Structural organization of alpha-synuclein fibrils studied by site-directed spin labeling.', *The Journal of biological chemistry*, 278(39), pp. 37530–5. doi: 10.1074/jbc.M305266200.

Dice, J. F. (1982) 'Altered degradation of proteins microinjected into senescent human fibroblasts.', *The Journal of biological chemistry*, 257(24), pp. 14624–7. Available at: https://www.jbc.org/content/257/24/14624.long.

Dimri, G. P. *et al.* (1995) 'A biomarker that identifies senescent human cells in culture and in aging skin in vivo.', *Proceedings of the National Academy of Sciences of the United States of America*, 92(20), pp. 9363–7. doi: 10.1073/pnas.92.20.9363.

Do, M. T. H. and Yau, K.-W. (2010) 'Intrinsically photosensitive retinal ganglion cells.', *Physiological reviews*. NIH Public Access, 90(4), pp. 1547–81. doi: 10.1152/physrev.00013.2010.

Dou, F. *et al.* (2003) 'Chaperones increase association of tau protein with microtubules.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(2), pp. 721–6. doi: 10.1073/pnas.242720499.

Druelle, C. *et al.* (2016) 'ATF6α regulates morphological changes associated with senescence in human fibroblasts', *Oncotarget*, 7(42), pp. 67699–67715. doi: 10.18632/oncotarget.11505.

Dubrovsky, Y. V, Samsa, W. E. and Kondratov, R. V (2010) 'Deficiency of circadian protein CLOCK reduces lifespan and increases age-related cataract development in mice.', *Aging*, 2(12), pp. 936–44. doi: 10.18632/aging.100241.

Duffy, J. F. and Wright, K. P. (2005) 'Entrainment of the Human Circadian System by Light', *Journal of Biological Rhythms*. Sage PublicationsSage CA: Thousand Oaks, CA, 20(4), pp. 326–338. doi: 10.1177/0748730405277983.

Eckel-Mahan, K. and Sassone-Corsi, P. (2013) 'Metabolism and the circadian clock converge.', *Physiological reviews*. American Physiological Society, 93(1), pp. 107-35. doi: 10.1152/physrev.00016.2012.

Eghlidi, D. H. *et al.* (2018) 'Gene expression profiling of the SCN in young and old rhesus macaques', *Journal of Molecular Endocrinology*, 61(2), pp. 57–67. doi: 10.1530/JME-18-0062.

Erhardt, C. *et al.* (2004) 'Modulation of photic resetting in rats by lesions of projections to the suprachiasmatic nuclei expressing p75 neurotrophin receptor', *European Journal of Neuroscience*. John Wiley & Sons, Ltd (10.1111), 19(7), pp. 1773–1788. doi: 10.1111/j.1460-9568.2004.03281.x.

Erjavec, N. *et al.* (2007) 'Accelerated aging and failure to segregate damaged proteins in Sir2 mutants can be suppressed by overproducing the protein aggregation-remodeling factor Hsp104p.', *Genes & development*, 21(19), pp. 2410–21. doi: 10.1101/gad.439307.

Escusa-Toret, S., Vonk, W. I. M. and Frydman, J. (2013) 'Spatial sequestration of misfolded proteins by a dynamic chaperone pathway enhances cellular fitness during stress', *Nature Cell Biology*, 15(10), pp. 1231–1243. doi: 10.1038/ncb2838.

Evangelopoulos, M. E., Weis, J. and Krüttgen, A. (2005) 'Signalling pathways leading to neuroblastoma differentiation after serum withdrawal: HDL blocks neuroblastoma differentiation by inhibition of EGFR.', *Oncogene*, 24, pp. 3309–3318. doi: 10.1038/sj.onc.1208494.

Evangelopoulos Maria, Wüller Stefan, W. J. (2009) 'A role of nitric oxide in neurite outgrowth of neuroblastoma cells triggered by mevastatin or serum reduction', *Neuroscience Letters*, 468(1), pp. 28–33. doi: 10.1016/j.neulet.2009.10.054.

Evangelou, K. *et al.* (2017) 'Robust, universal biomarker assay to detect senescent cells in biological specimens', *Aging Cell*, 16(1), pp. 192–197. doi: 10.1111/acel.12545.

Fabunmi, R. P. *et al.* (2000) 'Activity and regulation of the centrosome-associated proteasome.', *The Journal of biological chemistry*, 275(1), pp. 409–13. doi: 10.1074/jbc.275.1.409.

Feleciano, D. R. *et al.* (2019) 'Crosstalk Between Chaperone-Mediated Protein Disaggregation and Proteolytic Pathways in Aging and Disease.', *Frontiers in aging neuroscience*. Frontiers Media SA, 11, p. 9. doi: 10.3389/fnagi.2019.00009.

Fernández-Fernández, M. R. and Valpuesta, J. M. (2018) 'Hsp70 chaperone: a master player in protein homeostasis.', *F1000Research*. Faculty of 1000 Ltd, 7. doi: 10.12688/f1000research.15528.1.

Ferrari, E. *et al.* (1995) 'Neuroendocrine correlates of the aging brain in humans.', *Neuroendocrinology*, 61(4), pp. 464–70. doi: 10.1159/000126869.

Ferrari, E. *et al.* (1996) 'Chrono-neuroendocrine markers of the aging brain.', *Aging (Milan, Italy)*, 8(5), pp. 320–7. doi: 10.1007/BF03339588.

Ferrari, E. *et al.* (2001) 'Age-related changes of the adrenal secretory pattern: possible role in pathological brain aging.', *Brain research. Brain research reviews*, 37(1–3), pp. 294–300. doi: 10.1016/s0165-0173(01)00133-3.

Figueiro, M. G. *et al.* (2014) 'Tailored lighting intervention improves measures of sleep, depression, and agitation in persons with Alzheimer's disease and related dementia living in long-term care facilities.', *Clinical interventions in aging*, 9, pp. 1527–37. doi: 10.2147/CIA.S68557.

Forbes, D. *et al.* (2014) 'Light therapy for improving cognition, activities of daily living, sleep, challenging behaviour, and psychiatric disturbances in dementia', *Cochrane Database of Systematic Reviews*. John Wiley & Sons, Ltd, (2). doi: 10.1002/14651858.CD003946.pub4.

Fornace, A. J. *et al.* (1989) 'Ubiquitin mRNA is a major stress-induced transcript in mammalian cells.', *Nucleic acids research*, 17(3), pp. 1215–30. doi: 10.1093/nar/17.3.1215.

García-Mata, R. *et al.* (1999) 'Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera.', *The Journal of cell biology*, 146(6), pp. 1239–54. doi: 10.1083/jcb.146.6.1239.

Gardner, B. M. and Walter, P. (2011) 'Unfolded Proteins Are Ire1-Activating Ligands That Directly Induce the Unfolded Protein Response', *Science*, 333(6051), pp. 1891–1894. doi: 10.1126/science.1209126.

Genevaux, P., Georgopoulos, C. and Kelley, W. L. (2007) 'The Hsp70 chaperone machines of Escherichia coli: a paradigm for the repartition of chaperone functions.', *Molecular microbiology*, 66(4), pp. 840–57. doi: 10.1111/j.1365-2958.2007.05961.x.

