Effects of overexpression of nicotinamide phosphoribosyltransferase in mouse embryonic fibroblast cells on oxidative and endoplasmic reticulum stress-induced premature senescence

（マウス胎仔線維芽細胞における酸化および小胞体ストレス誘導性細胞老化に対する nicotinamide phosphoribosyltransferase 過剰発現の効果）

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Abstract

Cellular senescence is a complex physiological process characterized by persistent growth arrest followed by some alterations inside cells even under normal physiological conditions. Accumulation of senescent cells is a major feature of aged individuals, which in turn causes tissue and organismal aging. Cellular senescence can be categorized into two groups; one is the replicative senescence, and another is the stress-induced premature senescence. Replicative senescence is the result of telomere shortening after a limited number of cell divisions. Whereas, the stress-induced premature senescence is triggered by various endogenous or exogenous agents which cause stresses or damages to the cell such as oxidative stress, DNA damage, endoplasmic reticulum (ER) stress, oncogene activation, telomere damage, and X-ray irradiation.

Decrease in nicotinamide adenine dinucleotide (NAD^+) level is known to be one of the critical events for tissue and organismal aging, because replenishment of NAD^+ in organism ameliorates these processes. It is crucial to maintain NAD^+ homeostasis through balancing the mechanism of NAD^+ production and degradation. Previously, Khaidizar *et al.* (2017) reported that *nicotinamide phosphoribosyltransferase* (*Nampt)* transcript, encoding a rate-limiting enzyme for the dominant NAD^+ biosynthesis pathway, and NAD^+ decrease with passages of primary mouse embryonic fibroblasts (MEFs), and primary MEFs derived from *Nampt* overexpressing transgenic mice (*Nampt* TG-MEFs) delay the onset of replicative senescence *in vitro*. Increase and/or activation of NAMPT have become one of the attractive strategies to delay the onset of replicative senescent cells in an organism. Cells in the body are also continuously exposed various endogenous and exogenous stressors, which are known to promote the induction of stress-induced premature senescence. However, it is not understood yet whether *Nampt* TG-MEFs have resistances against stress-induced premature senescence. In this study, it was examined that *Nampt* overexpression also prevents stress-induced premature senescence *in vitro*.

In this study, I found that *Nampt* TG-MEFs had higher resistances toward both oxidative and ER stress-induced premature senescence. I first evaluated whether *Nampt* TG-MEFs could proliferate more than wild type MEFs (WT-MEFs) after the exposure of H_2O_2 as oxidative stress or tunicamycin as ER stress. Against both stresses, I found that *Nampt* TG-MEFs possessed higher proliferative capacity compared with WT-MEFs, suggesting that *Nampt* TG-MEFs possessed resistance to turn senescent by these stressors. Next, to address that possibility, senescence-associated β-galactosidase assay (SA-β-Gal assay) was performed. The results showed that the accumulation of senescent cells in *Nampt* TG-MEFs was lower than that of WT-MEFs. Moreover, by comparing two different *Nampt* TG-MEF lines, I found that MEFs expressing more NAMPT/NAD^+ possessed more resistance against H_2O_2 or tunicamycin-induced premature senescence, indicating the NAMPT/NAD^+ dose-dependency against stress-induced premature senescence. Based on these results, I concluded that *Nampt* TG-MEFs develop higher resistance to stress-induced premature senescence.

Further, to look into the molecular basis of ER stress-induced premature senescence in *Nampt* TG-MEFs, I evaluated expression levels of some ER stress-related genes in both genotypes. Physiologically, cells have quality control machinery to maintain protein homeostasis through the unfolded protein response (UPR) signalling pathway. This system guarantees prevention of cells from damage which lead to age-related diseases, apoptosis or senescence. ER stressor such as tunicamycin may disturb protein homeostasis inside cells which lead to apoptosis or senescence. Thus, I evaluated the relationship between the UPR pathways and ER stress-induced premature senescence. I first examined the spliced form of *Xbp1*, because the spliced form of *Xbp1* mRNA by IRE1α is known to trigger ER stress responses and can be used as an indicator for ER stress. After 3 hours of tunicamycin treatment, the spliced form of *Xbp1* was more in *Nampt* TG-MEFs than that in WT-MEFs. Interestingly, the spliced form of *Xbp1* at 24 hours was comparable between genotypes. These results suggested that *Nampt* TG-MEFs were more sensitive when responding to ER stress compared with WT-MEFs. Some other UPR-related genes were also upregulated in *Nampt* TG-MEFs after 3 hours of tunicamycin treatment compared to WT-MEFs. For example, I detected some key genes which regulate proper protein folding through chaperoning system such as *Bip* and enzyme dismutase such as *Pdi*. I also detected the UPR-related genes which play a role in cell adaptation through misfolding/unfolding protein degradation such as *Herpud1* and *Erdj4* or apoptosis such as *Chop* and *Gadd34*. These results proposed that *Nampt* TG-MEFs were more sensitive to ER stress and responded quickly to prevent cells from severe damage and causing senescence and probably apoptosis.

Collecting together, I concluded that the increase of NAMPT/NAD^+ level could protect stress-induced premature senescence *in vitro*. This study and a previous study by Khaidizar *et al.* (2017) were complimentary, suggesting that elevation of NAMPT activity or NAD^+ level *in vivo* can be developed as one therapeutic strategy to delay the onset of both replicative and premature senescence which later support healthy aging and longevity.

Keywords: NAMPT, NAD^+, premature senescence, oxidative stress, ER-stress, primary MEFs

List of contents

List of figures

Abbreviations:

ATF	Activating transcription factor
bFGF	Basic fibroblast growth factor
BIP	Binding immunoglobulin protein
CD38	Cluster of differentiation 38
Chop	C/EBP homologous protein
DTT	Dithiothreitol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
Edem1	ER degradation-enhancing alpha-mannosidase-like 1
EGF	Epithelial growth factor
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
Erdj4	ER degradation-enhancing alpha-mannosidase-like protein 1
ER stress	Endoplasmic reticulum stress
FBS	Fetal Bovine Serum
Gadd34	Growth arrest and DNA-damage-inducible 34
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Grp78	78 kDa glucose-regulated protein
H_2O_2	Hydrogen peroxide
Herpud1	Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1
HSF1	Heat-shock factor protein 1
Hyou1	Hypoxia up-regulated 1
IRE1α	Inositol-requiring enzyme 1α
MEFs	Mouse embryonic fibroblasts
MEM	Minimum essential medium
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
mRNA	Messenger ribose nucleic acid
NAD	Nicotinamide adenine dinucleotide

NMN	Nicotinamide mononucleotide
NMNAT	Nicotinamide mononucleotide-adenylyltransferase
PARP	poly ADP ribose polymerase
PBS	Phosphate buffer saline
PERK	PKR like ER kinase
PDI	Protein disulfide-isomerase
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribose nucleic acid
ROS	Reactive oxygen species
SA-β-Gal	Senescence-associated beta-galactosidase
SAHF	Senescence-associated heterochromatic foci
SASP	Senescence-associated secretory phenotype
SIRT	Sirtuin
SMCs	Smooth muscle cells
SOD	Superoxide dismutase
UPR	Unfolded protein response
Xbp1	X-box binding protein 1

1.0 Introduction

1.1 Cellular senescence

In 1961, Hayflick and Moorhead reported that some strains of human primary fibroblast showed growth cessation after several times of cell division (Hayflick & Moorhead, 1961), and now this phenomenon is called cellular senescence. Recent decades, senescence gains more attention especially to various pathophysiological alterations which involve the onset of insulin resistance, decreasing of β-cell function, the onset of chronic inflammation, and processes which influence homeostasis in response to diet and surrounding environment (Imai & Yoshino, 2013). Cellular senescence is a complex physiological process which is characterized by one clear feature that is constant growth arrest followed by some modifications inside the cell. Thus, the cell cannot proliferate even under physiological stimuli (Campisi & d'Adda di Fagagna, 2007; Pawlikowski *et al*., 2013; Petrova *et al*., 2016). This promotes to accumulate senescent cells in an aged organism (Schosserer *et al.*, 2017) and brings to consequence impairment of tissues regeneration and functions (Campisi & d'Adda di Fagagna, 2007; Khaidizar *et al*., 2017). Despite its adverse effects, cellular senescence has some beneficial effects to organisms. Senescence participates in tissue repairs and regenerations, suppression of tumorigenesis, elimination of unwanted cells such as damaged cells in cancer and aging (Campisi & d'Adda di Fagagna, 2007; Pawlikowski *et al*. 2013; Munoz-Espin & Serrano, 2014; Hernandez-Segura *et al*., 2018[a]), also in embryonic development (Munoz-Espin & Serrano, 2014; Schosserer *et al.*, 2017).

There are some specific physiological and morphological features in senescence such as high secretion of pro-inflammatory substances (Schosserer *et al.*, 2017; Hernandez-Segura *et al.,* 2018[a]), high reactive oxygen species (ROS) level (Rufini *et al*., 2013; Hernandez-Segura *et al*., 2018[a]), activation of anti-programmed cell death genes, endoplasmic reticulum stress, increasing of lysosomal activity, cell enlargement and flattened, nucleus enlargement, as well as chromatin fragmentation (Hernandez-Segura *et al*., 2018[a]). Senescent cells can be detected using a marker which shows positive to proliferation, since senescent cells show negative (Matjusaitis *et al*., 2016). Senescent cells also can be detected through its lysosomal activity using a quite popular assay, senescence-associated beta-galactosidase (SA-β-Gal) assay (Dimri *et al.,* 1995; Campisi & d'Adda di Fagagna. 2007; Pawlikowski *et al*., 2013; Munoz-Espin & Serrano, 2014; Matjusaitis *et al.*, 2016), DNA mutation/damage markers, and secretion of senescence-associated secretory phenotypes (SASPs) such as interleukins, chemokines, as well

as growth factors (Pawlikowski *et al*., 2013; Munoz-Espin & Serrano, 2014; Matjusaitis *et al*., 2016). In senescence, there is an alteration of genes expression which regulates proliferation, cell cycle, and tumor suppressors such as *p16*, *p21*, and *p53*. The expression level of *p16*, *p21*, and *p53* increase in senescence (Pawlikowski *et al.*, 2013; Munoz-Espin & Serrano, 2014; Matjusaitis *et al*., 2016). The appearance of senescence-associated heterochromatic foci (SAHF) in some cell types also can be used as a marker of senescence (Campisi & d'Adda di Fagagna, 2007; Pawlikowski *et al*., 2013; Munoz-Espin & Serrano, 2014). However, mouse senescent cells such as senescent fibroblasts do not show SAHF configuration (Kennedy *et al*., 2010).

Mouse fibroblasts use p19/p53 as the dominant pathway in senescence, whereas p16 may not show an important function in senescence signalling (Itahana *et al*., 2004; Kennedy *et al*., 2010). Narita (2007) reviewed that p53 and p16/pRb activity increased in senescence without considering its causes. p16 is essential to stimulate G1-S checkpoint (Mirzayans *et al*., 2012). The role of p16 is still debatable in cellular senescence. The previous study reported that lifespan increases after the loss of p53 and p16 expression (Huschtscha & Reddel, 1999). Recently Rhinn *et al.* (2019) reviewed that there are two major signalling pathways in senescence, internal and external pathways. The internal pathway consists of p16, p19, p21, and p53. Whereas, the external pathway consists of p53, p38, NF-κB, Cebpβ, and Gata4.

1.2 Replicative senescence

Somatic cells such as human fibroblast physiologically lose their proliferation capacity after several times of division. This condition refers as replicative senescence (Hayflick, 1979; Brookes *et al*., 2004; Cristofalo *et al.*, 2004) and is considered as the main factor in tumor suppression (Huschtscha & Reddel, 1999). Replicative senescence in human cells usually connects with telomere shortening since they lack telomerase, a ribonucleoprotein polymerase, activity (Herbig *et al*., 2004; Itahana *et al*., 2004; Kennedy *et al*., 2010). A telomere is the terminal part of a chromosome which gives genomic stability (Greider, 1996; Narita, 2007). This structure consists of G-rich DNA duplication, which shortens in each replication event and brings cell becomes senescence (Narita, 2007; Liu *et al*., 2019). On the other hand, telomere extension by the activity of telomerase promotes the onset of the tumor (Greider, 1996; Liu *et al*., 2019).

Instead of human, some other organisms such as cat, dog, sheep, horse, cow, beaver, and capybara also lack telomerase activity (Gorbunova & Seluanov, 2009). The rate of telomere shortening was known related with the lifespan of organisms. For example, the rate of telomere

shortening in goat is ~ 360 bp/year with average of lifespan ~ 20 years, whereas the rate of telomere shortening in human is ~ 70 bp/year with average of lifespan ~ 80 years (Whittemore *et al*., 2019). However, somatic cells of some organisms, such as mouse have telomerase activity and can maintain their proliferation capacity under physiological oxygen level *in vitro* (Gorbunova & Seluanov, 2009). Yang & Fogo (2010) stated that telomeres of mouse and rat are quite long and only undergo little reduction. Thus, aging and senescence in these organisms do not show telomeres dependency.

1.3 Stress-induced premature senescence

Instead of undergoing senescence naturally through telomere shortening as in replicative senescence, cells may experience senescence earlier than they should. In human cells, this senescence happens before the phase caused by telomere shortening. This is called as premature senescence (Kuilman *et al*., 2010). Moreover, cells/tissues are exposed continuously endogenous and exogenous agents which cause stress and cell defect. The cell has two choices to cope with this condition. At low stress level, cell may defence and recover. However, at severe condition, cell activates programmed cell death mechanism (apoptosis pathway) (Campisi & d'Adda di Fagagna, 2007; Pluquet *et al*., 2015). During adaptation to the stress, the cell may arrest its growth transiently or permanently. Thus, the condition which cells stop to proliferate permanently is called stress-induced premature senescence. Some stimulants can cause stress-induced premature senescence. There are some examples of those stimulants: oncogene activation (Campisi & d'Adda di Fagagna, 2007; Pawlikowski *et al.*, 2013; Pluquet *et al*., 2015), DNA-damage-response (chemotherapeutic drug) (Campisi & d'Adda di Fagagna 2007; Pluquet *et al.*, 2015; Bhatia-Dey *et al*., 2016), chromatin perturbation (i.e. epigenetic alterations, aneuploidy) (Campisi & d'Adda di Fagagna. 2007; Bhatia-Dey *et al.*, 2016), oxidative stress (Pawlikowski *et al.* 2013; Pluquet *et al.,* 2015; Bhatia-Dey *et al*., 2016), mitochondrial dysfunction, inflammation, energy sensing, altered metabolic regulation, chronic mitogenic signalling, dysfunctional extracellular matrix (Bhatia-Dey *et al.*, 2016), telomere damage (Pluquet *et al.,* 2015), and X-ray irradiation (Hewitt *et al.,* 2012).

1.4 Effects of H_2O_2 on stress-induced premature senescence

H_2O_2 plays some essential roles in a redox reaction, signalling pathways, and may regulates cell fate, life or death (Lennicke *et al.,* 2015; Sies *et al*., 2017). Vilema-Enriquez *et al.* (2016) stated that H_2O_2 could regulate cell proliferation depending on cell type and H_2O_2 concentration. H_2O_2 promotes cell growth cessation and cell death (Sies *et al*., 2017). This

compound also is widely known to induce senescence in various type of cells such as human fibroblast (Chen & Ames, 1994; Bladier *et al*., 1997; Duan *et al*., 2005; Furukawa *et al*., 2007), mouse osteoblast cell line, human osteoblast-like cell line (Li *et al*., 2009), human tumor cell (Yoshizaki *et al*., 2009), mouse gingival fibroblast (Kiyoshima *et al*., 2012), mesenchymal stem cell (Choo *et al*., 2014), and bone marrow mesenchymal stem cell (Li *et al*., 2017).

Chen & Ames (1994) studied that H_2O_2 prevented cell proliferation through irreversible DNA synthesis inhibition. Human fibroblasts failed to response to growth factor stimuli such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and serum after H_2O_2 treatment since inhibition of DNA synthesis cannot be recovered within four days. Decreasing proliferation capacity possibly is facilitated by p21 (de Haan, 1996; Bladier *et al.*, 1997). Moreover, senescent cell after H_2O_2 treatment also shows elevation of p21, p53 (Duan *et al*., 2005; Kiyoshima *et al*., 2012; Choo *et al*., 2014), and gadd45 (Duan *et al*., 2005). The molecular mechanism of H_2O_2 to induce senescence also involves p21, p53, and SIRT1 pathways (Furukawa *et al*., 2007). Li *et al.* (2009) studied that cell growth cessation in osteoblast after H_2O_2 treatment involved reducing cyclin B1 protein.

1.5 The unfolded protein response (UPR) pathway in the response of endoplasmic reticulum stress-induced premature senescence

The endoplasmic reticulum (ER) is an organelle which has prominent function in protein production, assembly, and maturation (Oslowski & Urano, 2011; Brown & Naidoo, 2012; Krajarng *et al*., 2015; Iurlaro & Munoz-Pinedo, 2015). Thus, it is equipped by a machinery system which guarantees correct protein folding. This machinery needs to maintain protein homeostasis, which ensures the balance between protein production and breakdown (Oslowski & Urano, 2011; Lafleur *et al*., 2013). However, a lot of factors such as infection of virus, toxins (Oslowski & Urano, 2011), nutrient deficiency, hypoxia (Martinez *et al*., 2017; Hetz & Papa, 2018), and imbalance of calcium or redox level (Martinez *et al*., 2017) can impair its function. This condition promotes and increases the accumulation of misfolded and unfolded protein which is called ER stress (Oslowski & Urano, 2011, Walter & Ron, 2011, Iurlaro & Munoz-Pinedo, 2015; Krajarng *et al.*, 2015; Hetz & Papa, 2018). Such situations activate the adaptation machinery system, called the unfolded protein response (UPR) pathways (Oslowski & Urano, 2011, Walter & Ron, 2011, Iurlaro & Munoz-Pinedo, 2015; Krajarng *et al.*, 2015; Hetz & Papa, 2018). The final effect of these stressors may depend on several factors such as the action pathway of stressor, concentration, duration of exposure (acute or chronic), cell type, nutrient status, and oxidative stress (Oslowski & Urano, 2011; Lafleur *et al*., 2013).

Once stressor comes, ER responses by activating the UPR pathway. This pathway triggers several mechanisms to overcome stress. One of the adaptive controls to overcome stress involves ER-associated degradation (ERAD) (Doultsinos *et al*., 2017). Thus, the UPR system is important to guarantee ER protein homeostasis (proteostasis). On the other hand, if stress is persistent, the UPR system activates apoptosis pathway (Brown & Naidoo, 2012; Iurlaro & Munoz-Pinedo, 2015; Doultsinos *et al*., 2017, Martinez *et al*., 2017; Amodio *et al*., 2018; Hetz & Papa, 2018). In aging, the UPR-related genes which regulate chaperoning and folding system, as well as target genes which play a role in ERAD were activated (Brown & Naidoo, 2012). Thus, studying the UPR pathway may promote the development of a new therapeutic method, especially in aging (Doultsinos *et al*., 2017).

In the ER machinery, there are chaperones which ensure correct protein assemblies, such as GRP78/BIP (78 kDa glucose-regulated protein/binding immunoglobulin protein), PDI (protein disulfide isomerase), and calnexin (Matos *et al*., 2015; Doultsinos *et al*., 2017). BIP is a chaperone which plays as an essential modulator during the UPR activation in ER stress. BIP assists protein folding. This protein expresses in a great amount; thus, can be used as an indicator of ER-stress (Oslowski & Urano, 2011). Further, PDI, an enzyme which catalyzes disulfide bond formation, is required to ensure disulfide bond formation during protein folding (Matos *et al.*, 2015; Doultsinos *et al.*, 2017). Whereas, calnexin, an ER transmembrane lectin, is needed to control glycoproteins production (Matos *et al*., 2015).

There are three major transmembrane proteins in the UPR system: inositol-requiring enzyme 1α (IRE1α), PKR like ER kinase (PERK), and activating transcription factor 6 (ATF6). During the normal condition, these proteins bind to GRP78/BIP. The binding dissociates when the ER gains stressor which increases misfolded or unfolded protein (Oslowski & Urano, 2011; Lafleur *et al*., 2013; Krajarng *et al*., 2015; Amodio *et al*., 2018). The first pathway involves dissociation of IRE1α from BIP (Amodio *et al.,* 2018). This protein links with pro-survival pathways during ER stress via modulation of various chaperones (Iurlaro & Munoz-Pinedo, 2015). After IRE1α is activated, it splices 26 nucleotides from the mRNA encoding the X-box-binding protein 1 (*Xbp1*) permitting the translation of *Xbp1* (Matos *et al*., 2015; Iurlaro & Munoz-Pinedo, 2015; Krajarng *et al*., 2015; Amodio *et al*., 2018). Spliced *Xbp1* (*Xbp1s*) controls many target genes in this pathway such as ER chaperones (*Bip*) and genes which regulate ER-associated degradation, such as *Erdj4* (Krajarng *et al*., 2015), *Edem*, and *Hrd1* (Oslowski & Urano, 2011) (Figure 1.).

The second pathway is PERK. This protein acts by decreasing protein translation and

modulating oxidative stress (Iurlaro & Munoz-Pinedo, 2015). After dissociation from BIP, PERK undergoes auto-phosphorylation and triggers in the ER membrane. PERK activates specific transcription factors such as ATF4. ATF4 activates the transcription of pro-apoptotic factors C/ EBP homologous protein (CHOP) and GADD34 (Oslowski & Urano, 2011; Brown & Naidoo, 2012; Iurlaro & Munoz-Pinedo, 2015; Krajarng *et al.*, 2015) (Figure 1.).

The third pathway is ATF6. After unbinding from BIP, this protein relocates to the Golgi apparatus and divides into its active form by site-1 and site-2 protease (S1P and S2P). Active ATF6 triggers transcription of many target genes such as *Bip, Grp94* (Iurlaro & Munoz-Pinedo, 2015; Krajarng *et al.*, 2015; Doultsinos *et al.*, 2017), *Chop, Xbp1* (Iurlaro & Munoz-Pinedo, 2015; Krajarng *et al.*, 2015), *Herpud1* (Doultsinos *et al.*, 2017), and *Pdi* (Brown & Naidoo, 2012; Doultsinos *et al.*, 2017) (Figure 1.).

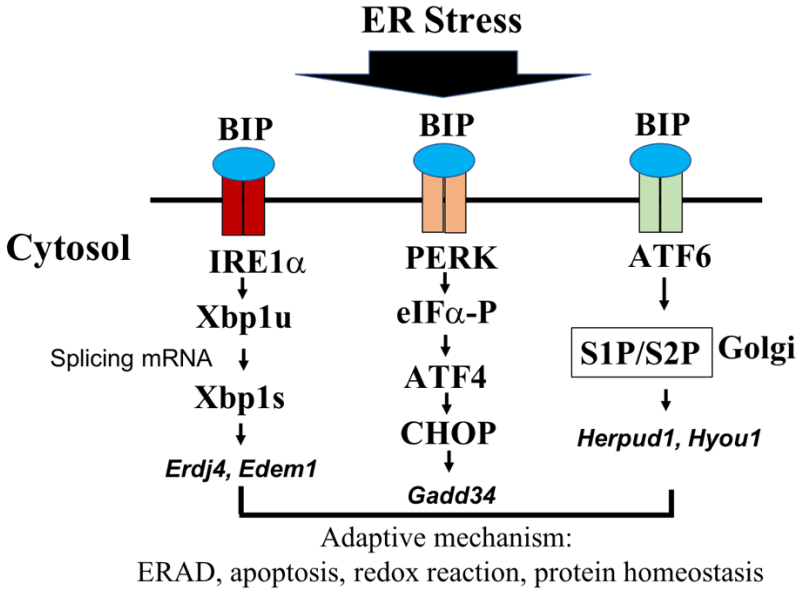

Figure 1. The three UPR pathways on ER stress responses. 1st pathway is IRE1α activation involves *Xbp1s* mRNA splicing as a transcription factor which triggers ERAD genes. 2nd pathway is PERK activation relates with the promotion of apoptotic pathway. 3rd pathway is ATF6 activation triggers ERAD genes. *Xbp1u*, unspliced *Xbp1*: *Xbp1s*, spliced *Xbp1* (Modified from Rutkowski & Kaufman, 2007; Oslowski & Urano, 2011; Hetz & Papa, 2018).

Decreasing proteostasis capability in ER-stress is a clear feature of aging. Senescence causes modification in protein metabolism which affects cell growth cessation and secretion of SAPSs (Pluquet *et al.*, 2015; Wiley & Campisi, 2016). Further, ER has two main mechanisms to attenuate senescence; first, accelerated misfolded or unfolded protein degradation through ERAD pathway, second, changes in the translational efficiency to avoid senescence (Wiley & Campisi, 2016). However, the mechanism in the UPR pathway remains unknown. ER-stress responses in correlation to many factors. Thus, the results may vary in some of studies. A lot of

studies reported that there was a relationship between the response of the UPR-related genes and senescence (Pluquet *et al*., 2015; Kannan *et al*., 2016). However, the studies about the relationship between the UPR and senescence still need further exploration.

Different results suggest that ER-stress is influenced by a lot of factors in the body, not only related to ER-machinery itself. ROS production also plays a role in ER stress-induced premature senescence. Rajesh *et al.* (2013) proved ROS elevation caused cell growth attenuation in PERK or eIF2αP-deficient primary cells. However, this condition could be repaired by antioxidant administration. This condition also found in pancreatic and breast cancer cells caused by cristacarpin, a metabolite of *Erythrina suberosa.* The compound triggers ER stress via ROS production. Cristacarpin increases p21 level followed by morphological changes such as enlarged and flattened cell as well as SAHF arrangement (Chakraborty *et al.*, 2016).

Some studies showed that the UPR machinery responses in different pathway either adaptation to cope ER stress or triggers apoptosis if the damage progresses continuously. Paz Gavilan *et al.* (2006) and Gavilan *et al.* (2009) reported that BIP, calnexin, and PDI were downregulated in the hippocampus of old rats whereas CHOP level was increased. Hussain & Ramaiah (2007) reported decreasing of ATF4 and BIP levels in the brain of old rats as well as increasing of PERK and GADD34. Whereas, aged macrophages showed alleviation of IRE1α activation and elevated sensibility to the apoptosis pathway in ER stress condition (Song *et al*., 2013). These findings suggested that the ability to connect the UPR pathway may be interrupted during aging (Martinez *et al.*, 2017). Increased levels of CHOP, ATF4, and XBP1s were observed in primary osteocytes from aged mice when compared to adult mice exposed to ER stress-inducing agents (Chalil *et al*., 2015). Similar results were reported in stromal cells from adipose tissue of aged mice, associated with augmented levels of BIP, CHOP, ATF6, and phosphorylated IRE1α (Ghosh *et a*l., 2015). CHOP level of the hindlimb muscular tissue of aged rats was also shown higher compared to that of adult rats (Baehr *et al*., 2016). Lenox *et al.* (2015) also studied that the UPR target genes also have a relationship with aging in the central nervous system. They found that phosphorylation of CHOP, ATF6, GADD34, ATF4, and eIF2a are upregulated in the retina of aged rats. Druelle *et al.* (2016) evaluated that senescence has a correlation with increasing ER stress level accompanied by elevation of ER size. The condition caused by an elevation of protein level in ER during senescence is mainly through ATF6 activation.

1.6 Senescence related to decreasing of NAD^+ level in the cell

NAD^+ plays an essential role in various biological processes/metabolic reactions such as glucose, lipid, and energy homeostasis (Lin & Guarente, 2003, Peek *et al*., 2013; Di Stefano & Conforti, 2013), cell communication, cell metabolism (Chini *et al*., 2017), post-translational protein modification, DNA repair, redox reaction, as well as multiple signalling pathways (De Figueiredo *et al*., 2011; Yaku *et al*., 2018). Decreasing NAD^+ level gains great attention because of association with the onset of age-associated diseases such as metabolic and neurodegenerative diseases, and many types of cancer (Imai, 2009; Yaku *et al*., 2018). NAD^+ level decreases as the organism become older (van der Veer *et al.,* 2007; Khaidizar *et al*., 2017; Ma *et al*., 2017). Ma *et al*. (2017) reported that mesenchymal stem cells from aged rats had a low level of NAMPT followed by declining of intracellular NAD^+ as well as SIRTUIN 1 (SIRT1) levels and activities. Moreover, Khaidizar *et al.* (2017) investigated that this phenotype also found in replicative senescence of mouse embryonic fibroblasts. This supports previous study which showed that there was reducing NAMPT activity accompanied by replicative senescence of human smooth muscle cells through decreasing of SIRT1 activity (van der Veer *et al*., 2007). Imai & Guarente (2014) suggested that the onset of aging is related to reducing SIRTUIN activity in nucleus and mitochondria, which caused a reduction of NAD^+.

Several factors cause decreasing of NAD^+ level. Imbalance of NAD^+ production and degradation can be caused by damage of NAMPT and PARP (poly ADP ribose polymerase), respectively (Imai & Guarente, 2014). Some endogenous and exogenous factors such as DNA damage, oxidative stress, senescence, and inflammation reduce NAD^+ level. This mechanism can be occurring vice versa. This means the reduction of NAD^+ level can be caused by the alteration in NAD^+ breakdown, production, or both (Chini *et al*., 2017) which leads to aging and disturbances of metabolic and physiological functions (Figure 2.).

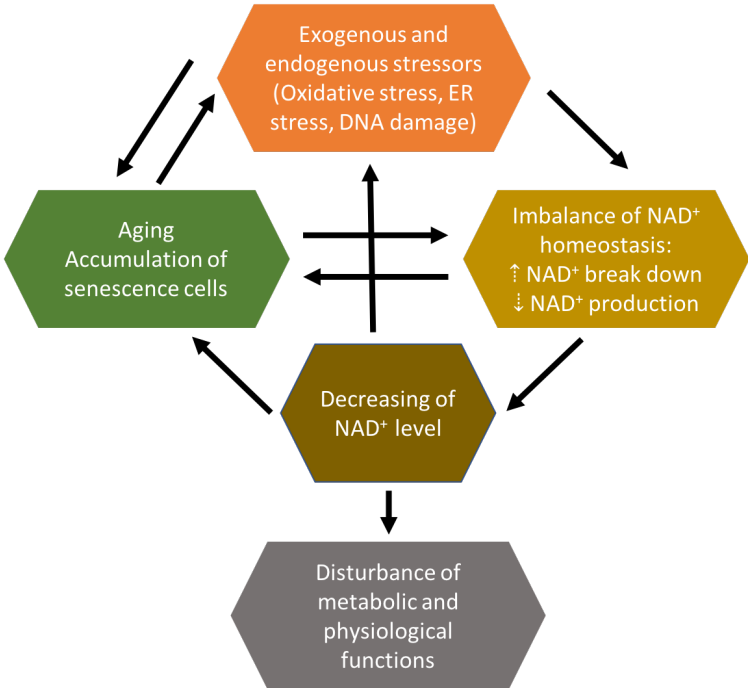

Figure 2. The connection between stressor, aging, and decreasing of NAD^+ level, which promotes metabolic and physiological disfunction (Modified from Chini *et al*., 2017).

1.7 Maintenance of NAD^+ homeostasis is a strategy to prevent senescence

In mammals, NAD^+ is maintained mostly through the salvage pathway. This pathway converts nicotinamide (NAM, one of vitamin B) to nicotinamide mononucleotide (NMN) by the rate-limiting enzyme, NAMPT. Further NMN is converted to NAD^+ by nicotinamide mononucleotide-adenylyltransferase (NMNAT) 1-3. NAD^+ is utilized by NAD^+-consuming enzymes such as SIRTUINs, PARPs, and cluster of differentiation (CD) 38 for several physiological and metabolic aspects (Houtkooper *et al.,* 2010; Nikiforov *et al*., 2015). Thus, it is crucial to maintain NAD^+ homeostasis through balancing the mechanism of NAD^+ synthesis and degradation (Chini *et al.*, 2017; Yaku *et al*., 2018) (Figure 3.).

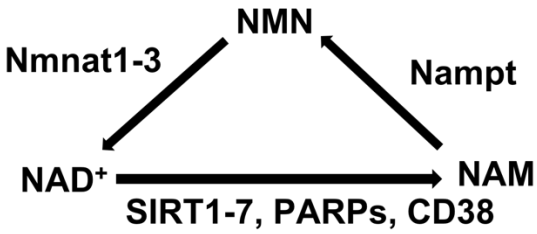

Figure 3. NAD^+ salvage pathway. NAD^+ homeostasis tightly controlled by its rate-limiting enzyme (NAMPT) and its consuming enzymes (SIRTUINs, PARPs, CD38) (Modified from Nakahata *et al*., 2009; Imai, 2010; Imai & Guarente, 2014).

Many strategies have already done to increase NAD^+ level in the body. The first strategy relates to limiting NAD^+ reduction or increasing NAD^+ level using its precursors such as NAM

or other supplementations. This can be one of the best and fast strategies (Imai & Guarente, 2014; Yaku *et al*., 2018). Kang *et al.* (2006) reported that NAM could stimulate life extension of human fibroblast. NAM is predicted to be involved in this process through the suppression of ROS production and mitochondrial activity. NAM supplementation could maintain the level of some cell cycle regulator proteins such as p53, p21, and pRb in human fibroblast. Increasing NAD^+ level by NMN and nicotinamide ribose (NR), another NAD^+ precursor, supplementation also can repair many age-associated pathophysiologies caused by NAD^+ reduction (Imai & Guarente, 2014). Increasing of NAD^+ metabolism extends lifetime in various organisms. Thus, it is obvious that NAD^+ regulation plays an essential role in aging and lifespan extension (Yaku *et al*., 2018).

The second strategy can be done using an internal mechanism which requires activation of NAMPT, a rate-limiting enzyme of NAD^+ production, which involves activation of SIRT1 to inhibit senescence. A previous study by van der Veer *et al.* (2007) found that the introduction of the *Nampt* gene into human smooth muscle cells (SMCs) can delay senescence. NAMPT-mediated SMCs lifespan extension was associated with increased activity of the NAD^+-dependent longevity enzyme SIRT1. Ho *et al.* (2009) also found that NAMPT activity influenced SIRT1 activity in smooth muscle cells (SMCs). Overexpression of *Nampt* delayed senescence of SIRT1-overexpressing SMCs by increasing SIRT1 activity. Khaidizar *et al.* (2017) studied this mechanism using primary mouse embryonic fibroblasts (MEFs). Raising NAMPT level is proofed to play a role in the delay of onset of replicative senescence with increasing SIRT1 activity.

Although almost all researches investigate the effect of NAD^+ on senescence were carried out about replicative senescence, most of the senescence is caused by stress-induced premature senescence such as oxidative or endoplasmic reticulum stress (Toussaint *et al*., 2002; de Magalhaes & Passos, 2018). Stressors may promote cellular senescence depend on the concentration of stressor, cell type, duration of exposure (de Magalhaes & Passos, 2018). Duan *et al.* (2005) and Pienkowska *et al.* (2020) stated that stress-induced premature senescence caused by stress such as oxidative stress after H_2O_2 exposures might have a different response from replicative senescence. Thus, it is still needed to reveal whether NAD^+ has resistances to stress-induced premature senescence in the mammalian cell. To address that, I use the fibroblast cells derived from *Nampt*-overexpressing mice, which were previously established in my laboratory (Khaidizar *et al.* 2017). This study is important to get a more comprehensive understanding of the role of NAMPT on senescence that would be essential to find suitable

therapies for mammalian age-associated diseases.

I found that *Nampt* overexpressing MEFs (*Nampt* TG-MEFs) resistant against stress-induced premature senescence caused by oxidative- and ER-stress, which showed higher cell growth, normal cell morphology, and lower SA-β-Gal positive cells compared with the counterpart WT-MEFs. *Nampt* TG-MEFs also needed a higher concentration of stressors to become senescence. Besides, *Nampt* TG-MEFs showed more resistance and response more quickly against ER stress.

1.8 Objectives

Senescence gains attention since the process is associated with aging and age-associated degenerative diseases. It is needed a proper strategy to prevent cellular senescence. Decreasing of NAD^+ level is known to be related to senescence in elderly both human and mouse cells. Thus, increasing of NAMPT activity or NAD^+ level can become a choice. Previously, van der Veer *et al.* (2007) and Khaidizar *et al.* (2017) proved that this strategy could postpone replicative senescence. However, the senescence response can be different between replicative and stress-induced premature senescence. Moreover, most of the senescence is caused by endogenous and exogenous stressors. Thus, the purpose of this study is studying the effects of overexpression of *Nampt* in mouse embryonic fibroblasts against oxidative and endoplasmic reticulum stress-induced premature senescence. Further, this can be a more comprehensive understanding related to the role of NAMPT/NAD^+ in senescence to promote healthy aging/longevity *in vivo*.