Geng, Y.-Q. *et al.* (2010) 'Senescence-associated beta-galactosidase activity expression in aging hippocampal neurons', *Biochemical and Biophysical Research Communications*, 396(4), pp. 866–869. doi: 10.1016/j.bbrc.2010.05.011.

Georgakopoulou, E. *et al.* (2012) 'Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues', *Aging*, 5(1), pp. 37–50. doi: 10.18632/aging.100527.

Groh, N. *et al.* (2017) 'Age-Dependent Protein Aggregation Initiates Amyloid-β Aggregation', *Frontiers in Aging Neuroscience*, 9, p. 138. doi: 10.3389/fnagi.2017.00138.

Gruber, H. E. *et al.* (2007) 'Senescence in cells of the aging and degenerating intervertebral disc: immunolocalization of senescence-associated beta-galactosidase in human and sand rat discs.', *Spine*, 32(3), pp. 321–7. doi: 10.1097/01.brs.0000253960.57051.de.

Haas, A. L. *et al.* (1982) 'Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation.', *The Journal of biological chemistry*, 257(5), pp. 2543–8. Available at: https://www.jbc.org/content/257/5/2543.

Hainfellner, J. A. *et al.* (1998) 'Coexistence of Alzheimer-type neuropathology in Creutzfeldt-Jakob disease.', *Acta neuropathologica*, 96(2), pp. 116–22. doi: 10.1007/s004010050870.

Hansen, M. *et al.* (2007) 'Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans', *Aging Cell*. John Wiley & Sons, Ltd (10.1111), 6(1), pp. 95–110. doi: 10.1111/j.1474-9726.2006.00267.x.

Hara, E. *et al.* (1996) 'Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence.', *Molecular and cellular biology*, 16(3), pp. 859–67. doi: 10.1128/mcb.16.3.859.

Hara, T. *et al.* (2006) 'Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice', *Nature*, 441(7095), pp. 885–889. doi: 10.1038/nature04724.

Harada, C. N., Natelson Love, M. C. and Triebel, K. L. (2013) 'Normal cognitive aging.', *Clinics in geriatric medicine*. NIH Public Access, 29(4), pp. 737–52. doi: 10.1016/j.cger.2013.07.002.

Harding, H. P. *et al.* (2000) 'Regulated translation initiation controls stress-induced gene expression in mammalian cells.', *Molecular cell*, 6(5), pp. 1099–108. doi: 10.1016/s1097-2765(00)00108-8.

Harnois, C. and Di Paolo, T. (1990) 'Decreased dopamine in the retinas of patients with Parkinson's disease.', *Investigative ophthalmology & visual science*, 31(11), pp. 2473–5. Available at: https://iovs.arvojournals.org/article.aspx?articleid=2161210.

Harper, D. G. *et al.* (2005) 'Disturbance of endogenous circadian rhythm in aging and Alzheimer disease.', *The American journal of geriatric psychiatry : official journal of the American Association for Geriatric Psychiatry*, 13(5), pp. 359–68. doi: 10.1176/appi.ajgp.13.5.359.

Harris, C. C. and Hollstein, M. (1993) 'Clinical Implications of the p53 Tumor-Suppressor Gene', *New England Journal of Medicine*, 329(18), pp. 1318–1327. doi: 10.1056/NEJM199310283291807.

Hartl, F. U. (1996) 'Molecular chaperones in cellular protein folding', *Nature*, 381(6583), pp. 571–580. doi: 10.1038/381571a0.

Hartl, F. U., Bracher, A. and Hayer-Hartl, M. (2011) 'Molecular chaperones in protein folding and proteostasis', *Nature*. Nature Publishing Group, 475(7356), pp. 324–332. doi: 10.1038/nature10317.

Hartl, F. U. and Hayer-Hartl, M. (2009) 'Converging concepts of protein folding in vitro and in vivo', *Nature Structural & Molecular Biology*. Nature Publishing Group, 16(6), pp. 574–581. doi: 10.1038/nsmb.1591.

Hartmann, A. *et al.* (1997) 'Twenty-four hour cortisol release profiles in patients with Alzheimer's and Parkinson's disease compared to normal controls: ultradian secretory pulsatility and diurnal variation.', *Neurobiology of aging*, 18(3), pp. 285–9. doi: 10.1016/s0197-4580(97)80309-0.

Hatfeld, C. F. *et al.* (2004) 'Disrupted daily activity/rest cycles in relation to daily cortisol rhythms of home-dwelling patients with early Alzheimer's dementia', *Brain*. doi: 10.1093/brain/awh129.

Hatori, M. *et al.* (2008) 'Inducible ablation of melanopsin-expressing retinal ganglion cells reveals their central role in non-image forming visual responses.', *PloS one*. Edited by M. Hendricks, 3(6), p. e2451. doi: 10.1371/journal.pone.0002451.

Hayflick, L. and Moorhead, P. S. (1961) 'The serial cultivation of human diploid cell strains.', *Experimental cell research*, 25, pp. 585–621. doi: 10.1016/0014-4827(61)90192-6.

Herbig, U. *et al.* (2004) 'Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a).', *Molecular cell*, 14(4), pp. 501–13. doi: 10.1016/s1097-2765(04)00256-4.

Herbig, U. *et al.* (2006) 'Cellular senescence in aging primates.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 311(5765), p. 1257. doi: 10.1126/science.1122446.

Hernandez-Segura, A., Nehme, J. and Demaria, M. (2018) 'Hallmarks of Cellular Senescence', *Trends in Cell Biology*, 28(6), pp. 436–453. doi: 10.1016/j.tcb.2018.02.001.

Hershko, A. *et al.* (1982) 'Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. Relationship to the breakdown of abnormal proteins.', *The Journal of biological chemistry*, 257(23), pp. 13964–70. Available at: https://www.jbc.org/content/257/23/13964.long.

Hershko, A. *et al.* (1983) 'Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown.', *The Journal of biological chemistry*, 258(13), pp. 8206–14. Available at: https://www.jbc.org/content/258/13/8206.long.

Hetz, C., Chevet, E. and Oakes, S. A. (2015) 'Proteostasis control by the unfolded protein response', *Nature Cell Biology*. Nature Publishing Group, 17(7), pp. 829–838. doi: 10.1038/ncb3184.

Hipp, M. S., Park, S.-H. and Hartl, F. U. (2014) 'Proteostasis impairment in protein-misfolding and -aggregation diseases.', *Trends in cell biology*. Elsevier, 24(9), pp. 506–14. doi: 10.1016/j.tcb.2014.05.003.

Hofman, M. A. and Swaab, D. F. (1995) 'Influence of aging on the seasonal rhythm of the vasopressin-expressing neurons in the human suprachiasmatic nucleus.', *Neurobiology of aging*, 16(6), pp. 965–71. doi: 10.1016/0197-4580(95)02016-0.

Hollien, J. *et al.* (2009) 'Regulated Ire1-dependent decay of messenger RNAs in mammalian cells', *The Journal of Cell Biology*, 186(3), pp. 323–331. doi: 10.1083/jcb.200903014.

Hood, S. and Amir, S. (2017) 'Neurodegeneration and the Circadian Clock', *Frontiers in Aging Neuroscience*. Frontiers, 9, p. 170. doi: 10.3389/fnagi.2017.00170.