1.9 Hypothesis

Nampt TG-MEFs have resistance against oxidative and ER stress-induced premature senescence caused by hydrogen peroxide (H_2O_2) and tunicamycin, respectively compared to WT-MEFs which are caused by high NAMPT activity followed by high NAD^+ level.

2.0 Materials and Methods

2.1 Generating transgenic mice

In this study, I used C57BL/6 mice. The mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and maintained under 12 hours light/12 hours dark cycles. Transgenic mice were generated using human *Nampt* coding sequence with HA tag at 3' region of pCMV, which subcloned into pCAGGS vector. The clone was digested by *Pvu*I and *Bam*HI. Further, the clone was microinjected into the pronuclei of fertilized eggs of C57BL/6 mice. TG mice were crossed with C57BL/6 female mice to produce primary MEFs (Khaidizar's thesis, 2017). My experiments were approved by the Animal Care Committee of Nara Institute of Science and Technology and performed in consent with the guideline that was established by the Science Council of Japan.

2.2 Primary mouse embryonic fibroblasts

Primary mouse embryonic fibroblasts were harvested from embryonic day 14.5 embryos. Protocols to establish and maintain *Nampt* TG- and WT- primary MEFs were followed procedure by Khaidizar *et al.* (2017). First, the head and intestinal organs of the mouse were removed. The mouse embryo was rinsed with 1x PBS and chopped on a petri dish. Chopped tissues were incubated with 2 ml of trypsin solution (0.05% trypsin, 0.53 mmol/l EDTA-4Na, Wako, Japan) for 10 min at 37°C with a flow of 5% CO_2. After that, added 8 ml of Dulbecco's Modified Eagle Media (DMEM) (4.5 g/L glucose) (Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate (Nacalai tesque), 1× MEM amino acids (GIBCO), 0.1 mM 2-mercaptoethanol (GIBCO), 10 U/ml penicillin/10 µg/ml streptomycin (Nacalai tesque), and 0.1 mg/ml primocin (InvivoGen). This media deactivated trypsin. The chopped tissues and media were powerfully pipetted several times to get a higher number of single cells. The cell suspension was let for 5 min at room temperature, and the supernatant was removed to a 10-cm dish and incubated at 37°C with a flow of 5% CO_2 for 1 day. These cells defined as passage 0. All cells used in this study were from passage 1.

2.3 Cell culture

Primary *Nampt* TG- and WT-MEFs were cultured in 10 ml culture dish nourish with Dulbecco's Modified Eagle Media (4.5 g/L glucose) enriched with 10% fetal bovine serum, 1 mM sodium pyruvate (Nacalai tesque), 1× MEM amino acids (GIBCO), 0.1 mM 2-

mercaptoethanol (GIBCO), 10 U/ml penicillin/10 μg/ml streptomycin, and 0.1 mg/ml primocin incubated at 37°C with a flow of 5% CO_2. After reached 70-90% confluency, the cells were trypsinized for 5 mins at the 37°C to detach from neighboring cells and 10 ml culture dish. 10 ml primary MEF media was added to deactivate trypsin. After that, gently pipetting cell suspension to detach them completely. The cell suspension was removed to the conical tube. For counting the cell number, then take 10 μl of cell suspension and mixed with 10 μl trypan blue in a microtube. Cell counting was done using counting cell chamber haemocytometer. The number of the cells were seeded based on the experiment design in each procedure.

2.4 Cell viability assay by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Cells were grown at 1,500 cells/well in 96-well plate and incubated at 37°C with a flow of 5% CO_2 for 24 hours prior treatment. After 24 hours culture, cells were treated with various concentration (25, 50, 100, 200, 400, and 600 μM) of hydrogen peroxide (H_2O_2) (Wako, Osaka, Japan) for 24 hours and trypsinized right after treatment or various concentration (2, 20, and 200 ng/ml) of tunicamycin (Sigma-Aldrich, St. Louis, MO, USA) for 3 hours and trypsinized after additional 21 hours culture. All treatments were made triplicate. Cell viability was measured using MTT assay based on manufacturer protocol (Nacalai tesque, Kyoto, Japan). This method used to evaluate cell viability by quantifying the dehydrogenase activity of mitochondria. 10 μl of MTT solution (5 mg/ml MTT in phosphate-buffered saline) was added to each well and the plate was incubated at 37°C with a flow of 5% CO_2 for 3 hours. Subsequently, 100 μl of solubilization solution (0.04 mol/l HCl in isopropanol) was added to each well, and the solution was pipetted repeatedly to dissolve the precipitated formazan. The plate was observed under the microscope to ensure that all formazan has dissolved. Finally, the absorbance was measured using a microplate reader (Mithras) at 570 nm wavelength. Cell viability represents as a percentage of viable cells.

2.5 Cell growth assay

Cells were grown at 4 x 10^4 cells in 35 mm dishes at and cultured for 24 hours at 37°C with a flow of 5% CO_2. Primary WT- and TG-MEFs were treated with various concentration (50, 200, and 400 μM) of hydrogen peroxide for 24 hours or various concentration (2, 20, and 200 ng/ml) of tunicamycin for 3 hours. Cells were washed twice with 1x PBS and cultured for another 6 days (day 7) with a single passaging after 3 days culturing (day 4). After trypsinization

at day 4 and 7, the cells were counted using trypan blue staining to analyze cell growth assay.

2.6 Senescence-associated β-galactosidase activity

The same cells which used for growth assay were used for SA-β-Gal assay. Cells were grown in 35 mm dishes at 4 x 10^4 cells and cultured for 24 hours at 37°C with a flow of 5% CO_2. Primary MEFs were treated with various concentration of hydrogen peroxide (25, 50, 100, 200, 400, and 600 μM) for 24 hours or tunicamycin (2, 20, and 200 ng/ml) for 3 hours. Cells were rinsed twice with 1x PBS and cultured for another 6 days with a single passaging 3 days after treatment. After trypsinization at day 7, the cells were seeded again in 35 mm dishes at 4 x 10^4 cells and cultured for 24 hours at 37°C with a flow of 5% CO_2 prior SA-β-Gal assay (Figure 4.). SA-β-Gal assay was done according to the protocol described by Debacq-Chainiaux *et al.* (2009). Cells were rinsed twice with 1x PBS, fixed with 2 ml the mixture of 2% formaldehyde and 0.2% glutaraldehyde in H_2O for 5 min at room temperature. After that, the cells were rinsed again with 1x PBS twice. Further, 2 ml of SA-β-Gal staining solution (40 mM sodium citrate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 150 mM sodium chloride, and 2 mM magnesium chloride) were added to the cells and incubated at 37°C without CO_2 for 18 hours with gentle agitation. Each sample was checked under an inverted microscope. Every sample was count for minimum of 200 cells. Positive senescence cells were stained blue. Those cells were counted and represented in percent. The cells were photographed.

2.7 RNA extraction and cDNA synthesis

2.7.1 RNA extraction

Cells were grown at 4x10^4 cells in 35 mm dishes and cultured for 24 hr at 37°C with a flow of 5% CO_2. MEFs treated with tunicamycin for 3 hours at the various concentration (2, 20, and 200 ng/ml) of tunicamycin. Total RNA was extracted from MEFs at 0 or 21 hours after tunicamycin treatment. The medium of the cells was aspirated. Cells were rinsed twice using 1x PBS to clean the cells from remaining medium. 1 ml Sepasol RNA-I Super G (Nacalai tesque, Kyoto, Japan) were added to the cells. All cells were scraped evenly and transferred to 1.5 ml tubes and maintained at -80°C before used.

For extracting the RNA, cells were thawed by left to stand at room temperature for 30 mins with gentle agitation. After cells melted and mixed evenly, 200 μl chloroform is added to each tube. The content was mixed by inverting the tubes vigorously several times and left to

stand for 10 mins.

Afterwards, the mixture was centrifuged at 12,000 x g for 15 mins at 4°C to obtain the aqueous phase that contains the RNA fractions. 500-600 µl of the aqueous phase was removed to new 1.5 ml Eppendorf tubes and further added with an equal volume of isopropanol. The mixture was again mixed by inverted the tube vigorously and left to stand at room temperature for 10 mins. Next, the mixture was centrifuged at 12,000 x g for 10 mins at 4°C to make the RNA become pellet. The supernatant was discarded and RNA pellet was rinsed by adding 75% ethanol, and followed by centrifugation at 12,000 x g for 5 mins at 4°C. The supernatant was discarded carefully without disturbing the pellet of RNA. The pellet was air-dried for 10 mins at room temperature before dissolved in purified water. The total amount of RNA was calculated. RNA quantification was carried out using Nanodrop spectrophotometer (Thermo Scientific). Purified RNA samples are kept at -20°C freezer for long term storage.

2.7.2 cDNA synthesis

One µg of total RNA was converted to complementary DNA (cDNA) by reverse-transcription PCR using Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with random primers following the manufacturer's instructions. Each reaction consists of 1 µg RNA extract, 1X First-Strand Buffer, 10 mM dNTPs, 250 ng random primers, and added with purified autoclaved water to a final volume of 20 µl. First, RNA samples were added to random primers, dNTPs, and water in a PCR strip and were heated to 65°C for 10 mins. After that, First-Strand Buffer, 10 mM DTT, and Reverse Transcriptase were added to each sample. The samples were then heated up to 42°C for 2 hours, followed by 70°C for 15 mins. The cDNA products were diluted with distillate water 5x and kept at -20°C freezer for long term storage.

2.8 Real-time quantitative PCR (qPCR)

Quantitative PCR was performed using SYBR green dye from KAPA SYBR FAST Universal 2× qPCR Master Mix (Nippon Genetics, Tokyo, Japan). Each reaction consists of 1X KAPA SYBR FAST qPCR Master Mix universal, 200 nM of forward and reverse primers, 20 ng of cDNA products and added water to a final volume of 10 µl. All reagents except the cDNA were prepared in a master mix and aliquoted into wells of a white multiwell plate before lastly adding the cDNA samples. qPCR was performed out using the Light Cycler 480 (Roche) under the following conditions: samples were heated to 95°C for initial denaturation 3 min, and followed by 40 cycles of denaturation at 95°C for 10s, hybridization at 60°C for 20s followed

by elongation at 72°C for 1s. Fold change of target gene transcripts was calculated using the absolute quantification method and normalized to *Gapdh* gene expression as an endogenous control. The sequences of the forward and reverse primers are listed in Table 1. as follows.

Table 1. Table of primers.

No.	Gene	Sequences
1.	*Gapdh*	FW: TTCACCACCATGGAGAAGGC
		RV: TTGTCATGGATGACCTTGGC
2.	*Nampt*	FW: TTGTTCAGTCCTGGTATCCAAT
		RV: CCTATGCCAGCAGTCTCTTG
3.	*Chop* *1	FW: CCACCACACCTGAAAGCAGAA
		RV: AGGTGAAAGGCAGGGACTCA
4.	*Edem* *1	FW: CTACCTGCGAAGAGGCCG
		RV: GTTCATGAGCTGCCCACTGA
5.	*Bip* *2	FW: CATGGTTCTCACTAAAATGAAAGG
		RV: GCTGGTACAGTAACAATG
6.	*Gadd34* *2	FW: GAGATTCCTCTAAAAGCTCGG
		RV: CAGGGACCTCGACGGCAGC
7.	*Erdj4*	FW: CTCCACAGTCAGTTTTCGTCTT
		RV: GGCCTTTTTGATTTGTCGCTC
8.	*Pdi*	FW: TGAACAGACAGCTCCGAAGAT
		RV: GCCGTCATAGTCAGATACACTCT
9.	*Hyou1* *3	FW: TACTCCCGTTCCTTGGCTGAAG
		RV: GGCTGTGGCAGTGTTGTCATTG
10.	*Herpud1* *4	FW: AGAACATCTCTAGGCCTGAG
		RV: TGCCTTGCATAGATCTGCTG
11.	*Calnexin* *5	FW: GGGAGTCTTGTCGTGGAATTG
		RV: TGCTTTCCAAGACGGCAGA

*1 Oslowski, C. M. & Urano, F. (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods Enzymol*, 490, 71–92.

*2 Rutkowski, D. T., Arnold, S. M., Miller, C. N., Wu, J., Li, J., Gunnison, K. M., Mori, K., Akha, A. A. S., Raden, D., & Kaufman, R. J. (2006). Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS Biology*,

4(11), e374.
*3 Walters, S. N., Luzuriaga, J., Chan, J. Y., Grey, S. T., & Laybutt, D. R. (2013). Influence of chronic hyperglycemia on the loss of the unfolded protein response in transplanted islets. *Journal of Molecular Endocrinology*, 51, 225–232.
*4 Le, T. M., Hashida, K., Ta, H. M., Takarada-Iemata, M., Kokame, K., Kitao, Y., & Hori, O. (2016). Deletion of Herpud1 enhances Heme Oxygenase-1 expression in a mouse model of Parkinson's disease. *Parkinson's Disease*, 2016, Article ID 6163934. https://doi.org/10.1155/2016/6163934.
*5 Martindale, J. J., Fernandez, R., Thuerauf, D., Whittaker, R., Gude, N., Sussman, M. A., & Glembotski, C. C. (2006). Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6. *Circulation Research*, 98, 1186-1193.

2.9 Statistical analysis

Values are reported as mean ± SEM. Statistical differences were determined by a Student's two-tailed *t* test. Statistical significance is displayed as * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$) or # ($p < 0.05$), ## ($p < 0.01$), or ### ($p < 0.001$).

3.0 Results

There are several strategies to increase NAD^+ level in the body to prevent age-related diseases and senescence. In this study, I used strategy to increase NAD^+ level in cells by overexpressing *Nampt* to inhibit senescence. Previously, the laboratory that I belong to showed that transgenic *Nampt*-overexpressing primary mouse embryonic fibroblasts (*Nampt* TG-MEFs) postponed replicative senescence (Khaidizar, *et al*., 2017). However, there is no clear report yet whether *Nampt* overexpressing or NAD^+ precursor administrated to cells also show similar resistance or mechanism to response stress-induced premature senescence. In this study, I used H_2O_2 and tunicamycin to induce stress-induced premature senescence. H_2O_2 is known as by-product in cellular metabolism, which is prominent to increase cellular ROS level (Guo *et al*., 2010). This compound is widely known as stress-induced premature senescence inducer (Duan *et al*., 2005). Whereas, tunicamycin is a drug which suppresses the UDP-N-acetylglucosamine-dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT) in the ER, therefore obstructs the primary stage of glycoprotein bioproduction in the ER. Tunicamycin treatment causes unfolded glycoprotein aggregations in ER, promoting to ER stress. Tunicamycin disturbs proper protein folding in cell thus result in ER stress which causes the onset of senescence (Oslowski & Urano, 2011).

3.1 *Nampt* overexpressing transgenic mice

Previously, Khaidizar *et al*. (2017) generated transgenic mice overexpressing HA-tagged human *Nampt* gene (Figure 4.), which is explained in section 2.1 of Materials and Methods. Kahidizar *et al.* (2017) established 4 different lines of *Nampt* TG-MEFs. Those lines increased NAD^+ levels playing a role to postpone replicative senescence in primary MEFs. The authors reported that line #4 has the highest NAMPT/NAD^+ among their counterpart and has the highest effect against senescence. Thus, in this study, I used *Nampt* TG-MEFs line #4 (refers as TG-MEFs) unless otherwise mentioned.

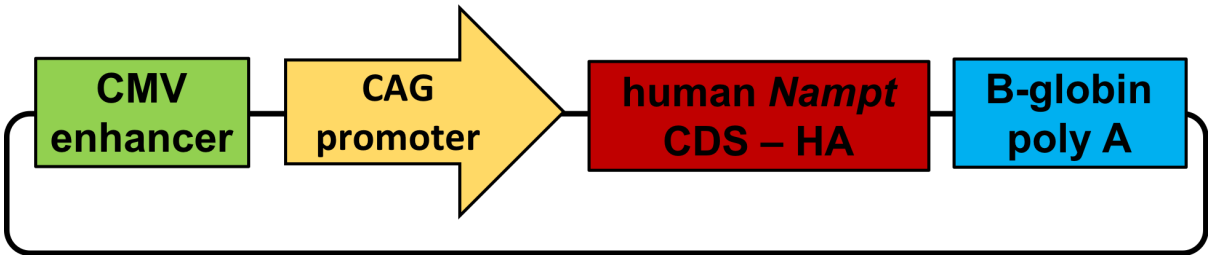

Figure 4. The construct of the expression vector of *Nampt* overexpressing transgenic C57BL/6 mice. Human *Nampt* gene tagged with HA to produce *Nampt* overexpressing transgenic mice. (Modified from Khaidizar's thesis, 2017).

3.2 *Nampt* overexpressing MEFs showed higher *Nampt* mRNA level compared to wild type MEFs

In this study, first I measured *Nampt* mRNA amount in MEFs by qPCR using the primer set, which could detect both mouse and human *Nampt*. I confirmed that relative *Nampt* mRNA amount in TG-MEFs line #4 was around 53 folds compared to WT-MEFs (Figure 5A.). This result was consistent with Khaidizar *et al*. (2017) result, which showed that NAMPT protein amount of TG-MEFs line #4 was the highest among the other counterparts. This result also confirmed for TG-MEFs line #1. I found that TG-MEFs line #1 had relative *Nampt* mRNA amount around 28 folds compared to WT MEFs (Figure 5B.) which were again consistent with Khaidizar *et al.* (2017) result. They reported that NAMPT protein level in TG-MEFs line #1 was the second-highest among the littermate. Later, I also used this TG-MEFs line #1 in some experiments for confirmation. Khaidizar *et al*. (2017) reported that *Nampt* overexpression in TG-MEFs caused increasing of NAD^+ level. Further, they found that *Nampt* overexpression played a role to postpone replicative senescence in primary mouse embryonic fibroblasts. High *Nampt* mRNA in TG-MEFs reflects high NAMPT amount along with high NAMPT activity which further may play a role in stress-induced premature senescence. In this study, I used *Gapdh* as a reference gene. *Gapdh* is an enzyme involved in the transformation of glyceraldehyde-3-phosphate (G3P) into 1,3-biphosphoglycerate (1,3-BPG) in glycolysis (Tristan *et al*., 2013). Hernandez-Segura *et al.* (2018)[b] reviewed that *Gapdh* widely used as a reference gene, either single (*Gapdh* only) or mix (*Gapdh* with other reference genes). This is supported by many studies that evaluated *Gapdh* is quite stable and can be used as a reference gene in the qPCR analysis (Barber *et al*., 2005; Zainuddin *et al*., 2010; Gonzalez-Bermudez *et al*., 2019; Yan *et al*., 2019).