Hoshino, T. *et al.* (2011) 'Suppression of Alzheimer's disease-related phenotypes by expression of heat shock protein 70 in mice.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(14), pp. 5225–34. doi: 10.1523/JNEUROSCI.5478-10.2011.

Hough, R., Pratt, G. and Rechsteiner, M. (1986) 'Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates.', *The Journal of biological chemistry*, 261(5), pp. 2400–8. Available at: https://www.jbc.org/content/261/5/2400.long.

Howcroft, T. K. *et al.* (2013) 'The role of inflammation in age-related disease', *Aging (Albany NY)*. Impact Journals, LLC, 5(1), p. 84. doi: 10.18632/AGING.100531.

Hsu, A.-L., Murphy, C. T. and Kenyon, C. (2003) 'Regulation of aging and age-related disease by DAF-16 and heat-shock factor.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 300(5622), pp. 1142–5.doi: 10.1126/science.1083701.

Hu, K. *et al.* (2004) 'Endogenous circadian rhythm in an index of cardiac vulnerability independent of changes in behavior.', *Proceedings of the National Academy of Sciences of the United States of America*, 101(52), pp. 18223–7. doi: 10.1073/pnas.0408243101.

Huang, Y.-L. *et al.* (2002) 'Age-associated difference in circadian sleep-wake and rest-activity rhythms.', *Physiology & behavior*, 76(4–5), pp. 597–603. doi: 10.1016/s0031-9384(02)00733-3.

Hwang, Jung Sun *et al.* (2007) 'Age-associated decrease in proteasome content and activities in human dermal fibroblasts: restoration of normal level of proteasome subunits reduces aging markers in fibroblasts from elderly persons.', *The journals of gerontology. Series A, Biological sciences and medical sciences*, 62(5), pp. 490–9. doi: 10.1093/gerona/62.5.490.

Itakura, E. and Mizushima, N. (2010) 'Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins.', *Autophagy*. Taylor & Francis, 6(6), pp. 764–76. doi: 10.4161/AUTO.6.6.12709.

Iwasa, H., Han, J. and Ishikawa, F. (2003) 'Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway.', *Genes to cells : devoted to molecular & cellular mechanisms*, 8(2), pp. 131–44. doi: 10.1046/j.1365-2443.2003.00620.x.

Jiang, Y. *et al.* (2012) 'GRP78 counteracts cell death and protein aggregation caused by mutant huntingtin proteins.', *Neuroscience letters*, 516(2), pp. 182–7. doi: 10.1016/j.neulet.2012.03.074.

Jin, S. and Levine, A. J. (2001) 'The p53 functional circuit.', *Journal of cell science*, 114(Pt 23), pp. 4139–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11739646

Johnson, I. P. (2015) 'Age-related neurodegenerative disease research needs aging models.', *Frontiers in aging neuroscience*. Frontiers Media SA, 7, p. 168. doi: 10.3389/fnagi.2015.00168.

Johnston, J. A., Ward, C. L. and Kopito, R. R. (1998) 'Aggresomes: a cellular response to misfolded proteins.', *The Journal of cell biology*, 143(7), pp. 1883–98. doi: 10.1083/jcb.143.7.1883.

Kaarniranta, K. *et al.* (2002) 'Neuronal cells show regulatory differences in the hsp70 gene response', *Molecular Brain Research*, 101(1–2), pp. 136–140. doi: 10.1016/S0169-328X(02)00179-1.

Kaarniranta, K *et al.* (2002) 'Neuronal cells show regulatory differences in the hsp70 gene response', *Molecular Brain Research*, 101(1–2), pp. 136–140. doi: 10.1016/S0169-328X(02)00179-1.

Kaganovich, D., Kopito, R. and Frydman, J. (2008) 'Misfolded proteins partition between two distinct quality control compartments', *Nature*, 454(7208), pp. 1088–1095. doi: 10.1038/nature07195.

Kalka, D. and Hoyer, S. (1998) 'Long-term cultivation of a neuroblastoma cell line in medium with reduced serum content as a model system for neuronal aging?', *Archives of Gerontology and Geriatrics Arch. Gerontol. Geriatr*, 27(27), pp. 251–268. doi: 10.1016/s0167-4943(98)00122-8.

Kampinga, H. H. *et al.* (2008) 'Guidelines for the nomenclature of the human heat shock proteins', *cell stress society*. doi: 10.1007/s12192-008-0068-7.

Kang, H. T. *et al.* (2011) 'Autophagy Impairment Induces Premature Senescence in Primary Human Fibroblasts', *PLoS ONE*. Edited by A. T. Y. Lau, 6(8), p. e23367. doi: 10.1371/journal.pone.0023367.

Kappé, G. *et al.* (2003) 'The human genome encodes 10 alpha-crystallin-related small heat shock proteins: HspB1-10.', *Cell stress & chaperones*, 8(1), pp. 53–61. doi: 10.1379/1466-1268(2003)8<53:thgecs>2.0.co;2.

Kawachi, I. *et al.* (1995) 'Prospective study of shift work and risk of coronary heart disease in women.', *Circulation*, 92(11), pp. 3178–82. doi: 10.1161/01.cir.92.11.3178.

Kim, Y. E. *et al.* (2013) 'Molecular Chaperone Functions in Protein Folding and Proteostasis', *Annual Review of Biochemistry*. Annual Reviews , 82(1), pp. 323–355. doi: 10.1146/annurev-biochem-060208-092442.

Klaips, C. L., Jayaraj, G. G. and Hartl, F. U. (2018) 'Pathways of cellular proteostasis in aging and disease.', *The Journal of cell biology*. Rockefeller University Press, 217(1), pp. 51–63. doi: 10.1083/jcb.201709072.

Klettner, A. (2004) 'The induction of heat shock proteins as a potential strategy to treat neurodegenerative disorders.', *Drug news & perspectives*, 17(5), pp. 299–306. doi: 10.1358/dnp.2004.17.5.829033.

Knutsson, A. *et al.* (1999) 'Shiftwork and myocardial infarction: a case-control study.', *Occupational and environmental medicine*, 56(1), pp. 46–50. doi: 10.1136/oem.56.1.46.

Kolker, D. E. *et al.* (2003) 'Aging Alters Circadian and Light-Induced Expression of Clock Genes in Golden Hamsters', *Journal of Biological Rhythms*.SAGE Publications, 18(2), pp. 159–169. doi: 10.1177/0748730403251802.

Komar, A. A., Lesnik, T. and Reiss, C. (1999) 'Synonymous codon substitutions affect ribosome traffic and protein folding during in vitro translation.', *FEBS letters*, 462(3), pp. 387–91. doi: 10.1016/s0014-5793(99)01566-5.

Komatsu, M. *et al.* (2006) 'Loss of autophagy in the central nervous system causes neurodegeneration in mice', *Nature*, 441(7095), pp. 880–884. doi: 10.1038/nature04723.

Kondratov, R. V *et al.* (2006) 'Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock.', *Genes & development*. Cold Spring Harbor Laboratory Press, 20(14), pp. 1868–73. doi: 10.1101/gad.1432206.

Krajnak, K. *et al.* (1998) 'Aging alters the rhythmic expression of vasoactive intestinal polypeptide mRNA but not arginine vasopressin mRNA in the suprachiasmatic nuclei of female rats.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 18(12), pp. 4767–74. doi: 10.1523/JNEUROSCI.18-12-04767.1998.