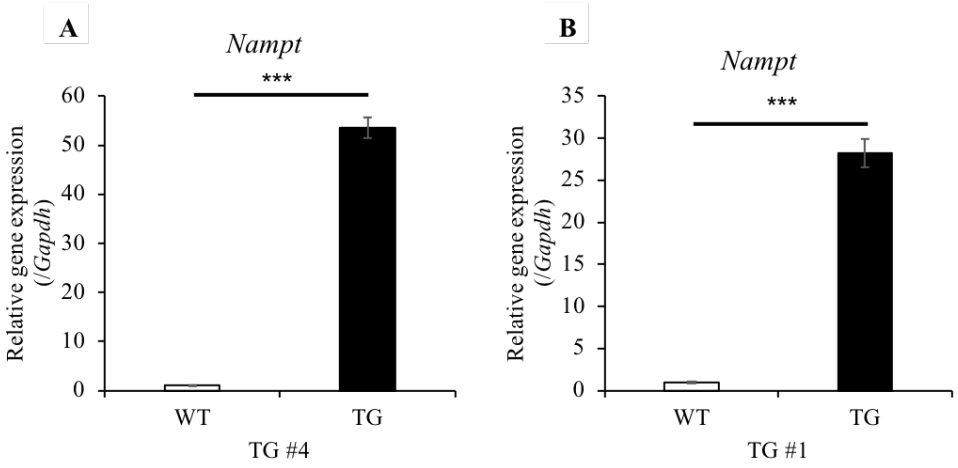

Figure 5. Relative *Nampt* mRNA level of TG-MEFs was higher compared to wild type MEFs. Moreover, relative *Nampt* mRNA level of TG-MEFs line #4 also higher compared to TG-MEFs line #1. A. TG-MEFs line #4 and WT-MEFs at passage 1 were extracted to collect total RNA and subjected to quantitative PCR analysis, B. TG-MEFs line #1 and WT-MEFs at passage 1 were extracted to collect total RNA and subjected to quantitative PCR analysis. *Gapdh* was used as reference gene, ***: $p < 0.001$ (Student's t-test), n=3.

3.3 *Nampt* overexpressing MEFs had higher cell growth under acute oxidative and ER stress compared to wild type MEFs

Previously Khaidizar *et al.* (2017) showed that *Nampt* TG-MEFs postponed replicative senescence. However, it remains unclear whether *Nampt* overexpressing MEFs also show similar resistance or mechanism to response stress-induced premature senescence in oxidative stress with H_2O_2 treatment and ER stress with tunicamycin treatment. Toussaint *et al*. (2002) and de Magalhaes & Passos (2018) stated that stressors such as UV irradiation, H_2O_2, and hyperoxia, can cause senescence and may become the major cause of senescence. Therefore, first, I evaluated the effect of H_2O_2 and tunicamycin on cell viability.

My results showed that after 24 hours of H_2O_2 treatment up to the concentration of 200 μM, cell viability was above 80%. Decreasing of cell viability clearly happened at the concentration of 400 μM. In this concentration, cell viability of WT-MEFs was 66.46 ± 6.32% whereas in TG-MEFs was 74.12 ± 8.68%. Further, at the highest treated concentration (600 μM), cell viability of WT-MEFs was 65.51 ± 5.26% whereas in that of TG-MEFs was 55.86 ± 5.7% (Figure 6A.). However, cell viability between WT- and TG-MEFs in almost all studied concentrations were not significantly different but decreased dose-dependently. I used passage 1 for this experiment. I thought that, in this condition, both WT- and TG-MEFs still had normal biochemical and physiological properties. Khaidizar *et al.* (2017) reported that MEFs started to cease cell proliferation around passage 6 and 7 for WT- and TG-MEFs, respectively.

Chen & Ames (1994) studied that H_2O_2 treatment on human fibroblast at the concentration between 50-300 μM relatively did not affect cell viability on the first day after treatment. Bladier *et al.* (1997) reported that up to the concentration of 100 μM, H_2O_2 treatment did not affect primary human fibroblasts. Other studies by Pizzaro *et al.* (2009) reported that H_2O_2 treatment at the concentration of 500-1000 μM reduced viabilities of rat neuroblastoma cell line more than 25%. Kiyoshima *et al.* (2012) reported that mouse gingival fibroblasts (MGFs) showed a similar result as Bladier *et al*. (1997). H_2O_2 treatment up to the concentration of 100 μM relatively did not affect cell viability. Similar to my result, those studies showed that H_2O_2 treatment affect cell viability dose-dependently both in human and mouse cells.

Although previously Khaidizar *et al.* (2017) reported that administration H_2O_2 for 24 hours at the concentration of 200-800 μM showed decreasing cell viability dose-dependently, they found that TG-MEFs have higher cell viability compared to WT-MEFs. However, they did that study at passage 3, and I did my experiment at passage 1. Previously, Khaidizar *et al.* (2017) found that NAMPT amount decreased along with the number of passages. Decreasing of NAMPT amount was accompanied by decreasing of NAD^+ level. I guessed that at passage 1 NAMPT/NAD^+ level of WT-MEFs is still high and comparable to TG-MEFs. This might be the reason why WT- and TG-MEFs viabilities at passage 1 were not significantly different between both genotypes.

Whereas, after 3 hours of tunicamycin treatment, cell viability in both genotypes was still above 90% up to the concentration of 20 ng/ml and decreased more than 30% at the concentration of 200 ng/ml (Figure 6B.). The response of tunicamycin depends on various factors such as cell type, concentration, and time of exposure. Chalil *et al.* (2015) showed that cell viability of mouse osteocyte after tunicamycin treatment at the concentration of 1-5 μg/ml for 17 hours was above 75%. Ji *et al.* (2018) showed that cell viability of intestinal epithelial cell treated with tunicamycin at the concentration of 5 μg/ml and above for 24 hours decreased more than 25%. Wu *et al.* (2018) found that in various human gastric epithelial cell lines treated with 200 ng/ml tunicamycin had cell viability above 75%. On those experiments, cell viability was decreased dose-dependently. Those previous reports used a higher dose of tunicamycin compared to my study. My result was also similar to the study by Shen *et al.* (2015) that showed after 24 hours of tunicamycin treatment up to the concentration 500 ng/ml on primary rat cardiomyocytes, cell viability was above 70%. Whereas the study by Nakahata *et al.* (2018) reported that tunicamycin treatment at the concentration of 20 ng/ml decrease MEFs cell viability more than 20%. Interestingly at that study, tunicamycin administration up to the

concentration of 200-2000 ng/ml did not show a significant difference in cell viability to that of the cells treated by 20 ng/ml of tunicamycin. These results suggest that cells need time to response stressor, which affect cell viability. Thus, cell viability between both genotypes were still comparable after short time of exposure.

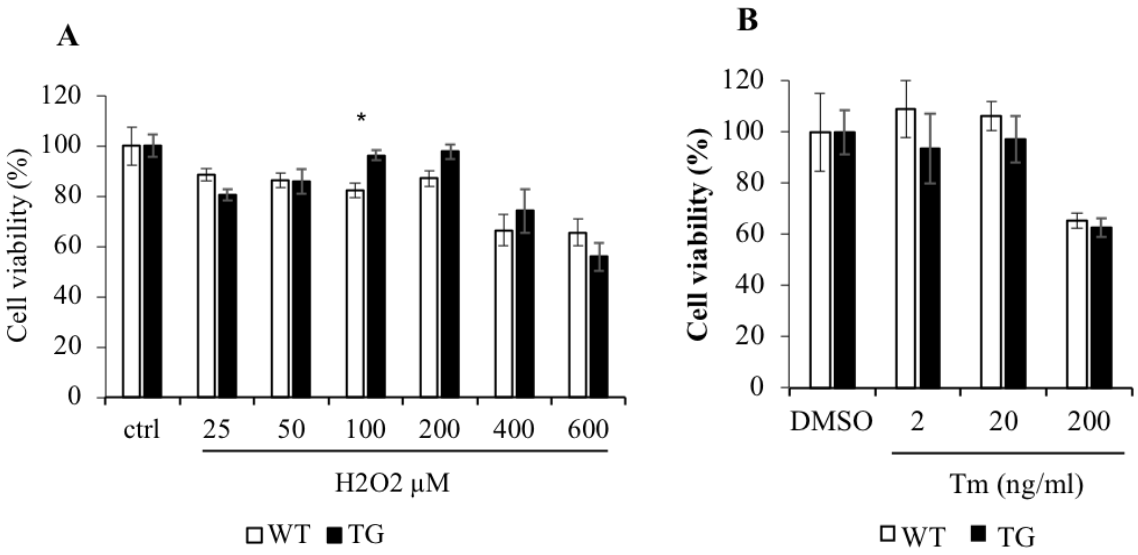

Figure 6. *Nampt* overexpressing MEFs (TG-MEFs) showed no significant difference in cell viability compared to wild type MEFs after acute oxidative and ER stress condition. A. Cell viability was assessed at passage 1 under the various concentration of H_2O_2 treatment for 24 hours, B. Cell viability was assessed at passage 1 under the various concentration of tunicamycin treatment for 3 hours followed by 21 hours incubation. Cell viability was analyzed using MTT assay and represent as a percentage compared to the control group. H_2O_2: hydrogen peroxide, Tm: tunicamycin, *: $p < 0.05$ (Student's t-test), n=3 (Data published by Nuriliani *et al*., 2020).

A major characteristic of senescence is growth arrest. Khaidizar *et al*. (2017) reported that there was a decreasing of cell proliferative capacity in senescence. They proofed that TG-MEFs had higher cell growth compared to WT-MEFs under continuous H_2O_2 (50 µM) treatment beginning from passage 2. In this project, I used a single treatment of various concentration of H_2O_2. The results showed that at the concentration of 50 µM H_2O_2 treatment, proliferation capacity of WT- and TG-MEFs were comparable and continuously proliferated up to day 7. However, TG-MEFs growth seemed slightly higher compared to that of WT-MEFs. My result showed that after H_2O_2 treatment at the concentration of 100 µM WT-MEFs proliferation capacity seemed ceased at day 4. However, TG-MEFs proliferation capacity remained high until day 7. At the concentration of 400 µM H_2O_2, WT-MEFs totally diminished its proliferation capacity. In contrast, TG-MEFs proliferation remained up to day 7. However, TG-MEFs proliferation capacity was reduced compared to at the concentration of 100 µM H_2O_2

treatment (Figure 7.). The results showed that TG-MEFs required a higher concentration of H_2O_2 to induce cell growth arrest compared to WT-MEFs. Furthermore, this result was consistent with previous reports by Chen & Ames (1994) and Bladier *et al*. (1997) which showed that under sub-lethal H_2O_2 concentration treated condition, human fibroblasts decreased its proliferative capacity.

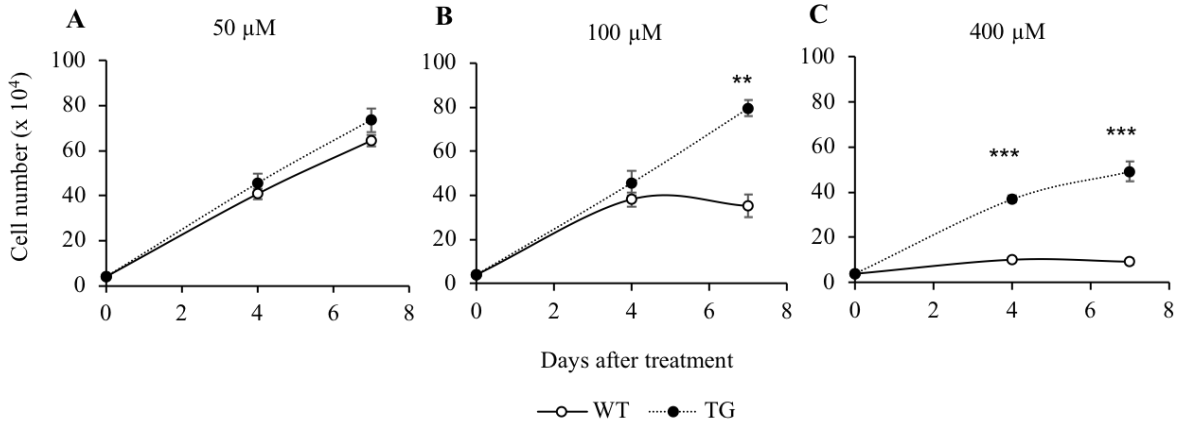

Figure 7. *Nampt* overexpressing MEFs (TG-MEFs) had higher cell growth under acute oxidative stress-induced premature senescence compared to wild type MEFs. A-C: cell growth was assessed start from passage 1 under the various concentration of H_2O_2 treatment for 24 hours. Cells were stained by trypan blue exclusion. Viable cells were unstained whereas death cells were stained blue, **: $p < 0.01$, ***: $p < 0.001$ (Student's t-test), n=3 (Data published by Nuriliani *et al*., 2020).

I studied whether this effect is specific to oxidative stress or not. Thus, I evaluated WT- and TG-MEFs cell growth in ER-stress condition after 3 hours of tunicamycin treatment. The results showed that at the concentration of 2 and 20 ng/ml of tunicamycin treatment, WT- and TG-MEFs cell growth was arrested at day 4, although proliferation capacity was higher in TG-MEFs. At the concentration of 200 ng/ml, tunicamycin treatment affected both genotypes more than lower tunicamycin concentration. At this concentration, TG-MEFs still proliferated more than WT-MEFs (Figure 8.). My results were consistent with previous studies which showed that tunicamycin treatment in various concentration and exposure time decreased proliferation capacity of human breast cancer cells (Zhong *et al*., 2017; Serrano-Negron *et al*., 2018).

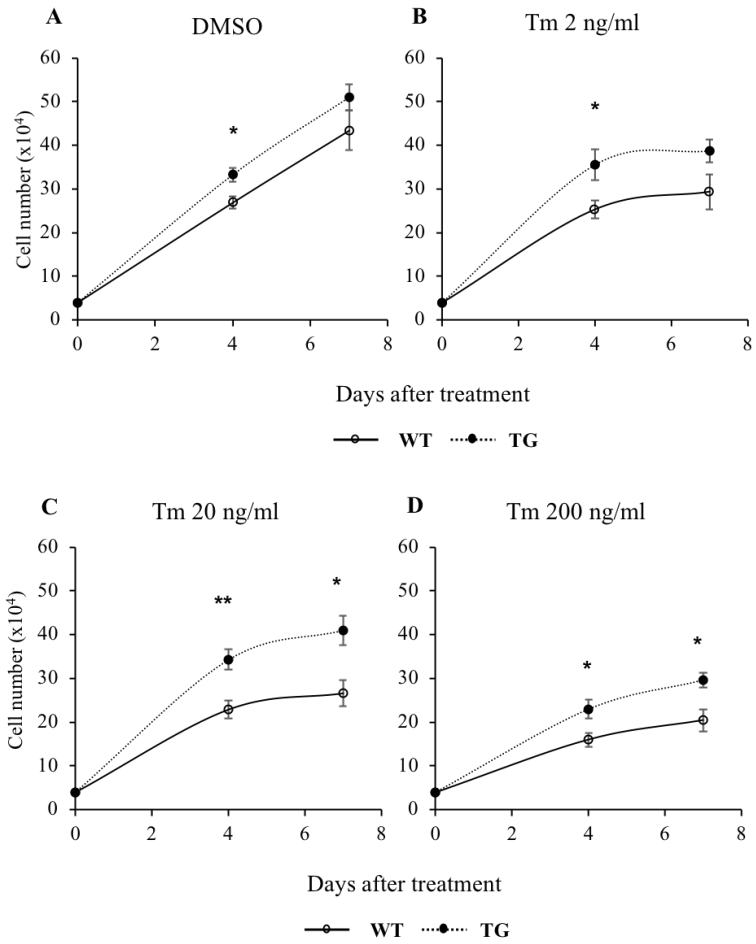

Figure 8. *Nampt* overexpressing MEFs (TG-MEFs) had higher cell growth under acute ER stress-induced premature senescence compared to wild type MEFs. A-D: cell growth was assessed start from passage 1 under the various concentration of tunicamycin treatment for 3 hours. Cells were stained by trypan blue exclusion. Viable cells were unstained whereas death cells were stained blue, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ (Student's t-test), n=3 (Data published by Nuriliani *et al*., 2020).

3.4 *Nampt* overexpressing MEFs had resistance to stress-induced premature senescence compared to wild type MEFs

Since I found that oxidative and ER stress using H_2O_2 and tunicamycin treatment decreased cell growth. Further, I evaluated whether that arrest is caused by senescence. One reliable method to detect senescence is using a senescence-associated β-galactosidase assay which reflects lysosomal activity (Campisi & d'Adda di Fagagna, 2007; Debacq-Chainiaux *et al*., 2009; Pawlikowski *et al*., 2013; Munoz-Espin & Serrano, 2014; Matjusaitis *et al*., 2016). Dimri *et al.* (1995) and Itahana *et al*. (2013) reported that many human cells expressed β-galactosidase. This enzymatic activity can be detected at pH 6, so can be a valuable marker to evaluate senescence cells *in vitro* and *in vivo*. Moreover, this marker can be used to distinguish

from quiescent and terminally differentiated cells, which do not show any positive result to this marker.

The results showed that 3 days culture after H_2O_2 treatment up to the concentration of 200 μM SA-β-Gal positive cells in both WT- and TG-MEFs remained low (< 5%) comparable with the control groups in the same genotype. At the control group, positive cells of WT-MEFs and TG-MEFs were 1 ± 0.52% and 0.71 ± 0.19%, respectively. However, at the concentration of 400 μM H_2O_2, WT-MEFs showed positive around 7.51 ± 2.16%, and TG-MEFs showed positive around 3.05 ± 0.99% without significant difference although positive cells in WT-MEFs was a little bit higher. At the concentration of 600 μM H_2O_2, WT-MEFs showed positive around 10.46 ± 0.46%, and TG-MEFs showed positive around 3.72 ± 0.57% with significant difference. SA-β-Gal positive cells in the WT-MEFs at the concentration of 400 μM H_2O_2 and more were significantly higher than the control group. However, significantly increase of SA-β-Gal positive cells in TG-MEFs clearly detected at the concentration of 600 μM H_2O_2, but the number was relatively low (Figure 9A.).