Kramer, A. *et al.* (2001) 'Regulation of Daily Locomotor Activity and Sleep by Hypothalamic EGF Receptor Signaling', *Science*, 294(5551), pp. 2511–2515. doi: 10.1126/science.1067716.

Kriehuber, T. *et al.* (2010) 'Independent evolution of the core domain and its flanking sequences in small heat shock proteins', *The FASEB Journal*. Federation of American Societies for Experimental Biology Bethesda, MD, USA, 24(10), pp. 3633–3642. doi: 10.1096/fj.10-156992.

Krishnamurthy, J. *et al.* (2004) 'Ink4a/Arf expression is a biomarker of aging', *Journal of Clinical Investigation*, 114(9), pp. 1299–1307. doi: 10.1172/JCI22475.

Krishnan, B. *et al.* (2001) 'A new role for cryptochrome in a Drosophila circadian oscillator.', *Nature*, 411(6835), pp. 313–7. doi: 10.1038/35077094.

Kudo, T. *et al.* (2011) 'Circadian dysfunction in a mouse model of Parkinson's disease', *Experimental Neurology*, 232(1), pp. 66–75. doi: 10.1016/j.expneurol.2011.08.003.

Kuilman, T. *et al.* (2008) 'Oncogene-Induced Senescence Relayed by an Interleukin-Dependent Inflammatory Network', *Cell*, 133(6), pp. 1019–1031. doi: 10.1016/j.cell.2008.03.039.

Labbadia, J. and Morimoto, R. I. (2015) 'The Biology of Proteostasis in Aging and Disease', *Annual review of biochemistry*. NIH Public Access, 84, p. 435. doi: 10.1146/ANNUREV-BIOCHEM-060614-033955.

Lakin-Thomas, P. L. (2006) 'Transcriptional Feedback Oscillators: Maybe, Maybe Not...', *Journal of Biological Rhythms*. Sage PublicationsSage CA: Thousand Oaks, CA, 21(2), pp. 83–92. doi: 10.1177/0748730405286102.

Lam, P. Y. *et al.* (2009) 'Elevated neuronal nitric oxide synthase expression during ageing and mitochondrial energy production.', *Free radical research*, 43(5), pp. 431–9. doi: 10.1080/10715760902849813.

Lee, B. Y. *et al.* (2006) 'Senescence-associated β-galactosidase is lysosomal β-galactosidase', *Aging Cell*, 5(December 2005), pp. 187–195. doi: 10.1111/j.1474-9726.2006.00199.x.

Lee, C. C. (2005) 'The Circadian Clock and Tumor Suppression by Mammalian Period Genes', in *Methods in enzymology*, pp. 852–861. doi: 10.1016/S0076-6879(05)93045-0.

Lee, G. J. and Vierling, E. (2000) 'A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein.', *Plant physiology*, 122(1), pp. 189–98. doi: 10.1104/pp.122.1.189.

Leverenz, J. B. *et al.* (2008) 'Empiric Refinement of the Pathologic Assessment of Lewy-Related Pathology in the Dementia Patient', *Brain Pathology*, 18(2), pp. 220–224. doi: 10.1111/j.1750-3639.2007.00117.x.

Lin, A. W. *et al.* (1998) 'Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling.', *Genes & development*, 12(19), pp. 3008–19. doi: 10.1101/gad.12.19.3008.

Lindquist, S. (1986) 'The Heat-Shock Response', *Annual Review of Biochemistry*, 55(1), pp. 1151–1191. doi: 10.1146/annurev.bi.55.070186.005443.

Linskens, M. H. *et al.* (1995) 'Cataloging altered gene expression in young and senescent cells using enhanced differential display.', *Nucleic acids research*, 23(16), pp. 3244–51. doi: 10.1093/nar/23.16.3244.

Liu, Y. *et al.* (1998) 'How temperature changes reset a circadian oscillator.', *Science (New York, N.Y.)*, 281(5378), pp. 825–9. doi: 10.1126/science.281.5378.825.

Liu, Y. *et al.* (2009) 'Expression of *p16* ^{INK4a} in peripheral blood T-cells is a biomarker of human aging', *Aging Cell*, 8(4), pp. 439–448. doi: 10.1111/j.1474-9726.2009.00489.x.

Lloyd, A. C. (2002) 'Limits to lifespan', *Nature Cell Biology*, 4(2), pp. E25–E27. doi: 10.1038/ncb0202-e25.

López-Otín, C. *et al.* (2013) 'The hallmarks of aging.', *Cell*. Europe PMC Funders, 153(6), pp. 1194–217. doi: 10.1016/j.cell.2013.05.039.

Majeski, A. E. and Fred Dice, J. (2004) 'Mechanisms of chaperone-mediated autophagy', *The International Journal of Biochemistry & Cell Biology*, 36(12), pp. 2435–2444. doi: 10.1016/j.biocel.2004.02.013.

Manal, M. *et al.* (2016) 'Inhibitors of histone deacetylase as antitumor agents: A critical review', 67, pp. 18–42. doi: 10.1016/j.bioorg.2016.05.005.

Mann, D. M. and Jones, D. (1990) 'Deposition of amyloid (A4) protein within the brains of persons with dementing disorders other than Alzheimer's disease and Down's syndrome.', *Neuroscience letters*, 109(1–2), pp. 68–75. doi: 10.1016/0304-3940(90)90539-l.

Masters, C. L. *et al.* (1985) 'Amyloid plaque core protein in Alzheimer disease and Down syndrome.', *Proceedings of the National Academy of Sciences of the United States of America*, 82(12), pp. 4245–9. doi: 10.1073/pnas.82.12.4245.

McClellan, A. J. *et al.* (2007) 'Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches.', *Cell*, 131(1), pp. 121–35. doi: 10.1016/j.cell.2007.07.036.

McCurry, S. M. *et al.* (2011) 'Increasing walking and bright light exposure to improve sleep in community-dwelling persons with Alzheimer's disease: results of a randomized, controlled trial.', *Journal of the American Geriatrics Society*. NIH Public Access, 59(8), pp. 1393–402. doi: 10.1111/j.1532-5415.2011.03519.x.

McNamara, P. *et al.* (2001) 'Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock.', *Cell*, 105(7), pp. 877–89. doi: 10.1016/s0092-8674(01)00401-9.

Melk, A. *et al.* (2004) 'Expression of p16INK4a and other cell cycle regulator and senescence associated genes in aging human kidney.', *Kidney international*, 65(2), pp. 510–20. doi: 10.1111/j.1523-1755.2004.00438.x.

Merlino, G. *et al.* (2010) 'Daytime sleepiness is associated with dementia and cognitive decline in older Italian adults: a population-based study.', *Sleep medicine*, 11(4), pp. 372–7. doi: 10.1016/j.sleep.2009.07.018.

Merrow, M. *et al.* (2015) 'Using Circadian Entrainment to Find Cryptic Clocks', *Methods in Enzymology*. Academic Press, 551, pp. 73–93. doi: 10.1016/BS.MIE.2014.10.028.

Merrow, M., Brunner, M. and Roenneberg, T. (1999) 'Assignment of circadian function for the Neurospora clock gene frequency', *Nature*. Nature Publishing Group, 399(6736), pp. 584–586. doi: 10.1038/21190.