Previously, as Khaidizar *et al*. (2017) showed that TG-MEFs delayed replicative senescence, I would like to know whether a prolonged culture of TG-MEFs up to 6 days after treatment of oxidative stress remains resistance to senescence. At the control group, positive cells of WT- and TG-MEFs still low around 2.96 ± 0.56% and 1.3 ± 0.24%, respectively. Further, I found that at the lowest concentration in this study, 25 μM of H_2O_2 SA-β-Gal positive cells in WT-MEFs were significantly different compared to TG-MEFs. However, the number of senescent cells remained low up to the concentration of 50 μM. WT-MEFs were 3.44 ± 0.49% and TG-MEFs were 1.46 ± 0.37% in 50 μM. SA-β-Gal positive cells increased dose-dependently in WT-MEFs. At the concentration of 100 and 200 μM, SA-β-Gal positive cells were 7.35 ± 0.41% and 8.11 ± 0.91%, respectively in WT-MEFs. At the concentration of 400 and 600 μM, SA-β-gal positive cells were 20.14 ± 1.71% and 34.31 ± 1.42%, respectively. However, the SA-β-Gal positive cells of TG-MEFs remained low, at the concentration of 400 μM were 2.2 ± 0.32% and increased up to 11.96 ± 0.7% at the concentration of 600 μM (Figure 9B.). These results support my hypothesis that TG-MEFs have resistance to oxidative stress-induced premature senescence. In my study, I determined the concentration was enough to induce senescence when SA-β-Gal positive cell numbers were at least 2 folds compared to that of control treatment. Thus, 400 μM of H_2O_2 was enough to induced senescence in WT-MEFs whereas TG-MEFs need 600 μM of H_2O_2 to induce senescence.

At the ER-stress treatment using tunicamycin significantly difference of SA-β-Gal positive cells between both genotypes were found at the concentration of 200 ng/ml. At that concentration, SA-β-Gal positive cells in WT-MEFs were 12.59 ± 1.19%, and positive cells in TG-MEFs were 6.21 ± 1.04%. At the same genotype, I found that the number of SA-β-Gal positive cells of WT-MEFs were significantly increased at the concentration 20 and 200 ng/ml. Whereas, in TG-MEFs, the number of positive cells was relatively similar to the control event at the concentration of 200 ng/ml. At the concentration of 20 ng/ml, positive cells in TG-MEFs were significantly different compared to control. The number of TG-MEFs positive cells at the concentration of 20 ng/ml were around 7.18 ± 1.32%, whereas the control cells were around 3.29 ± 0.35% (Figure 9C.). My results showed that positive cells in WT- and TG-MEFs' control groups were 2.71 ± 0.82% and 3.29 ± 0.35%, respectively. Again, I determined that treated concentration is enough to induce senescence if can increase SA-β-Gal positive cells at least 2 folds compared to the control group. Thus, the concentration of 200 ng/ml tunicamycin was enough to induce senescence in WT-MEFs, but no treated concentrations of tunicamycin were enough to induce senescence in TG-MEFs.

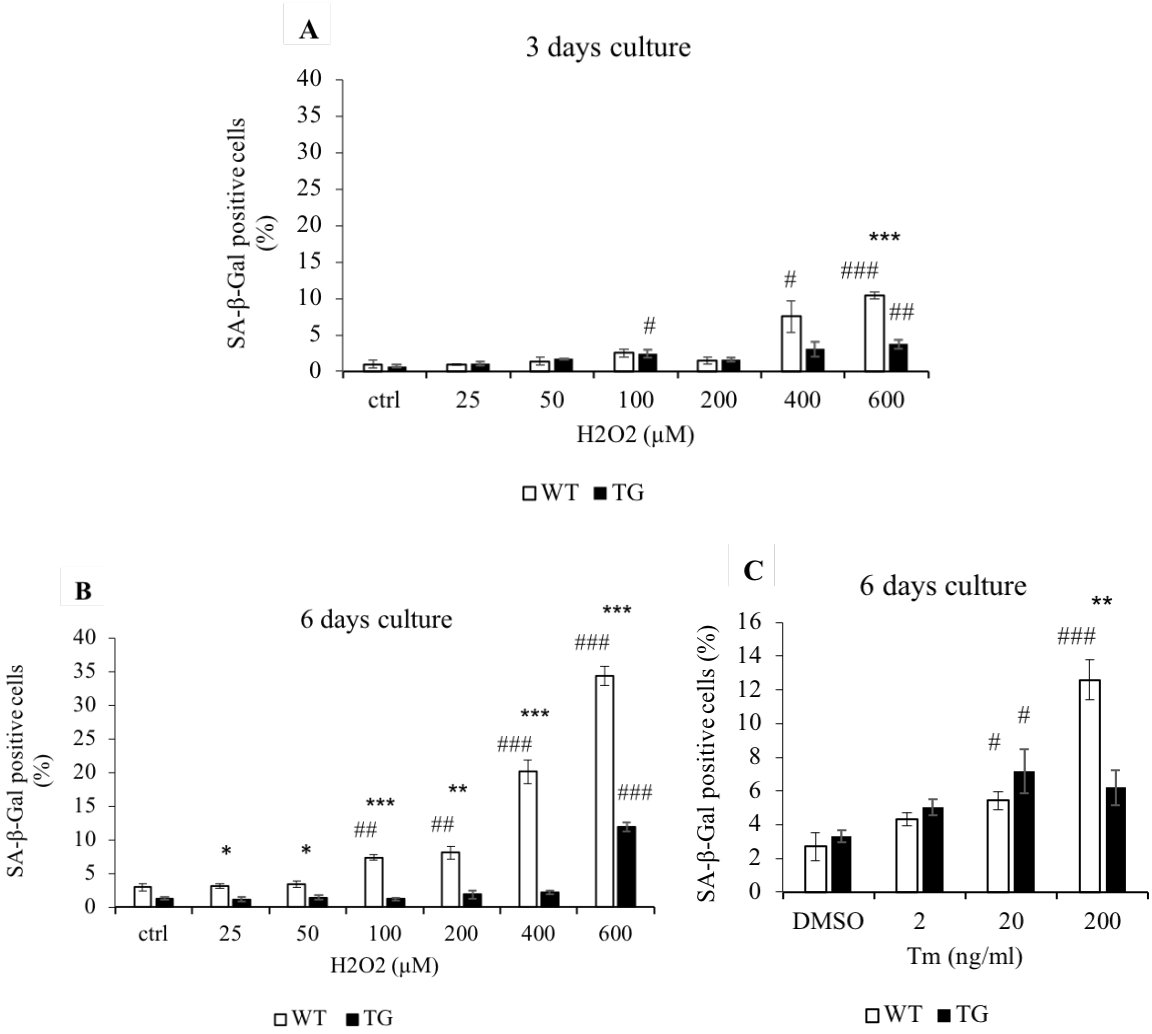

Figure 9. The number of SA-β-Gal positive cells in *Nampt* overexpressing MEFs (TG-MEFs) line #4 were lower compared to wild type MEFs under acute oxidative and ER-stress condition. A. Under various concentration of H_2O_2, 3 days culture after treatment, B. Under various concentration of H_2O_2, 6 days culture after treatment, C. Under various concentration of tunicamycin, 6 days culture after treatment. H_2O_2, hydrogen peroxide; Tm, tunicamycin; *: $p < 0.05$, **: $p < 0.01$: ***: $p < 0.001$ (WT vs TG), #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ (each genotype compares to its control), Student's t-test, n = 3 (Data published by Nuriliani *et al*., 2020).

I performed the same experiments using TG-MEFs line #1 to confirm the result of TG-MEFs line #4. The results showed that TG-MEFs line #1 had a similar pattern with TG-MEFs line #4. TG-MEFs line #1 also showed resistance to senescence compared to WT-MEFs both in oxidative stress using H_2O_2 and ER-stress using tunicamycin. At the H_2O_2 treatment, I found that 200 μM of H_2O_2 was enough to induce senescence in WT-MEFs both 3 and 7 days after treatment (Figure 10A-B.). At tunicamycin treatment, 200 ng/ml was required to induce senescence in WT-MEFs. At the same genotype, I found that the number of SA-β-Gal positive cells of WT-MEFs were significantly increased from control DMSO at the concentration 20

and 200 ng/ml. Whereas, in TG-MEFs, the number of positive cells was relatively similar to the control event at the concentration of 200 ng/ml. Positive cells in TG-MEFs at the concentration of 20 ng/ml were statistically different compared to control, but not enough to induce senescence. The number of positive TG-MEFs at the concentration of 20 ng/ml were around 7.46 ± 0.71%, whereas the control cells were around 5.1 ± 0.43% (Figure 10C.). Again, that concentration was not enough to induce senescence because I determined that treated concentration is enough to induce senescence if can increase SA-β-Gal positive cells at least 2 folds compared to the control group.

Moreover, by comparing between TG-MEFs line #4 and TG-MEFs line #1, I found that 400 μM H_2O_2 was enough to induce senescence in TG-MEFs line #1 whereas, in TG-MEFs line #4 required 600 μM H_2O_2. This means TG-MEFs line #4 requires a higher concentration of stressor to induce senescence.

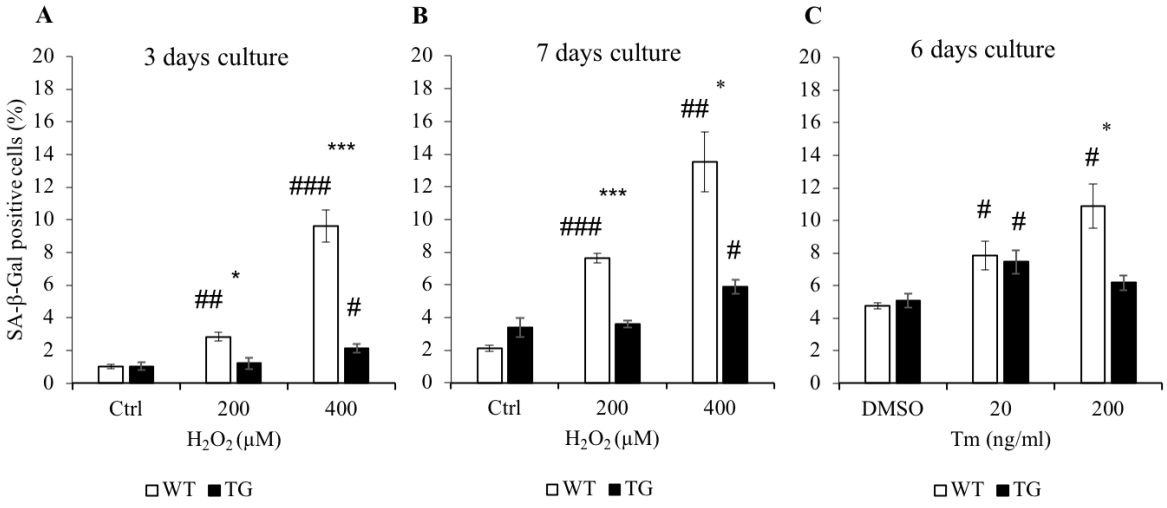

Figure 10. The number of SA-β-Gal positive cells in *Nampt* overexpressing MEFs (TG-MEFs) line #1 were lower compared to wild type MEFs under acute oxidative and ER-stress condition. A. Under various concentration of H_2O_2, 3 days culture after treatment, B. Under various concentration of H_2O_2, 7 days culture after treatment, C. Under various concentration of tunicamycin, 6 days culture after treatment. H_2O_2, hydrogen peroxide; Tm, tunicamycin; *: $p < 0.05$, ***: $p < 0.001$ (WT vs TG), #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ (each genotype compares to its control), Student's t-test, n = 3 (Data published by Nuriliani *et al*., 2020).

Further, I found discrepancies in this study. The result showed TG-MEFs cell growth decreased at the concentration of 400 μM H_2O_2. However, the number of SA-β-Gal positive cells at that concentration remained low, similar to control and the lower concentration of H_2O_2 groups. These results suggested that at the concentration of 400 μM H_2O_2 proliferation capacity of TG-MEFs was impaired. However, the cells did not directly become senescence; instead, they ceased in a quiescent-like state. These results were consistent with the previous report by

Khaidizar *et al.* (2017), which reported that TG-MEFs remained in quiescent-like condition longer compared to WT-MEFs.

I also found that WT-MEFs cell growth at the concentration of 20 ng/ml tunicamycin treatment decreased at day 4 and 7 compared to that of TG-MEFs. Whereas SA-β-Gal positive cells were not significantly different between both genotypes. Again, this is probably because WT-MEFs stopped growing and remained in a quiescent-like condition.

A special characteristic of senescence also could be seen in the cell morphology alteration such as enlarged and flattened shape as well as enlargement of nuclei (Bladier *et al*., 1997; Kiyoshima *et al.,* 2012). In oxidative stress-induced premature senescence by H_2O_2 and ER-stress-induced senescence by tunicamycin, the WT cells showed more enlarged and flattened cells. However, TG cells showed regular and fusiform shape (Figure 11. & 12.).

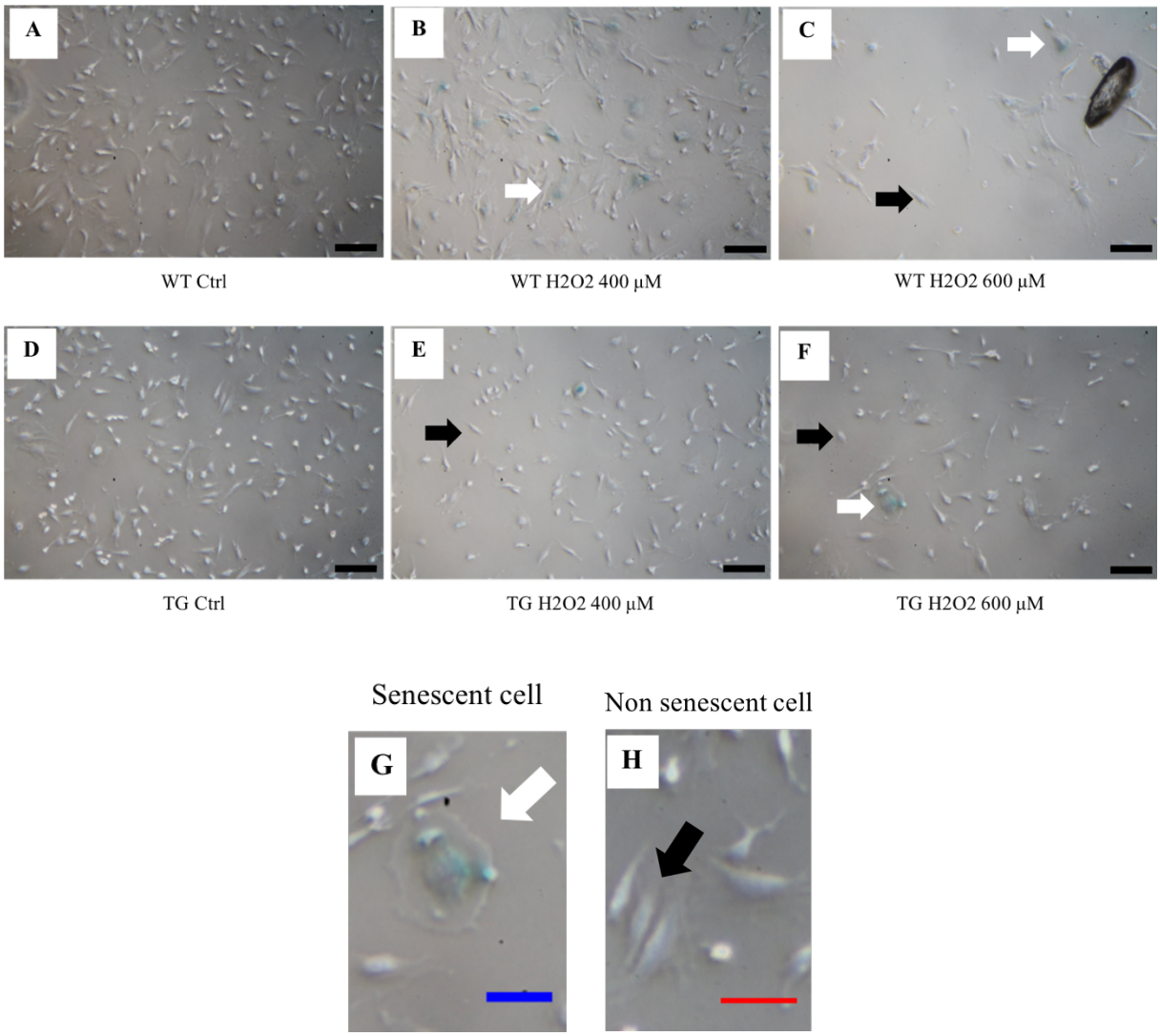

Figure 11. *Nampt* overexpressing MEFs (TG-MEFs) showed regular and fusiform cell morphology, whereas wild type MEFs showed enlargement and flattened cell morphology after treated by a various concentration of H_2O_2. A-C: WT-MEFs, D-F: TG-MEFs, G: insert photo shows a single senescent cell with enlargement and flattened morphology. H: insert photo shows a single non-senescent cell with regular and fusiform morphology. Bright-field image of SA-β-Gal positive cells, scale bar: 200 µm (black); 100 µm (blue and red), H_2O_2, hydrogen peroxide; black arrow, non-senescent cell; white arrow, senescent cell.

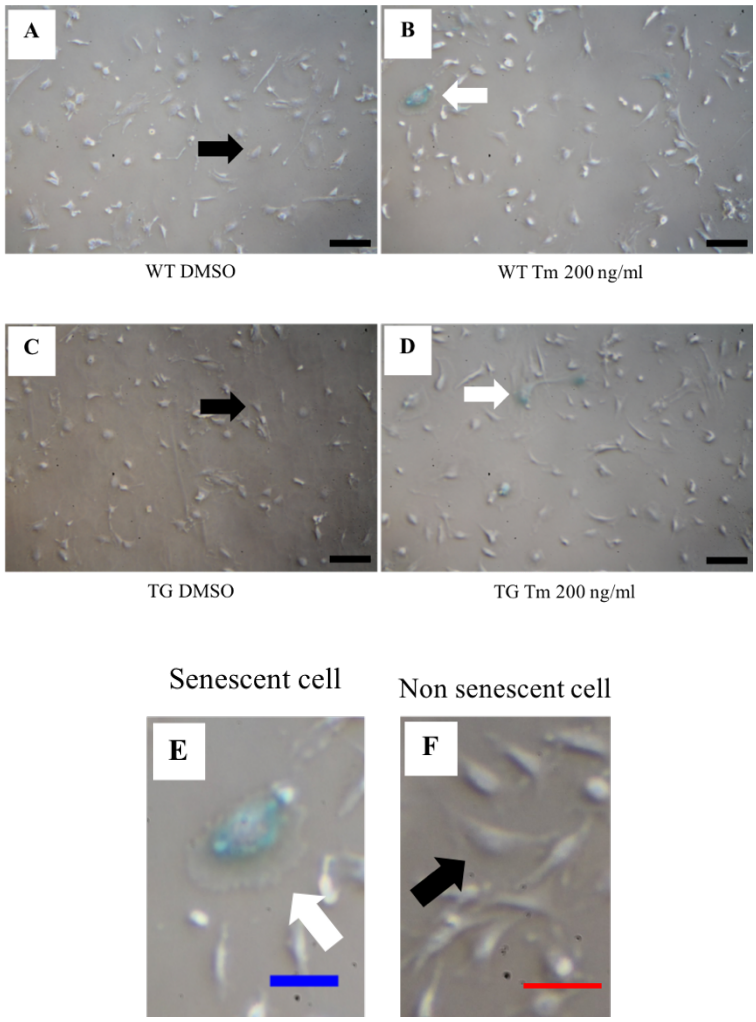

Figure 12. *Nampt* overexpressing MEFs (TG-MEFs) showed regular and fusiform cell morphology, whereas wild type MEFs showed enlargement and flattened cell morphology after treated by a various concentration of tunicamycin. A-B: WT-MEFs, C-D: TG-MEFs, E: insert photo shows a single senescent cell with enlargement and flattened morphology. F: insert photo shows a single non-senescent cell with regular and fusiform morphology. Bright-field image of SA-β-Gal positive cells, scale bar: 200 μm (black); 100 μm (blue and red), Tm, tunicamycin; black arrow, non-senescent cell; white arrow, senescent cell.