Mogk, A. *et al.* (2003) 'Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation.', *Molecular microbiology*, 50(2), pp. 585–95. doi: 10.1046/j.1365-2958.2003.03710.x.

Morán Luengo, T. *et al.* (2018) 'Hsp90 Breaks the Deadlock of the Hsp70 Chaperone System.', *Molecular cell*, 70(3), pp. 545-552.e9. doi: 10.1016/j.molcel.2018.03.028.

La Morgia, C. *et al.* (2016) 'Melanopsin retinal ganglion cell loss in Alzheimer disease', *Annals of Neurology*, 79(1), pp. 90–109. doi: 10.1002/ana.24548.

Morris, C. J., Yang, J. N. and Scheer, F. A. J. L. (2012) 'The impact of the circadian timing system on cardiovascular and metabolic function.', *Progress in brain research*. NIH Public Access, 199, pp. 337–58. doi: 10.1016/B978-0-444-59427-3.00019-8.

Muchowski, P. J. *et al.* (2000) 'Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils.', *Proceedings of the National Academy of Sciences of the United States of America*, 97(14), pp. 7841–6. doi: 10.1073/pnas.140202897.

Muchowski, P. J. and Wacker, J. L. (2005) 'Modulation of neurodegeneration by molecular chaperones', *Nature Reviews Neuroscience*. Nature Publishing Group, 6(1), pp. 11–22. doi: 10.1038/nrn1587.

Müller, T. (2012) 'Drug therapy in patients with Parkinson's disease.', *Translational neurodegeneration*. BioMed Central, 1(1), p. 10. doi: 10.1186/2047-9158-1-10.

Munro, J. *et al.* (2004) 'Histone deacetylase inhibitors induce a senescence-like state in human cells by a p16-dependent mechanism that is independent of a mitotic clock.', *Experimental cell research*, 295(2), pp. 525–38. doi: 10.1016/j.yexcr.2004.01.017.

Musi, N. *et al.* (2018) 'Tau protein aggregation is associated with cellular senescence in the brain', *Aging Cell*, 17(6), p. e12840. doi: 10.1111/acel.12840.

Nagoshi, E. *et al.* (2004) 'Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells.', *Cell*, 119(5), pp. 693–705. doi: 10.1016/j.cell.2004.11.015.

Nagy, M. *et al.* (2016) 'Extended survival of misfolded G85R SOD1-linked ALS mice by transgenic expression of chaperone Hsp110.', *Proceedings of the National Academy of Sciences of the United States of America*, 113(19), pp. 5424–8. doi: 10.1073/pnas.1604885113.

Nakamura, A. J. *et al.* (2008) 'Both telomeric and non-telomeric DNA damage are determinants of mammalian cellular senescence', *Epigenetics & Chromatin*, 1(1), p. 6. doi: 10.1186/1756-8935-1-6.

Nicolaides, N. C. *et al.* (2014) 'Circadian endocrine rhythms: the hypothalamic-pituitary-adrenal axis and its actions.', *Annals of the New York Academy of Sciences*. NIH Public Access, 1318, pp. 71–80. doi: 10.1111/nyas.12464.

Nillegoda, N. B. *et al.* (2017) 'Evolution of an intricate J-protein network driving protein disaggregation in eukaryotes.', *eLife*, 6. doi: 10.7554/eLife.24560.

Ogryzko, V. V *et al.* (1996) 'Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent.', *Molecular and cellular biology*, 16(9), pp. 5210–8. doi: 10.1128/mcb.16.9.5210.

Omata, Y. *et al.* (2014) 'Age-induced reduction of autophagy-related gene expression is associated with onset of Alzheimer's disease.', *American journal of neurodegenerative disease*, 3(3), pp. 134–42. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4299720/.

Parker, C. S. and Topol, J. (1984) 'A Drosophila RNA polymerase II transcription factor binds to the regulatory site of an hsp 70 gene.', *Cell*, 37(1), pp. 273–83. doi: 10.1016/0092-8674(84)90323-4.

Parker, S. M. *et al.* (2008) 'Airway epithelial cell senescence in the lung allograft.', *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, 8(7), pp. 1544–9. doi: 10.1111/j.1600-6143.2008.02284.x.

Parrinello, S. *et al.* (2003) 'Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts.', *Nature cell biology*, 5(8), pp. 741–7. doi: 10.1038/ncb1024.

Paz Gavilán, M. *et al.* (2006) 'Cellular environment facilitates protein accumulation in aged rat hippocampus', *Neurobiology of Aging*, 27(7), pp. 973–982. doi: 10.1016/j.neurobiolaging.2005.05.010.

Peschek, J. *et al.* (2013) 'Regulated structural transitions unleash the chaperone activity of αB-crystallin.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(40), pp. E3780-9. doi: 10.1073/pnas.1308898110.

Pittendrigh, C. S. (1960) 'Circadian rhythms and the circadian organization of living systems.', *Cold Spring Harbor symposia on quantitative biology*, 25, pp. 159–84. doi: 10.1101/sqb.1960.025.01.015.

Te Poele, R. H. *et al.* (2002) 'DNA damage is able to induce senescence in tumor cells in vitro and in vivo.', *Cancer research*, 62(6), pp. 1876–83. Available at: https://cancerres.aacrjournals.org/content/62/6/1876.long.

Pollak, C. P. and Perlick, D. (1991) 'Sleep problems and institutionalization of the elderly.', *Journal of geriatric psychiatry and neurology*, 4(4), pp. 204–10. doi: 10.1177/089198879100400405.

Poon, H. F. *et al.* (2006) 'Quantitative proteomics analysis of differential protein expression and oxidative modification of specific proteins in the brains of old mice', *Neurobiology of Aging*, 27(7), pp. 1010–1019. doi: 10.1016/j.neurobiolaging.2005.05.006.

Prahlad, V., Cornelius, T. and Morimoto, R. I. (2008) 'Regulation of the cellular heat shock response in Caenorhabditis elegans by thermosensory neurons.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 320(5877), pp. 811–4. doi: 10.1126/science.1156093.

Prolo, L. M. (2005) 'Circadian Rhythm Generation and Entrainment in Astrocytes', *Journal of Neuroscience*, 25(2), pp. 404–408. doi: 10.1523/JNEUROSCI.4133-04.2005.

Ralph, M. R. *et al.* (1990) 'Transplanted suprachiasmatic nucleus determines circadian period.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 247(4945), pp. 975–8. doi: 10.1126/SCIENCE.2305266.

Reeve, A., Simcox, E. and Turnbull, D. (2014a) 'Ageing and Parkinson's disease: why is advancing age the biggest risk factor?', *Ageing research reviews*. Elsevier, 14(100), pp. 19–30. doi: 10.1016/j.arr.2014.01.004.

Reeve, A., Simcox, E. and Turnbull, D. (2014b) 'Ageing and Parkinson's disease: Why is advancing age the biggest risk factor?', *Ageing Research Reviews*, 14, pp. 19–30. doi: 10.1016/j.arr.2014.01.004.

Reinke, H. *et al.* (2008) 'Differential display of DNA-binding proteins reveals heat-shock factor 1 as a circadian transcription factor', *Genes and Development*, 22(3), pp. 331–345. doi: 10.1101/gad.453808.