3.5 *Nampt* overexpressing MEFs still showed resistance to oxidative stress-induced premature senescence after the prolonged culture time compared to its control in the same genotype

To convince whether TG-MEFs have resistance to stress-induced premature senescence, I used oxidative stress treatment group and compared the same genotype in different days of culture. The results showed that TG-MEFs dramatically showed resistance to oxidative stress-induced premature senescence compared to WT-MEFs in various concentration from the lowest (25 μM H_2O_2) to the highest concentration (600 μM H_2O_2). From the lowest concentration of treatment 25 μM H_2O_2, I found that WT-MEFs were significant

different, increasing the number of SA-β-Gal cells between 3 and 6 days after treatment. Moreover, the number of positive cells increased dose-dependently, especially at 6 days after treatment (Figure 13A.). Whereas, in TG-MEFs, almost all treated concentration did not show any significant difference either at 3 or 6 days after treatment, except at the highest concentration 600 µM H_2O_2 (Figure 13B.).

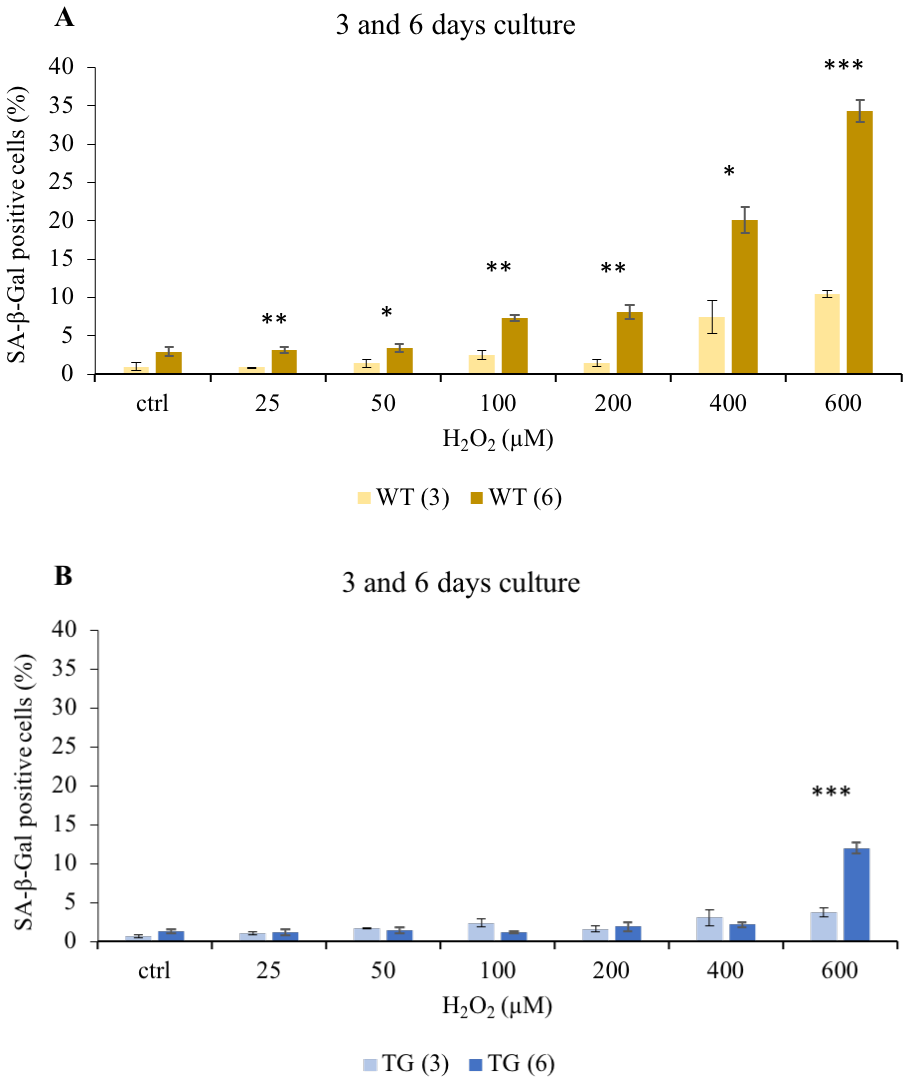

Figure 13. *Nampt* overexpressing MEFs (TG-MEFs) showed resistance to stress-induced premature senescence compared to wild type MEFs. The graphs represent the number of SA-β-Gal positive cells 3 and 6 days after H_2O_2 treatment among the same genotype. A. WT-MEFs, B. TG-MEFs. H_2O_2, hydrogen peroxide. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ (Student's t-test), n = 3.

This result strengthens the evidence that TG-MEFs are more tolerant to oxidative stress-induced premature senescence. Moreover, TG-MEFs require higher H_2O_2 concentration to induce senescence compared to WT-MEFs. My study used cells from passage 1. Cells were subculture every 3 days. That mean 6 days after treatment, cells were at passage 3. Previously,

Khaidizar *et al*. (2017) reported that primary MEFs underwent senescence starting around passage 7. Thus, cells at passage 3 are categorized at early middle phase. This supports that senescent cells in my study were stress-induced premature senescent cells, not replicative senescent cells.

3.6 *Nampt* overexpressing MEFs were more responsive to ER stress compared to wild type MEFs

Inside cells, the balance of protein production is regulated by special machinery in the endoplasmic reticulum. Endogenous or exogenous ER stressors such as tunicamycin may cause disturbance of ER homeostasis, which lead to ER stress. This stress triggers the UPR pathway, which in turn protects the cell from damage. There are 2 mechanisms inside cell to prevent the cell from damage. First, adaptive mechanism is activated if the damage is not too severe. Second is programmed cell death (apoptosis pathway) which is promoted if the damage continues and becomes severe.

Previously, some studies on the UPR pathway regulation caused by ER stress focused on cell growth inhibition (Serrano-Negron *et al.*, 2017; Hou *et al*., 2018; Wu *et al*., 2018) and cell death mechanism (Lin *et al*., 2012; Kishino *et al*., 2017; Zhong *et al*., 2017; Serrano-Negron *et al*., 2017; Hou *et al*., 2018; Ji *et al*., 2018; Wu *et al*., 2018). However, the study of how ER stress activates the UPR pathway which promotes senescence remain unclear. In this study, I evaluated the effect of tunicamycin on the UPR pathway related to senescence process. I studied how TG-MEFs respond to ER stress compared to that of WT-MEFs in senescence. *Xbp1* is known as a gene in the UPR system, which is very responsive to stress. Yoshida *et al.* (2001) reported that *Xbp1* splicing process is essential to trigger the UPR target genes as a response to ER-stress.

Spliced form of *Xbp1* mRNA is known to trigger ER stress responses and can be used as an indicator for ER stress (Yoshida *et al*., 2001). Therefore, I measured the amounts of spliced *Xbp1* mRNA (*Xbp1s*) and total *Xbp1* mRNA (*Xbp1t,* mixed of spliced and unspliced *Xbp1*) in both WT- and TG-MEFs at two-time points. First, I measured right after 3 hours of tunicamycin treatment and the second is 21 hours after tunicamycin treatment, at a condition where the cells are permitted to repair after the tunicamycin treatment. My results showed that 3 hours after tunicamycin treatment at the concentration of 2 and 20 ng/ml, both *Xbp1s* and *Xbp1t* relative mRNA amounts were not elevated in WT- and TG-MEFs (Figure 14A.). However, *Xbp1s* and *Xbp1t* relative mRNA amounts were increased in both genotypes at the concentration of 200 ng/ml tunicamycin. TG-MEFs showed higher amount of *Xbp1s* and *Xbp1t*

compared to WT-MEF cells. Moreover, the ratio of *Xbp1s* to *Xbp1t* was also higher in TG-MEFs. These results suggest that TG-MEFs express more amount of *Xbp1* and have higher *Xbp1* splicing efficiency compared to WT-MEFs in responses to ER stress. However, 21 hours after tunicamycin treatment (24-hour time point), *Xbp1s* and *Xbp1t* amounts between WT- and TG-MEFs were comparable (Figure 14B). The results of SA-β-Gal assay and *xbp1* mRNA expression propose that TG-MEFs are more responsive to ER stress compared with WT-MEFs to trigger proper protein folding and improves proteostasis.

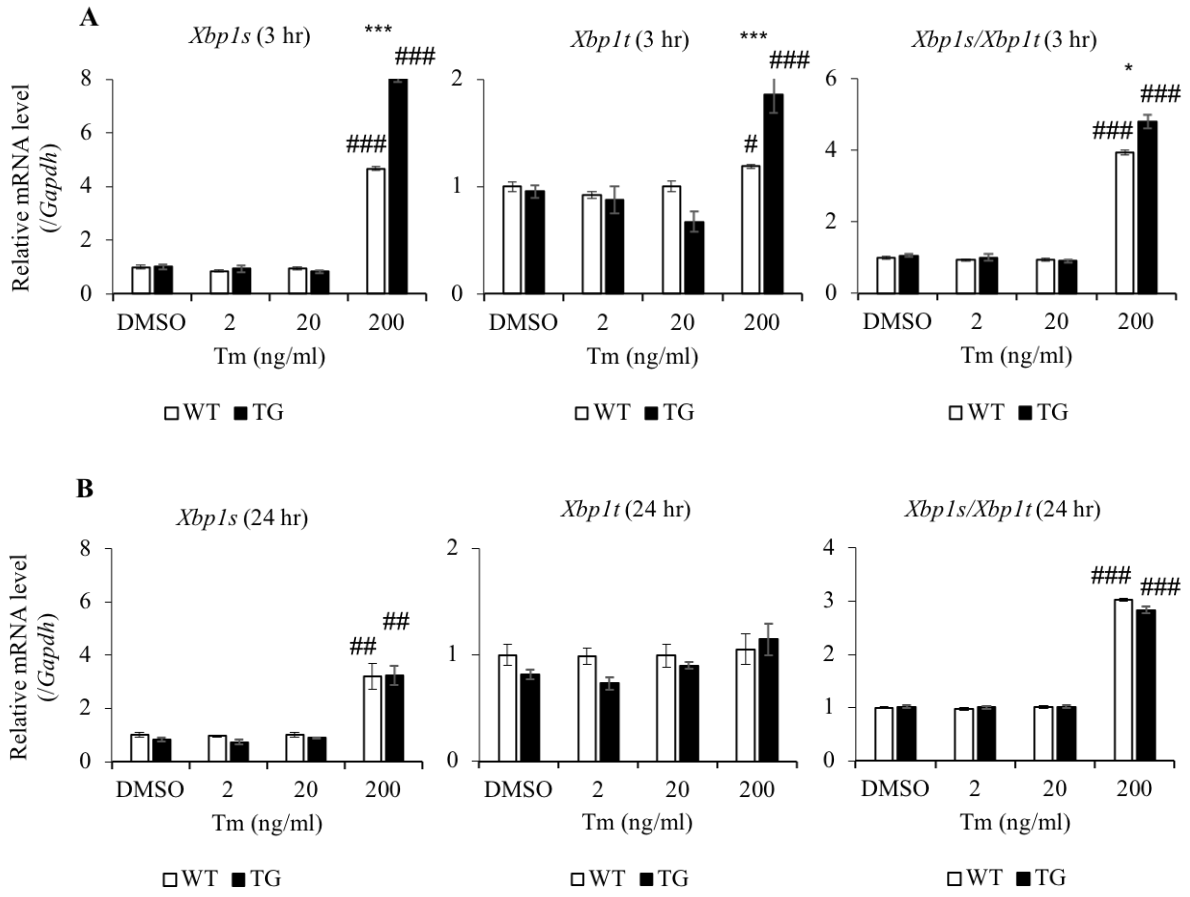

Figure 14. Relative *Xbp1s, Xbp1t*, and the ratio of *Xbp1s/Xbp1t* mRNA levels of *Nampt* overexpressing MEFs (TG-MEFs) were higher compared to wild type MEFs at 3-hour time point but not at 24-hour time point. Total RNA was extracted at A. 3-hour time point and B. 24-hour time point and subjected to qPCR analysis, *Gapdh* was used as reference gene, *: $p < 0.05$, ***: $p < 0.001$ (WT vs TG), #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ (each genotype compares to its control), Student's t-test, n = 3 (Data published by Nuriliani *et al*., 2020).

Rutkowski & Kaufman (2007) and Walter & Ron (2011) reported that once endoplasmic reticulum undergoes imbalance, it triggers the UPR system through 3 different pathways which direct cell to reach its homeostasis condition or promote programmed cell death if stress condition cannot be restored. ER stress greatly relates to other aging or associated

degenerative diseases thus, it still needs further studies on how the UPR plays a role in senescence. Here I checked some of the UPR target genes to screen how TG cells response to ER stress compared to WT cells 3 hours after treatment. In ER stress condition, the cell activates some key chaperones such as BIP, PDI, and calnexin. These chaperones function to ensure proper protein folding. Further, I examined the expression of the UPR target genes in WT- and TG-MEFs. My results showed that some of the UPR target genes that I evaluated in this study (*Bip, Pdi, Chop, Gadd34, Herpud1*, and *Erdj4*) in TG-MEFs were upregulated 3 hours after tunicamycin treatment (Figure 15A-B.). My results showed that some of key chaperones such as *Bip* and *Pdi* mRNA levels increased in response to stress higher in TG-MEFs compared to WT-MEFs. BIP is one of the major chaperones to cope with misfolded or unfolded protein caused by stress (Olowski & Urano, 2011; Brown & Naidoo, 2012). Some studies investigated that there was a decline of BIP level in aged mice (Hussain & Ramaiah, 2007; Gavilan *et al.,* 2009) and higher BIP level in young mice (Naidoo *et al.,* 2009). PDI, an enzyme which is needed in the mechanism of disulphide bond formation in protein assembly was also significantly higher in TG-MEFs group after 200 ng/ml of tunicamycin treatment. Whereas, there were no differences *calnexin* mRNA levels in WT- and TG-MEFs in tunicamycin treatment (Figure 15C.) although at the highest concentration, calnexin in TG-MEFs was slightly higher compared to that in WT-MEFs. In the UPR system, there are 3 pathways which respond to the stress differently regarding the exposure time and severity of stress. Then the cells activate adaptation or cell death mechanism (Rutkowski & Kaufman, 2007; Oslowski & Urano, 2011). Among the 3 UPR pathways, there are IRE1, PERK, and ATF6, which detach from BIP protein under ER stress condition (Walter & Ron, 2011; Iurlaro & Munoz-Pinedo, 2016). Thus, I determined how each of these pathways responses to ER stress in TG-MEFs. I detected mRNA levels of some of the UPR target genes on each pathway. The first pathway, IRE1 pathway is the most conserved pathway which is found in simple organism until the complex organism such as mammals (Tsuru *et al*., 2016; Amodio *et al*., 2018). Some of its target genes are *Edem1* and *Erdj4,* which involve in ERAD (Oslowski & Urano, 2011; Krajarng *et al*., 2015). My results showed that *Erdj4* mRNA levels was higher in TG-MEFs compared to WT-MEFs at the highest concentration of tunicamycin treatment (Figure 15B.) but *Edem1* not. The *Edem1* mRNA level in TG-MEFs was similar to WT-MEFs and showed an almost similar amount of mRNA levels among all concentration. There was only slightly increasing of *Edem1* mRNA level in TG-MEFs compared to WT-MEFs in the lowest concentration of tunicamycin (Figure 15C.), and at the highest concentration, TG-MEFs was slightly higher compared to WT-MEFs although there is no significant difference. Based on these results, I assumed that TG-

MEFs might be more sensitive to the appearance of misfolded or unfolded protein which produces during ER stress, thus stimulates activation of ER-association protein degradation genes to reduce stress and return the ER to its homeostasis condition. The second pathway, PERK, particularly plays a role in cell death mechanism, which involves *Chop* and *Gadd34* as downstream target genes (Krajarng *et al*., 2015). Interestingly, although cell death mechanism found in more severe condition, here I found a similar trend with the previous pathway. CHOP and GADD34 mRNA levels of TG-MEFs were significantly higher compared to WT-MEFs (Figure 15A-B.). Usually, the cell responses to adaptation or cell death mechanism regarding length or severity of stress. Interestingly, my results showed that TG-MEFs also seem to activate cell death mechanism higher than WT-MEFs. Whereas, in the 3rd pathway, ATF6's targets genes such as *Herpud1* and *Hyou1* (Shoulders *et al*., 2013) (Figure 15A. & C.) also showed higher mRNA level at the concentration of 200 ng/ml in TG-MEFs compared to WT-MEFs. Thus, it seemed that TG-MEFs response the stress more sensitive compared to WT-MEFs. Based on these results, I assumed that in *Nampt* overexpression condition might cause increasing in cell sensitivity to response the stress. Moreover, I found that the basal level expression of those genes was comparable in both genotypes. These results suggest that TG-MEFs are more responsive to ER stress and response quickly to prevent cells from ER stress-induced damage which promotes senescence and might be apoptosis.

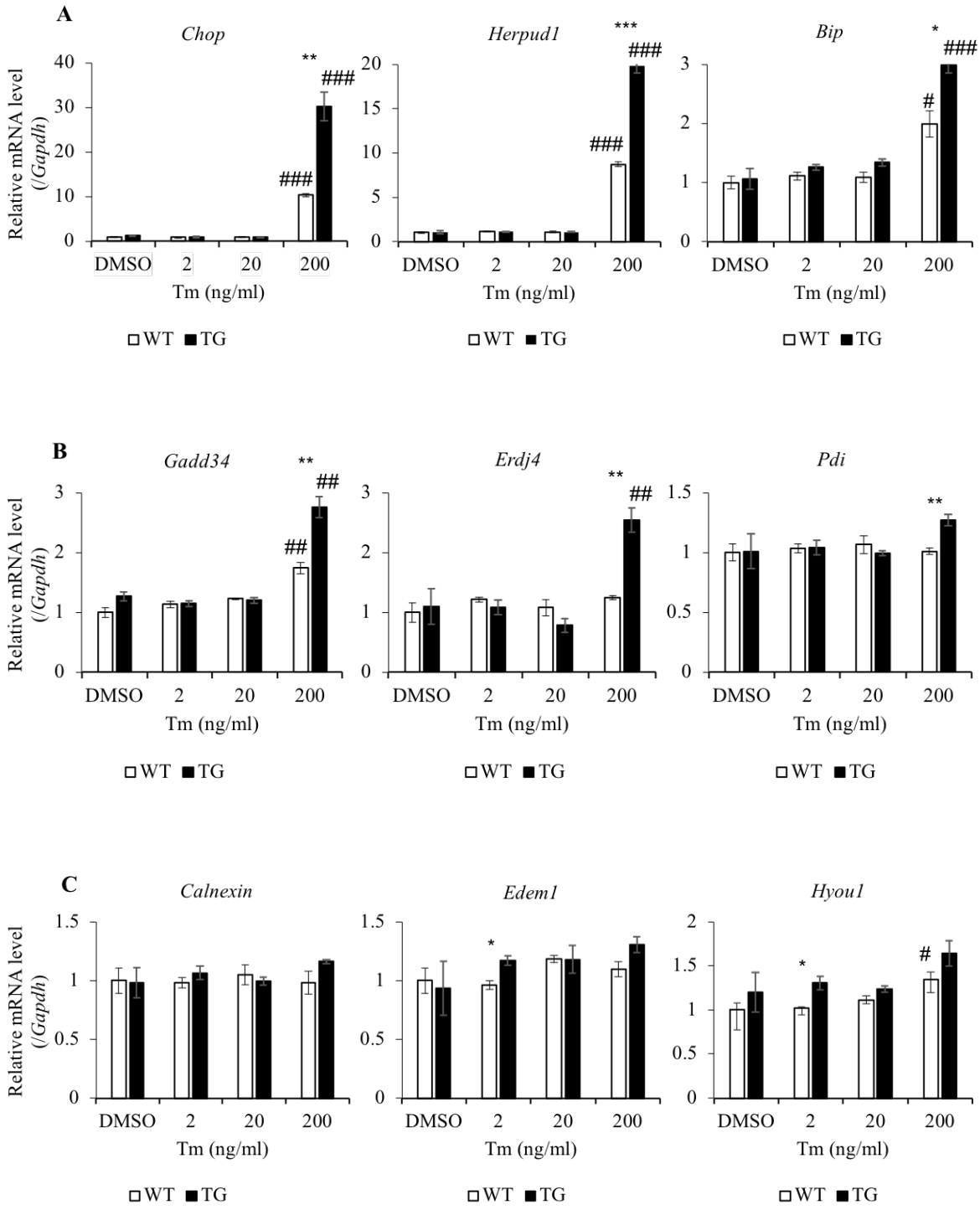

Figure 15. *Nampt* overexpressing MEFs (TG-MEFs) had higher genes expression and more sensitive responses to ER stress treated with tunicamycin compared to wild type MEFs. Total RNA extracted from primary MEFs after 3 hours of tunicamycin treatment. The samples were subjected to qPCR analysis. A. Relative mRNA level of *Chop, Herpud1*, and *Bip*, B. Relative mRNA level of *Gadd34, Erdj4,* and *Pdi*, C. Relative mRNA level of *Calnexin*, *Edem1*, and *Hyou1*. *Gapdh* was used as reference gene, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ (WT vs TG), #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ (each genotype compares to its control), Student's t-test, n = 3 (Data published by Nuriliani *et al*., 2020).

Overall, my finding suggests that TG-MEFs are resistant to stress-induced premature senescence compared to WT-MEFs. Moreover, to overcome stress, especially in ER stress caused by tunicamycin treatments, TG-MEFs raise their mRNA levels of some genes which involve in 3 majors of the UPR pathways. However, TG-MEFs tend to activate all of those the 3 UPR pathways unselectively like those in WT-MEFs. These results showed that at the higher concentration, cell can response and activate the UPR pathway quickly to cope with stress condition.

Results shown in this study revealed that *Nampt* TG-MEFs have higher resistance against oxidative and ER stress-induced premature senescence *in vitro*, most likely by upregulating antioxidant gene and UPR genes, respectively, via NAMPT/NAD^+ axis, suggesting that the increment of NAMPT enzymatic activity or NAD^+ *in vivo* could protect cells from stress-induced premature senescence, thereby potentially keeping organisms healthy.

4.0 Discussion

4.1 *Nampt* overexpressing MEFs had resistance against oxidative and ER stress-induced premature senescence

My results gave evidence that *Nampt* overexpressing primary mouse embryonic fibroblasts (MEFs) had resistance against oxidative and ER stress-induced premature senescence caused by hydrogen peroxide and tunicamycin, respectively. Moreover, I found that TG-MEFs required a higher concentration of the stressors to promote stress-induced premature senescence. This is supported by the results of cell growth and SA-β-Gal assay results. Further, I found that on stress-induced premature senescence, some of the UPR target genes were upregulated both in WT- and TG-MEFs. However, TG-MEFs responded more sensitively to the stress.

In this study, first I evaluated that single treatment of various concentration of H_2O_2 for 24 hours and tunicamycin for 3 hours did not show any significant difference on cell viability of WT- and TG-MEFs at passage 1. I speculated that NAMPT level and activity in cells/tissues did not deplete suddenly. NAMPT level might decrease with age in cells/tissues. In the culture of WT-MEFs, this phenomenon might happen during subculture. Khaidizar *et al*. (2017) found that the cell viability of TG-MEFs have more resistance to H_2O_2 treatment compared to that of WT-MEFs at passage 3. They reported that NAMPT/NAD^+ level decreased during the passage. I thought that probably at passage 1 NAMPT/NAD^+ level of WT-cells was comparable to TG-MEFs. Whereas, at passage 3 NAMPT/NAD^+ level of WT-cells might lower compare to passage 1. Thus, MEFs of both genotypes at passage 1 might have similar power to deal with the stress.

One aspect which reflects senescence condition involves cell growth arrest. Duan *et al.* (2005) investigated that in senescence, cell lost its proliferation capacity. I found that *Nampt* overexpression in primary MEFs might maintain its proliferation capacity after H_2O_2-exposure. These results were consistent with the previous study by Khaidizar *et al*. (2017) in replicative senescence. In the lower concentration of H_2O_2 treatment (50 μM), I found that WT-MEFs still maintained their proliferation capacity until day 7 and comparable with TG-MEFs. Whereas, at the concentration of 100 μM H_2O_2 treatment, WT-cells growth reduced dramatically at day 7. Further, WT-MEFs cell growth completely lessens at the concentration of 400 μM H_2O_2 treatment. In this condition, TG-MEFs proliferation capacity remained although reduced. Whereas, after tunicamycin treatment, proliferation capacities in both WT- and TG-MEFs

decreased compared to the control group of the same genotype. However, WT-MEFs proliferation capacity was lower compared to TG-MEFs.

In WT-MEFs, H_2O_2 and tunicamycin induced senescence dose-dependently. However, TG-MEFs tend to maintain a low number of senescence cells in all concentrations. This supports the idea that TG-MEFs are resistant to senescence. Interestingly, these results were more clearly in oxidative stress-induced senescence after I prolonged the culturing time. TG-MEFs had a lower number of SA-β-Gal positive cells at 3 and 6 days after H_2O_2 treatment almost in all treated concentrations (Figure 13.).

There are some possibilities of resistance against stress-induced premature senescence in TG MEFs. In stress-induced premature senescence, proliferation inhibition might be caused by high ROS level in cells milieu. Thus, high NAMPT/NAD^+ in TG-MEFs probably play a role to activate antioxidant enzymes which scavenge ROS. Other possibilities, H_2O_2 and tunicamycin, might cause DNA damage which inhibits DNA synthesis. Cells would stop proliferate to repair the defect and might survive in a quiescent condition. If the repair process fail, cells would enter senescence phase. Probably in TG-MEFs, the cells kept under quiescent condition to allow them to repair the defect. This might explain the discrepancy that I found between the result of reducing cell proliferation capacity and low SA-β-Gal positive cells in TG-MEFs. Further, higher NAMPT/NAD^+ level accompanied by increasing of SIRT1 level might play a role in cell cycle regulation through modulation of cell cycle genes such as *p53* and *p21*.

Some of the previous studies reported that NAMPT/NAD^+ played an important role to ensure the longevity of organism (from a simple organism such as yeast to the complex organism such as mammals) either through raising NAD^+ metabolism or supplementation directly or indirectly (supplementation of NAD^+ precursors) (Kang *et al.*, 2006; Borradaile & Pickering, 2009; Evans *et al*., 2010; Yaku *et al.,* 2018). Houtkooper *et al.* (2010) and Nikiforov *et al.* (2015) stated that NAD^+ is a ubiquitous compound involved in several physiological and metabolic aspects in organisms. NAMPT is a rate-limiting enzyme which crucial to maintain cellular NAD^+ level (Imai, 2010). NAD^+ homeostasis is maintained tightly through several enzyme mechanisms which involved SIRTUIN family such as SIRT1, as one of the NAD^+-consuming enzymes and NAMPT (Imai & Yoshino, 2013). *Nampt* overexpression has many beneficial impacts to the organism such as elevation of proliferation capacity, delay of senescence, and inhibition of increasing of ROS (Borradaile & Pickering, 2009).

On the other hand, decreasing NAD^+ level might cause adverse effect to the organism related to senescence. Some studies reported that decreasing NAD^+ level was seen in older

organisms (Massudi *et al*., 2012; Chini *et al*., 2017; Yaku *et al*., 2018). Ma *et al*. (2017) studied that low NAMPT level accompanying by decreasing cellular NAD^+ was found in aged mesenchymal stem cells. Van der Veer *et al.* (2007) and Khaidizar *et al.* (2017) reported that declining of NAD^+ level related with the occurrence of replicative senescence in human and mouse cells. Cellular NAD^+ level could be reduced by several factors such as DNA damage, oxidative stress, senescence, and inflammation, or this can occur vice versa (Chini *et al*., 2017). In reality, our body continuously faces stressors both endogenous (telomere damage and genotoxic stress) and exogenous (UV exposure, oxidative stress, drugs, or X-ray irradiation) stressors which may affect cellular NAD^+ level. Thus, these agents continuously expose to cell, it causes stress-induced premature senescence instead of replicative senescence.

H_2O_2 is one of the most known agents which causes stress-induced premature senescence. Chen & Ames (1994) reported that H_2O_2 caused senescence-like growth arrest in human diploid fibroblast F65 cells. Other studies related to H_2O_2 caused premature senescence was investigated by Bladier *et al.* (1997) in primary human fibroblast cell line, Duan *et al.* (2005) in human diploid fibroblasts, Furukawa *et al.* (2007) in human fibroblast TIG3, Kiyoshima *et al.* (2012) in mouse gingival fibroblasts, Suo *et al.* (2013) in human umbilical vein endothelial cells, and Choo *et al.* (2014) in human mesenchymal stem cells. Other agents such as tunicamycin which causes ER stress have been reported to induce senescence and affects cell viability in various cancer cells (Serrano-Negron *et al.,* 2017; Hou *et al.,* 2018; and Wu *et al*., 2018) as well as normal cell such as mouse embryonic fibroblasts (Rutkowski *et al.,* 2006; Nakahata *et al*., 2018).

Khaidizar *et al.* (2017) found that the resistance against replicative senescence is related to lower ROS production caused by the higher capacity of antioxidant genes such as *sod2* and *catalase* to scavenge ROS (Supplement 1.). Although Liochev (2013) reported that to become senescence, high ROS level is not always required. However, ROS production can be a marker to detect senescence. Previously, Kang *et al.* (2006) reported that reducing ROS level plays a role to prolong primary human fibroblasts lifespan in relation to NAD^+ regulation. Premature senescence is caused by an exogenous stressor such as X-ray irradiation (Hewitt *et al*., 2012), DNA damage, mitochondrial dysfunction, ER stress (van Deursen *et al*., 2014; Pluquet *et al*., 2015; Ziegler *et al*., 2015) accompanied by high ROS level. Rufini *et al.* (2013) found that high ROS level promoted cells to become senescence, which could be repaired by activation of antioxidant enzymes. Soto-Gamez *et al.* (2019) studied that ER stress increased ROS level and inhibited proliferation. Moreover, Chen & Ames (1994) reported that after a single exposure of various sub-lethal concentration of H_2O_2 could cause decreasing of DNA synthesis, although

proliferation capacity and protein synthesis were unaffected. However, after several days, the decreasing ability of DNA synthesis promotes proliferation inhibition. Another study by Pizzaro *et al.* (2009) also confirmed that H_2O_2 might lead to DNA damage and allowed cells to stop at S phase in cell cycle. Thus, it proposes that after expose by stressor cell does not directly loose its living capacity.

Although my study did not reveal overall molecular mechanism aspects, there were some reports which confirmed that NAMPT/NAD^+/SIRT1 levels and activities are involved in resistance to senescence. Ma *et al.* (2017) confirmed that decreasing NAMPT level of aged rats is related with decreasing of NAD^+ level and SIRT1 expression as well as activity. This result was confirmed that administration of NAMPT inhibitor, FK866, to young MSCs induced senescence. The mechanism was associated with declining of NAD^+ level and SIRT1 activity. Previously, Ho *et al.* (2009) suggested that the replicative life span of smooth muscle cells dramatically elevated by SIRT1 if N*ampt* is overexpressed. This could happen because the process of SIRT1 deacetylation highly depended on NAMPT. Pizzaro *et al*. (2009) confirmed that SIRT1 involved in p53 regulation. They found that following oxidative stress, SIRT1 expression rapidly increased. Further, the declining of SIRT1 expression paralleled with an elevation of p53 acetylation. Rufini *et al.* (2013) confirmed that SIRT1 expression completely decreased in senescence cells. SIRT1 is important to regulate its main substrate, p53. Cell resistance to stress and senescence were depended on the regulation of p53 deacetylation.

Moreover, in most of stressors, proliferative inhibition involves the regulation of cell-cycle dependent genes such as *p16, p21, pRb*, and *p53* (Ben-Porath & Weinberg, 2005; Salama *et al*., 2014; Paez-Ribes *et al.,* 2019). Van der Veer *et al*. (2007) studied that the role of NAMPT to prevent senescence may be facilitated by elevation of SIRT1 deacetylase activity. This process is important to keep p53 level low hence prevent from senescence. Thus in human aging, delaying of senescence may involve a link of Nampt-SIRT1-p53. Furukawa *et al.* (2007) also found that human fibroblast, TIG3 could be induced to undergo senescence under oxidative stress condition. In such condition, the authors found that decreasing of NAD^+ level along diminishing of SIRT1 deacetylation capacity caused augmentation of p53. Whereas, high p53 acetylation level influenced the high expression of *p21*. Khaidizar *et al.* (2017) found that there were increasing of *p16, p19*, and *p21* mRNA amount passage dependent manner in WT-MEFs started from passage 4, whereas the levels in TG-MEFs remained constant. This suggested that cell-cycle dependent genes upregulated in replicative senescence.

Furthermore, in ER-stress, the cell cycle regulation also involves p16, p21, p53, and pRb pathways. Some of the previous studies reported that tunicamycin increases p53 expression

in human breast (MCF7) and cervical (HeLa) cancer cells through NF-κB signalling. They found that activity and phosphorylation of p53 elevated along with its accumulation in nuclear during ER stress (Lin *et al.,* 2012). Rufini *et al.* (2013) described that there are 2 major cell-cycle regulation pathways: p53/p21 and p16/pRb. They assured that activation of p53 triggers the expression of p21 as a pro-senescence target, which is responsible for allowing cell enter G1 phase arrest.

Van Deursen *et al*. (2014) and Herranz & Gil (2018) indicated that the senescence process happened gradually. Various stressors which cause stress-induced premature senescence such as oxidative stress, DNA damage, telomere damage, and mitochondrial disfunction will promote p53/p21 and p16 pathways. Initially, these pathways are responsible for modulating transient cell-cycle arrest (quiescent phase). In this phase, the cell is allowed to recover its condition. Inoue *et al.* (2017) reported that *p21* overexpression could increase cell survival after ER stress induction. This activation happened through ATF4 pathway, which further prevents senescence. Soto-Gamez *et al.* (2019) assured that initially, ER stress triggers p16 and p53 pathways to prevent cell growth and allow the cell to repair the damage. However, if the stress continues and become severe, the cell in the quiescent phase promotes to enter senescence. This might explain my results that after treatment using H_2O_2 or tunicamycin, WT-MEFs proliferation becomes slower although the number of senescence cells (which are indicated in SA-β-Gal positive cells) are still low. Again, this result supports my assumption that senescence becomes gradually.

My results are consistent with the previous study by Chen & Ames (1994). They showed that human fibroblasts become senescence 3 days after hydrogen peroxide treatment. After several days, those cells appeared similar to senescence cell morphology. Kiyoshima *et al.* (2012) reported that 20 μM H_2O_2 treatment for 2 hours in mouse gingival fibroblast showed increasing of senescence cells, especially at 6 days after treatment. The number of senescence cells increased day dependent manner.

Bladier *et al.* (1997), Kiyoshima *et al*. (2012), and Hernandez-Segura *et al.* (2018)[a] stated that senescence cell had an irregular shape, and enlarged as well as flattened appearance. WT-MEFs clearly showed morphological alteration compared to TG-MEFs (Figure 11. &12.). WT-MEFs morphology became flattened and enlarged.

In short, my study suggest that since SIRTUIN activation happens along with increasing of NAMPT activity as well as increasing of NAD^+ level, sensitivity of TG-MEFs to response oxidative stress are regulated through NAMPT/NAD^+/SIRTUIN axis (Figure 16.).