Reitz, C., Brayne, C. and Mayeux, R. (2011) 'Epidemiology of Alzheimer disease', *Nature Reviews Neurology*, 7(3), pp. 137–152. doi: 10.1038/nrneurol.2011.2.

Riemersma-van der Lek, R. F. *et al.* (2008) 'Effect of bright light and melatonin on cognitive and noncognitive function in elderly residents of group care facilities: a randomized controlled trial.', *JAMA : the journal of the American Medical Association*, 299(22), pp. 2642–2655. doi: 10.1001/jama.299.22.2642.

Rivera, I. *et al.* (2018) 'Modulation of Alzheimer's amyloid β peptide oligomerization and toxicity by extracellular Hsp70.', *Cell stress & chaperones*. Springer, 23(2), pp. 269–279. doi: 10.1007/s12192-017-0839-0.

Rizzolo, K. *et al.* (2017) 'Features of the Chaperone Cellular Network Revealed through Systematic Interaction Mapping', *Cell Reports*, 20(11), pp. 2735–2748. doi: 10.1016/j.celrep.2017.08.074.

Robles, S. J. and Adami, G. R. (1998) 'Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts.', *Oncogene*, 16(9), pp. 1113–23. doi: 10.1038/sj.onc.1201862.

Rock, K. L. *et al.* (1994) 'Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules.', *Cell*, 78(5), pp. 761–71. doi: 10.1016/s0092-8674(94)90462-6.

Rodier, F. *et al.* (2011) 'DNA-SCARS: distinct nuclear structures that sustain damage induced senescence growth arrest and inflammatory cytokine secretion.', *Journal of cell science*, 124(Pt 1), pp. 68–81. doi: 10.1242/jcs.071340.

Roenneberg, T., Daan, S. and Merrow, M. (2003) 'The Art of Entrainment', *Journal of Biological Rhythms*, 18(3), pp. 183–194. doi: 10.1177/0748730403018003001.

Roenneberg, T. and Merrow, M. (2007) 'Entrainment of the Human Circadian Clock', *Cold Spring Harbor Symposia on Quantitative Biology*, 72(1), pp. 293–299. doi: 10.1101/sqb.2007.72.043.

Ross, C. A. and Poirier, M. A. (2004) 'Protein aggregation and neurodegenerative disease', *Nature Medicine*. Nature Publishing Group, 10(S7), pp. S10–S17. doi: 10.1038/nm1066.

Saarikangas, J. and Barral, Y. (2015) 'Protein aggregates are associated with replicative aging without compromising protein quality control', *eLife*, 4, pp. 1–24. doi: 10.7554/eLife.06197.

Saez, I. and Vilchez, D. (2014) 'The Mechanistic Links Between Proteasome Activity, Aging and Age-related Diseases.', *Current genomics*. Bentham Science Publishers, 15(1), pp. 38–51. doi: 10.2174/1389202915011403061133344.

Sahu, R. *et al.* (2011) 'Microautophagy of Cytosolic Proteins by Late Endosomes', *Developmental Cell*, 20(1), pp. 131–139. doi: 10.1016/j.devcel.2010.12.003.

Saini, C. *et al.* (2012) 'Simulated body temperature rhythms reveal the phase-shifting behavior and plasticity of mammalian circadian oscillators', *Genes & Development*, 26(6), pp. 567–580. doi: 10.1101/gad.183251.111.

Sala, A. J., Bott, L. C. and Morimoto, R. I. (2017) 'Shaping proteostasis at the cellular, tissue, and organismal level.', *The Journal of cell biology*. The Rockefeller University Press, 216(5), pp. 1231–1241. doi: 10.1083/jcb.201612111.

Saretzki, G. (2010) 'Cellular senescence in the development and treatment of cancer.', *Current pharmaceutical design*, 16, pp. 79–100. doi: 10.2174/138161210789941874.

Satinoff, E *et al.* (1993) 'Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones?', *The American journal of physiology*, 265(5 Pt 2), pp. R1216-22. doi: 10.1152/ajpregu.1993.265.5.R1216.

Satinoff, E. *et al.* (1993) 'Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones?', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 265(5), pp. R1216–R1222. doi: 10.1152/ajpregu.1993.265.5.R1216.

Satlin, a. *et al.* (1995) 'Circadian locomotor activity and core-body temperature rhythms in Alzheimer's disease', *Neurobiology of Aging*, 16(5), pp. 765–771. doi: 10.1016/0197-4580(95)00059-N.

Scheiermann, C. *et al.* (2012) 'Adrenergic nerves govern circadian leukocyte recruitment to tissues.', *Immunity*. NIH Public Access, 37(2), pp. 290–301. doi: 10.1016/j.immuni.2012.05.021.

Schmitt, C. A. *et al.* (2002) 'A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy.', *Cell*, 109(3), pp. 335–46. doi: 10.1016/s0092-8674(02)00734-1.

Schulz, T. C. *et al.* (2004) 'Differentiation of Human Embryonic Stem Cells to Dopaminergic Neurons in Serum-Free Suspension Culture', *Stem Cells*, 22(7), pp. 1218–1238. doi: 10.1634/stemcells.2004-0114.

Seeds, N. W. *et al.* (1970) 'Regulation of axon formation by clonal lines of a neural tumor.', *Proceedings of the National Academy of Sciences of the United States of America*, 66(1), pp. 160–7. doi: 10.1073/pnas.66.1.160.

Serrano, M. *et al.* (1997) 'Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.', *Cell*, 88(5), pp. 593–602. doi: 10.1016/s0092-8674(00)81902-9.

Severino, J. *et al.* (2000) 'Is β-Galactosidase Staining a Marker of Senescence in Vitro and in Vivo?', *Experimental Cell Research*, 257(1), pp. 162–171. doi: 10.1006/excr.2000.4875.

Shalgi, R. *et al.* (2013) 'Widespread Regulation of Translation by Elongation Pausing in Heat Shock', *Molecular Cell*, 49(3), pp. 439–452. doi: 10.1016/j.molcel.2012.11.028.

Shang, F., Gong, X. and Taylor, A. (1997) 'Activity of ubiquitin-dependent pathway in response to oxidative stress. Ubiquitin-activating enzyme is transiently up-regulated.', *The Journal of biological chemistry*, 272(37), pp. 23086–93. doi: 10.1074/jbc.272.37.23086.

Sharpless, N. E. *et al.* (2001) 'Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis.', *Nature*, 413(6851), pp. 86–91. doi: 10.1038/35092592.

Sharpless, N. E. and Sherr, C. J. (2015) 'Forging a signature of in vivo senescence', *Nature Reviews Cancer*. Nature Publishing Group, 15(7), pp. 397–408. doi: 10.1038/nrc3960.

Shelton, D. N. *et al.* (1999) 'Microarray analysis of replicative senescence.', *Current biology : CB*, 9(17), pp. 939–45. doi: 10.1016/s0960-9822(99)80420-5.

Shi, Y., Mosser, D. D. and Morimoto, R. I. (1998) 'Molecular chaperones as HSF1-specific transcriptional repressors.', *Genes & development*. Cold Spring Harbor Laboratory Press, 12(5), pp. 654–66. doi: 10.1101/gad.12.5.654.

Shochat, T. *et al.* (2000) 'Illumination levels in nursing home patients: effects on sleep and activity rhythms', *Journal of Sleep Research*. John Wiley & Sons, Ltd (10.1111), 9(4), pp. 373–379. doi: 10.1046/j.1365-2869.2000.00221.x.

Siller, E. *et al.* (2010) 'Slowing bacterial translation speed enhances eukaryotic protein folding efficiency.', *Journal of molecular biology*, 396(5), pp. 1310–8. doi: 10.1016/j.jmb.2009.12.042.

Sittler, A. *et al.* (2001) 'Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease.', *Human molecular genetics*, 10(12), pp. 1307–15. doi: 10.1093/hmg/10.12.1307.

Smith, P. K. *et al.* (1985) 'Measurement of protein using bicinchoninic acid', *Analytical Biochemistry*, 150(1), pp. 76–85. doi: 10.1016/0003-2697(85)90442-7.

Song, H. *et al.* (2015) 'Aβ-induced degradation of BMAL1 and CBP leads to circadian rhythm disruption in Alzheimer's disease', *Molecular Neurodegeneration*. BioMed Central, 10(1), p. 13. doi: 10.1186/s13024-015-0007-x.

Spencer, P. S. *et al.* (2012) 'Silent substitutions predictably alter translation elongation rates and protein folding efficiencies.', *Journal of molecular biology*, 422(3), pp. 328–35. doi: 10.1016/j.jmb.2012.06.010.

Stathopulos, P. B. *et al.* (2004) 'Sonication of proteins causes formation of aggregates that resemble amyloid.', *Protein science : a publication of the Protein Society*, 13(11), pp. 3017–27. doi: 10.1110/ps.04831804.

Stokkan, K. A. *et al.* (2001) 'Entrainment of the circadian clock in the liver by feeding.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 291(5503), pp. 490–3. doi: 10.1126/science.291.5503.490.

Sund-Levander, M., Forsberg, C. and Wahren, L. K. (2002) 'Normal oral, rectal, tympanic and axillary body temperature in adult men and women: A systematic literature review', *Scandinavian Journal of Caring Sciences*, 16(2), pp. 122–128. doi: 10.1046/j.1471-6712.2002.00069.x.

Suwazono, Y. *et al.* (2008) 'Shift Work Is a Risk Factor for Increased Blood Pressure in Japanese Men', *Hypertension*. Lippincott Williams & Wilkins, 52(3), pp. 581–586. doi: 10.1161/HYPERTENSIONAHA.108.114553.

Swaab, D. F., Fliers, E. and Partiman, T. S. (1985) 'The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia.', *Brain research*, 342(1), pp. 37–44. doi: 10.1016/0006-8993(85)91350-2.

Sykiotis, G. P. and Bohmann, D. (2010) 'Stress-activated cap'n'collar transcription factors in aging and human disease.', *Science signaling*, 3(112), p. re3. doi: 10.1126/scisignal.3112re3.

Taipale, M., Jarosz, D. F. and Lindquist, S. (2010) 'HSP90 at the hub of protein homeostasis: emerging mechanistic insights', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 11(7), pp. 515–528. doi: 10.1038/nrm2918.

Takahashi, J. S. (2004) 'Finding new clock components: past and future.', *Journal of biological rhythms*. Howard Hughes Medical Institute, 19(5), pp. 339–47. doi: 10.1177/0748730404269151.

Tamaru, T. *et al.* (2011) 'Synchronization of Circadian Per2 Rhythms and HSF1-BMAL1:CLOCK Interaction in Mouse Fibroblasts after Short-Term Heat Shock Pulse', *PLoS ONE*. Public Library of Science, 6(9). doi: 10.1371/JOURNAL.PONE.0024521.

Tatum, M. C. *et al.* (2015) 'Neuronal Serotonin Release Triggers the Heat Shock Response in C. elegans in the Absence of Temperature Increase', *Current Biology*. Elsevier, 25(2), pp. 163–174. doi: 10.1016/J.CUB.2014.11.040.

Tawo, R. *et al.* (2017) 'The Ubiquitin Ligase CHIP Integrates Proteostasis and Aging by Regulation of Insulin Receptor Turnover.', *Cell*, 169(3), pp. 470-482.e13. doi: 10.1016/j.cell.2017.04.003.

Taylor, R. C. and Dillin, A. (2011) 'Aging as an event of proteostasis collapse.', *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Laboratory Press, 3(5). doi: 10.1101/cshperspect.a004440.

Terao, Y. *et al.* (2001) 'Sodium butyrate induces growth arrest and senescence-like phenotypes in gynecologic cancer cells.', *International journal of cancer*, 94(2), pp. 257–67. doi: 10.1002/ijc.1448.

Terman, A. (1995) 'The effect of age on formation and elimination of autophagic vacuoles in mouse hepatocytes.', *Gerontology*, 41 Suppl 2(2), pp. 319–26. doi: 10.1159/000213753.

Tominaga, T. *et al.* (2019) 'Senescence-associated-β-galactosidase staining following traumatic brain injury in the mouse cerebrum', *PLOS ONE*. Edited by G. Biagini. Public Library of Science, 14(3), p. e0213673. doi: 10.1371/journal.pone.0213673.

Trinklein, N. D. *et al.* (2004) 'The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response.', *Molecular biology of the cell*. American Society for Cell Biology, 15(3), pp. 1254–61. doi: 10.1091/mbc.e03-10-0738.

Tsujimoto, T. *et al.* (1990) 'Circadian rhythms in depression. Part I: Monitoring of the circadian body temperature rhythm.', *Journal of affective disorders*, 18(3), pp. 193–7. doi: 10.1016/0165-0327(90)90036-8.

Uchikado, H. *et al.* (2006) 'Alzheimer Disease With Amygdala Lewy Bodies', *Journal of Neuropathology and Experimental Neurology*, 65(7), pp. 685–697. doi: 10.1097/01.jnen.0000225908.90052.07.

Uhlen, M. *et al.* (2015) 'Tissue-based map of the human proteome', *Science*. American Association for the Advancement of Science, 347(6220), pp. 1260419–1260419. doi: 10.1126/science.1260419.

Ungerstedt, J. S. *et al.* (2005) 'Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 102(3), pp. 673–8. doi: 10.1073/pnas.0408732102.

Van Veggel, M., Westerman, E. and Hamberg, P. (2018) 'Clinical Pharmacokinetics and Pharmacodynamics of Panobinostat', *Clinical Pharmacokinetics*, 57(1), pp. 21–29. doi: 10.1007/s40262-017-0565-x.

Vgontzas, A. N. *et al.* (2003) 'Impaired nighttime sleep in healthy old versus young adults is associated with elevated plasma interleukin-6 and cortisol levels: physiologic and therapeutic implications.', *The Journal of clinical endocrinology and metabolism*, 88(5), pp. 2087–95. doi: 10.1210/jc.2002-021176.

Videnovic, A. *et al.* (2014) ‘’The clocks that time us’--circadian rhythms in neurodegenerative disorders.’, *Nature reviews. Neurology*. NIH Public Access, 10(12), pp. 683–93. doi: 10.1038/nrneurol.2014.206.

Videnovic, A. *et al.* (2017) ‘Timed Light Therapy for Sleep and Daytime Sleepiness Associated With Parkinson Disease: A Randomized Clinical Trial.’, *JAMA neurology*, 74(4), pp. 411–418. doi: 10.1001/jamaneurol.2016.5192.

Vihervaara, A. and Sistonen, L. (2014) ‘HSF1 at a glance.’, *Journal of cell science*, 127(Pt 2), pp. 261–6. doi: 10.1242/jcs.132605.

Vos, M. J. *et al.* (2008) ‘Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families.’, *Biochemistry*, 47(27), pp. 7001–11. doi: 10.1021/bi800639z.

Waldeck, K. *et al.* (2016) ‘Long term, continuous exposure to panobinostat induces terminal differentiation and long term survival in the TH-MYCN neuroblastoma mouse model’, *International Journal of Cancer*. John Wiley & Sons, Ltd, 139(1), pp. 194–204. doi: 10.1002/ijc.30056.

Wang, J. L. *et al.* (2015) ‘Suprachiasmatic neuron numbers and rest-activity circadian rhythms in older humans.’, *Annals of neurology*. NIH Public Access, 78(2), pp. 317–22. doi: 10.1002/ana.24432.

Wegele, H., Müller, L. and Buchner, J. (2004) ‘Hsp70 and Hsp90--a relay team for protein folding.’, *Reviews of physiology, biochemistry and pharmacology*. Berlin, Heidelberg: Springer Berlin Heidelberg, 151, pp. 1–44. doi: 10.1007/s10254-003-0021-1.

Weissová, K. *et al.* (2016) 'Moderate Changes in the Circadian System of Alzheimer's Disease Patients Detected in Their Home Environment.', *PloS one*. Edited by N. Cermakian, 11(1), p. e0146200. doi: 10.1371/journal.pone.0146200.

Welsh, D. K. *et al.* (2004) 'Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression.', *Current biology : CB*, 14(24), pp. 2289–95. doi: 10.1016/j.cub.2004.11.057.

Wiley, C. D. *et al.* (2017) 'Analysis of individual cells identifies cell-to-cell variability following induction of cellular senescence', *Aging Cell*, 16(5), pp. 1043–1050. doi: 10.1111/acel.12632.

Wilhelmus, M. M. M. *et al.* (2006) 'Small heat shock proteins inhibit amyloid-beta protein aggregation and cerebrovascular amyloid-beta protein toxicity.', *Brain research*, 1089(1), pp. 67–78. doi: 10.1016/j.brainres.2006.03.058.

Winden, K. D. *et al.* (2009) 'The organization of the transcriptional network in specific neuronal classes', *Molecular Systems Biology*, 5, p. 291. doi: 10.1038/msb.2009.46.

Wright, K. P. *et al.* (2013) 'Entrainment of the human circadian clock to the natural light-dark cycle.', *Current biology : CB*. NIH Public Access, 23(16), pp. 1554–8. doi: 10.1016/j.cub.2013.06.039.

Wu, C. (1984) 'Activating protein factor binds in vitro to upstream control sequences in heat shock gene chromatin.', *Nature*, 311(5981), pp. 81–4. doi: 10.1038/311081a0.

Yan, Y. *et al.* (2017) 'NS1 of H7N9 Influenza A Virus Induces NO-Mediated Cellular Senescence in Neuro2a Cells', *Cellular Physiology and Biochemistry*. Karger Publishers, 43(4), pp. 1369–1380. doi: 10.1159/000481848.

Yang, W., Ang, L. C. and Strong, M. J. (2005) 'Tau protein aggregation in the frontal and entorhinal cortices as a function of aging.', *Brain research. Developmental brain research*, 156(2), pp. 127–38. doi: 10.1016/j.devbrainres.2005.02.004.

Young, J. C. *et al.* (2004) 'Pathways of chaperone-mediated protein folding in the cytosol', *Nature Reviews Molecular Cell Biology*, 5(10), pp. 781–791. doi: 10.1038/nrm1492.

Young, K. W. and Greenwood, C. E. (2001) 'Shift in diurnal feeding patterns in nursing home residents with Alzheimer's disease.', *The journals of gerontology. Series A, Biological sciences and medical sciences*, 56(11), pp. M700-6. doi: 10.1093/gerona/56.11.m700.

Zheng, X. *et al.* (2016) 'Dynamic control of Hsf1 during heat shock by a chaperone switch and phosphorylation.', *eLife*. eLife Sciences Publications, Ltd, 5. doi: 10.7554/eLife.18638.

Zhong, G. *et al.* (2013) 'The relationship between thermoregulation and REM sleep behaviour disorder in Parkinson's disease.', *PloS one*. Edited by E. Planel, 8(8), p. e72661. doi: 10.1371/journal.pone.0072661.

Zhou, J. N., Hofman, M. A. and Swaab, D. F. (1995) 'VIP neurons in the human SCN in relation to sex, age, and Alzheimer's disease.', *Neurobiology of aging*, 16(4), pp. 571–6. doi: 10.1016/0197-4580(95)00043-e.

Zhou, Q. *et al.* (2009) 'Activation of c-Jun-N-terminal kinase and decline of mitochondrial pyruvate dehydrogenase activity during brain aging.', *FEBS letters*, 583(7), pp. 1132–40. doi: 10.1016/j.febslet.2009.02.043.

Zou, J. *et al.* (1998) 'Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1.', *Cell*. Elsevier, 94(4), pp. 471–80. doi: 10.1016/S0092-8674(00)81588-3.

Zuiderweg, E. R. P., Hightower, L. E. and Gestwicki, J. E. (2017) 'The remarkable multivalency of the Hsp70 chaperones.', *Cell stress & chaperones*, 22(2), pp. 173–189. doi: 10.1007/s12192-017-0776-y.

9. Abbreviations

AD	Alzheimer disease
ALS	Autophagy-lysosome system
BMAL1	Arnt-like protein 1
°C	Celsius
CRY	Cryptochromes
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
SCN	Suprachiasmatic nucleus
HSPs	Heat shock proteins
LBH589	Panobinostat
ND	Neurodegenerative diseases
PBS	Phospate Buffered Saline
PD	Parkinson disease
PER	Period homologue proteins
PN	Proteostasis network
smHSPs	Small heat shock proteins
UPS	Ubiquitin-proteasome system
VIP	Vasoactive intestinal peptide

10. Acknowledgments

I would like to express my sincere gratitude to my supervisor Dr. Mirjam Geibel, who supported me through all phases of this thesis. I was blessed with a patient, intelligent and wonderful supervisor.

Also I would like to thank my doctoral advisor Professor Martha Merrow. A woman I look up to with her wide knowledge about science and the circadian clock. Thank you for the helpful input and your great support.

Furthermore I would like to thank all the great people from the Institute of Medical psychology; Astrid Bauer and Tanja Popp, who showed me all the tricks in implementing experiments; Professor Maria Robles, Dr. Cornelia Madeti, Dr. Bala Koritala and Dr. Zheng Chen for the constructive help. Everyone supported me from day one and made my time in the Institute of Medical Psychology very special.

I am also very thankful getting the chance to be supported by the program FöFoLe (Förderung für Forschung und Lehre) of the Ludwig Maximilians University, a program which made my work and this thesis possible.

Special thanks to my family, who went through every ups and downs during my doctoral thesis with encouragement and support.

There were many more people who supported me through this time, thank you!