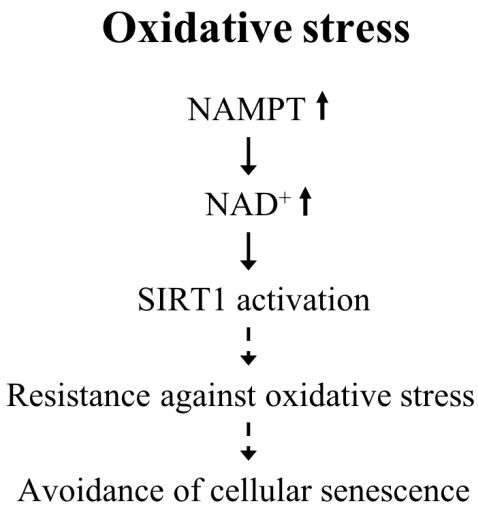

Figure 16. *Nampt* overexpressing MEFs (TG-MEFs) response to oxidative stress. Activation of NAMPT/NAD^+/SIRTUIN axis may promote resistance of TG-MEFs against oxidative stress to avoid cellular senescence.

4.2 *Nampt* overexpressing MEFs sensitively responded to ER stress by upregulating some of the UPR target genes

Instead of TG-MEFs resistance by evaluating cell growth and the number of senescent cells, furthermore, I also assessed the molecular mechanism on how *Nampt* overexpression MEFs responded to ER stress which triggered senescence. My results showed that TG-MEFs efficiently spliced *Xbp1* mRNA compared to WT-MEFs 3 hours after tunicamycin treatment. *Xbp1* is a responsive and sensitive gene in response to ER-stress (Yoshida *et al.,* 2001). Its splicing process, especially through IRE1α pathway, is important to promote activation of the other UPR target genes which play a role in endoplasmic reticulum-associated degradation (Yoshida *et al*., 2001; Kanemoto *et al.,* 2005; Tsuru *et al*., 2016; Bang *et al*., 2019). Further, this mechanism allowed activation of those UPR target genes to impair ER stress which might be permitted cell to recover proteostasis (Tsuru *et al.*, 2016). I postulated that efficiency and sensitivity of *Xbp1* regulation in TG-MEFs might be regulated through increasing of SIRTUIN activity because of high NAMPT/NAD^+ level.

Once stressor enters to the endoplasmic reticulum, this organelle promotes the UPR activation to cope with ER stress. The UPR reduces ER burden by increasing the level of ER chaperone and foldase, which function to eradicate improper protein folding by lowering genes which encode protein production (Schroder & Kaufman, 2005). Although stimulation of the UPR is well defined, it remains unclear the mechanisms of how the UPR response, which can selectively permit for adaptation (Rutkowski *et al.,* 2006). Blazanin *et al.* (2017) stated that the

relationship of ER stress and the UPR pathway in premature senescence remains unclear, especially in the primary cell. The early phase of the UPR consists of a rapid response to reduce the influx of proteins into the ER to allow the cell to regain ER homeostasis. Therefore, evaluation of the UPR downstream target genes can be one of useful marker to study ER stress (Oslowski & Urano, 2011) since the level IRE1α, PERK, and ATF6 in the body were low. IRE1α is the most conserved pathway in ER stress response. Activation of this pathway may cause mRNA splicing of *Xbp1*. This step is one of the main markers of ER stress (Oslowski & Urano, 2011; Iularlo & Munoz-Pinedo, 2015; Amodio *et al*., 2018).

Many studies also reported that *Xbp1* regulation involved the role of SIRTUIN family. SIRTUIN family is a group of protein which play a role in NAD^+ homeostasis modulation. Thus, the ER stress attenuation in TG-MEFs may cause by some possibilities related to NAD^+-dependent SIRTUIN activation. Increasing SIRT1 expression promotes deacetylation of HSF1 (heat-shock factor protein 1), which attenuates ER stress (Ghemrawi *et al.,* 2013). Shin *et al*. (2013) stated that Xbp1 activate SIRT7, which functions to repress ER stress through Myc activation. Xbp1 deacetylation by SIRT6 also found to suppress ER stress (Bang *et al*., 2019).

Some studies reported that there is an alteration in mRNA or protein level of the UPR related genes/proteins upon ER stress. My results showed that there are increasing of chaperone and some of the target genes in the UPR pathways especially in TG-MEFs right after 3 hours of treatment such as *Bip, Pdi, Chop, Gadd34, Herpud1*, and *Erdj4*. My results suggested that TG-MEFs are more responsive to stress and required a higher concentration of stressor. Moreover, TG-MEFs had a quick response to ER stress which might have benefit to maintain inner cell milieu against senescence. Again, I propose that responsiveness and sensitivity of TG-MEFs against ER stress-induced premature senescence are involved increasing of SIRTUIN activity because of high NAMPT/NAD^+ level. My results also showed that the UPR target genes from the 3 pathways responded unselectively to ER stress. Thus, I could not deny that these 3 systems were not working alone but interrelated one another.

ER stress is regulated by transmembrane proteins such as PERK, IRE1α, and ATF6. All of the three transmembrane proteins are activated through dissociation with GRP78 when there is an imbalanced of unfolded proteins and chaperones (Oslowski & Urano, 2011; Krajarng *et al*., 2015; Amodio *et al*., 2018). BIP is a central regulator of the UPR stress sensors as well as an ER chaperone to assist protein folding. BIP is highly expressed in the ER and can be used as an ER marker. PDI is involved in oxidative protein folding in the ER lumen, and its expression is induced by ER stress (Oslowski & Urano, 2011). Brown & Naidoo (2012) stated

that the impairment of chaperoning system plays an important role on the aging process and age-associated diseases. Ghost *et al.* (2015) found that many proteins in the UPR pathway, such as BIP, CHOP, and Xbp1 were elevated in old adipose stromal cells. Shen *et al*. (2015) evaluated that *Bip* and *Chop* are quickly upregulated after tunicamycin treatment in primary neonatal rat cardiomycetes. The peak of those genes reached after 24 hours and started to decline after that. Moreover, in ER stress, the level of BIP, calnexin, PDI, and Ero1 was altered to adapt with a senescence-associated imbalance in proteostasis (Matos *et al*., 2015).

Increasing of NAD^+/NAMPT activity correlates with increasing of SIRTUIN activity. Thus, ER stress resistance of TG-MEFs in stress-induced premature senescence may involve the role of SIRTUIN family. Some studies reported that SIRT1 involves in ER stress regulation through the PERK pathway, which modulates ATF4 and CHOP expression to cope with ER stress (Chan *et al*., 2017). Liu-Byan (2015) studied that modulation of cartilage homeostasis involves elevation of AMPK activity which further increased NAD^+ level and promoted the elevation of SIRT1 activity. Further homeostasis regulation involved in the mitigation of ER stress through PERK pathway by restricting CHOP expression. In Caco-2 cells and the colon tissues of mice treated SRT1720, an activator of SIRT1, inhibit ER stress-mediated apoptosis by decreasing BIP and CHOP (downstream pathway of PERK), cleaved caspase-12, cleaved caspase-9, and cleaved caspase-3 (Ren *et al*., 2019). Prola *et al*. (2017) reported that SIRT1 prevents cardiovascular disease by impairing PERK/eIF2α pathway activation in ER stress-induced apoptosis. However, Koga *et al*. (2015) found the opposing role of SIRT1 in ER stress. They discovered that SIRT1 induction by ER stress was performed through the PI3K-Akt-GSK3 pathway, not the other 3 main UPR pathways. Further, they evaluated that inhibition of SIRT1 expression by EX527 in ER stress reduced cellular damage in vitro and hepatocellular injury in vivo. Zhang *et al.* (2016) studied that SIRT3 involved in ER stress regulation of diabetic rat. SIRT3 inhibited apoptosis of pancreatic β-cells. Moreover, knocking down SIRT3 elevated palmitate-induced BIP acetylation as well as upregulated IRE1α, PERK, ATF6, and CHOP. Palmitate triggered apoptosis of pancreatic β-cells. Futhermore, SIRT3 overexpression also prevented the hyperacetylation of BIP.

In this study, I could show that TG-MEFs had higher resistance to stress-induced premature senescence and more sensitive to response ER stress. Thus, my project revealed that increasing NAD^+ level by inserting "foreign" *Nampt* (human *Nampt*) in the genome of the host organism (mouse) could be a valuable strategy, to protect cells/tissues from adverse effects of oxidative and ER stress especially related to senescence. Using this strategy, researchers also

can evaluate beneficial and or adverse effects of inserting "foreign" *Nampt* related to aging in host organism along of the development time until death. However, the limitation of my study is that there is no clear molecular mechanism related to the increasing of *Nampt* activity accompanied by increasing NAD^+ level. Therefore, the next study related to the role of SIRTUIN family, for example, treatment using SIRT1 inhibitors will be one example, since this protein family regulation connects with NAMPT activation/inhibition.

In summary related to ER stress, I speculated that NAMPT/NAD^+ axis in TG-MEFs regulates stress through increasing sensitivity of the UPR signalling pathway or deacetylation of HSF1/BIP/histone by activation of SIRTUIN 1/3/6/7 (Figure 17.).

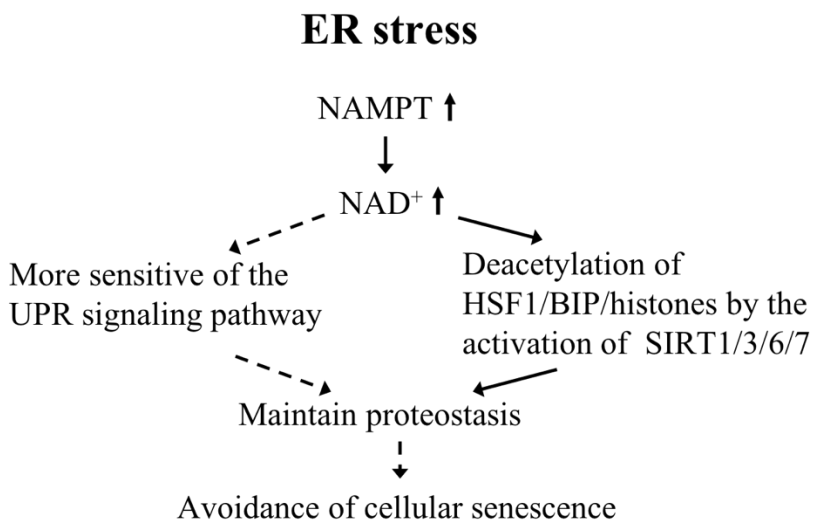

Figure 17. *Nampt* overexpressing MEFs (TG-MEFs) response to ER stress. Activation of NAMPT/NAD^+ axis may promote resistance of TG-MEFs against ER stress through proteostasis maintenance to avoid cellular senescence. Resistance mechanism against senescence may regulate at least through two different pathways. First, by increasing sensitivity of the UPR signalling pathway. Second, by activation of SIRTUIN family such as SIRT 1/3/6/7 which deacetylate HSF1/BIP/histone to reduce ER stress.

Collecting together with the previous report by Khaidizar *et al.* (2017), my study suggested that NAMPT/NAD^+ activation might prevent primary MEFs from oxidative and ER stress-induced premature senescence *in vitro* in relevant with replicative senescence. My study gave more comprehensive data that increasing of NAD level *in vitro* and probably *in vivo* can be used as one strategy to develop healthy aging/longevity.

5.0 Concluding Remarks

In this study, I made an effort to gain a comprehensive understanding regarding the role of NAMPT/NAD^+ in senescence. Previously, Khaidizar *et al.* (2017) reported that *Nampt* overexpression postpones replicative senescence of primary mouse embryonic fibroblast. Their study supported the previous study by van der Veer *et al*. (2007), which showed *Nampt* overexpression postpone replicative senescence in human smooth muscle cells. Since, cells continuously expose to stressors, it is important to evaluate how *Nampt* overexpressing MEFs response to stress-induced premature senescence.

My results proved that stress-induced premature senescence could be caused by oxidative and ER stress in acute exposure of hydrogen peroxide and tunicamycin, respectively. *Nampt* overexpression may alter NAD^+ homeostasis in the cell which permit the cell to tolerate high NAD^+ condition. This may give advantages through cell cycle regulation since one of the NAD^+-consuming enzymes, SIRT1 is activated in a high NAD^+ level. Moreover, SIRT1 is important in p53 regulation. p53/p21 pathway is one of the main pathways which responsible in cell cycle modulation. Deacetylation of p53 by SIRT1, which inhibit growth arrest can be one of the reasons why *Nampt* overexpressing MEFs more resistance to the stress-induced premature senescence compare to that of WT-MEFs. Besides, NAMPT activation seems related to higher antioxidant enzymes production, which scavenges ROS produced by senescence cells. Further, I found that TG-MEFs required a higher level of the stressor to induced senescence.

In this study, I also evaluated how the UPR target genes of ER stress play a role in stress-induced premature senescence. My finding suggested that TG-MEFs response to stress-induced premature senescence more sensitive compare to that of WT-MEFs. This allows cells to response stress quickly and allows cells to cope with the stress. Again, I suggested that SIRTUIN family may play a role in this mechanism to alleviate ER stress. This supported by some previous studies involved the role of SIRTUIN family. Hence, my finding supported that *Nampt* overexpressing mouse embryonic fibroblasts had resistance to stress-induced premature senescence. Moreover, molecular study related to the role of SIRTUIN family is needed to reveal how NAMPT/NAD^+/SIRT axis regulate cells response to stress-induced premature senescence.

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7.0 References

Amodio, G., Moltedo, O., Faraonio, R., & Remondelli, P. (2018). Targeting the endoplasmic reticulum unfolded protein response to counteract the oxidative stress-induced endothelial dysfunction. *Oxidative Medicine and Cellular Longevity*, 2018, Article ID 4946289. https://doi.org/10.1155/2018/4946289.

Baehr, L. M., West, D. W. D., Marcotte, G., Marshall, A. G., De Sousa, L. G., Baar, K., & Bodine, S. C. (2016). Age-related deficits in skeletal muscle recovery following disuse are associated with neuromuscular junction instability and ER stress, not impaired protein synthesis. *Aging,* 8(1), 127-146.

Bang, I. H., Kwon, O. K., Hao, L., Park, D., Chung, M-J., Oh, B-C., Lee, S., Bae, E. J., & Park, B-H. (2019). Deacetylation of XBP1s by sirtuin 6 confers resistance to ER stress-induced hepatic steatosis. *Experimental & molecular medicine,* 51, 107. https://doi.org/10.1038/s12276-019-0309-0.

Barber, R.D., Harmer, D. W., Coleman, R. A., & Clark, B. J. (2005). GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics,* 21, 389–395. Doi:10.1152/physiolgenomics.00025.2005.

Ben-Porath, I. & Weinberg, R. A. (2005). The signals and pathways activating cellular senescence. *The international journal of biochemistry & cell biology,* 37, 961-976.

Bhatia-Dey, N., Kanherkar, R. R., Stair, S. E., Makarev, E. O. & Csoka, A. B. (2016) Cellular Senescence as the Causal Nexus of Aging. *Frontiers in genetics,* 7, 13.

Bladier, C., Wolvetang, E. J., Hutchinson, P., de Haan, J. B., & Kola, I. (1997). Response of a primary human fibroblast cell line to H_2O_2 senescence-like growth arrest or apoptosis?. *Cell Growth & Differentiation*, 8, 589-598.

Blazanin, N., Son, J., Craig-Lucas, A. B, John, C. L., Breech, K. J., Podolsky, M. A., & Glick, A. B. (2017). ER stress and distinct outputs of the IRE1α RNase control proliferation and senescence in response to oncogenic Ras. *PNAS*, 114(37), 9900-9905.

Borradaile, N. M. , Pickering, J. G. (2009). Nicotinamide phosphoribosyltransferase imparts human endothelial cells with extended replicative lifespan and enhanced angiogenic capacity in a high glucose environment. *Aging Cell*, 8(2), 100-112. Doi: 10.1111/j.1474-9726.2009.00453.x.

Brookes, S., Rowe, J., del Arroyo, A. G., Bond, J., & Peters, G. (2004). Contribution of $p16^{INK4a}$ to replicative senescence of human fibroblasts. *Experimental Cell Research*, 298, 549– 559. Doi:10.1016/j.yexcr.2004.04.035.

Brown, M. K. & Naidoo, N. (2012). The endoplasmic reticulum stress response in aging and age-related diseases. *Frontier in Physiology*, 3, 1-10. Doi: 10.3389/fphys.2012.00263.

Campisi, J. & d'Adda di Fagagna, F. (2007). Cellular senescence: when bad things happen to good cells. *Molecular Cell Biology*, 8, 729-740.

Chakraborty, S., Rasool, R., Kumar, S., Nayak, D., Rah, B., Katoch, A., Amin, H., Ali, A., & Goswami, A. (2016). Cristacarpin promotes ER stress-mediated ROS generation leading to premature senescence by activation of $p21^{waf-1}$. *Age*, 38(62). Doi: 10.1007/s11357-016-9922-1.

Chalil, S., Jaspers, R. T., Manders, R. J., Klein-Nulend, J., Bakker, A. D., & Deldicque, L. (2015). Increased endoplasmic reticulum stress in mouse osteocytes with aging alters Cox-2 response to mechanical stimuli. *Calcif Tissue Int*, 96, 123–128. Doi:10.1007/s00223-014-9944-6

Chan, S. M. H., Zhao, X., Elfowiris, A., Ratnam, C., & Herbert, T. P. (2017). The Role of De novo protein synthesis and SIRT1 in ER stress-induced Atf4 and Chop mRNA expression in mammalian cells. *Biochimie,* 138, 156-167. Doi: 10.1016/j.biochi.2017.04.018.

Chen, Q. & Ames, B. N. (1994). Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. *Proc. Natl. Acad. Sci. USA*, 91, 4130-4134.

Chini, C. C. S., Tarrago, M. G., & Chini, E. N. (2017). NAD and the aging process: Role in life, death and everything in between. *Molecular and Cellular Endocrinology,* 455, 62e74.

Choo, K. B., Tai, L., Hymavathee, K. S., Wong, C. Y., Nguyen, P. N. N., Huang, C-J., Cheong. S. K., & Kamaru, T. (2014). Oxidative stress-induced premature senescence in Wharton's Jelly-derived mesenchymal stem cells. *International Journal of Medical Sciences,* 11(11), 1201-1207. doi: 10.7150/ijms.8356.

Cristofalo, V. J., Lorenzini, A., Allen, R.G., Torres, C., & Tresini, M. (2004). Replicative senescence: a critical review. *Mechanisms of Ageing and Development,* 125, 827–848. Doi:10.1016/j.mad.2004.07.010.

Debacq-Chainiaux, F., Erusalimsky, J. D., Campisi, J., & Toussaint, O. (2009). Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. *Nature Protocols*, 4, 1798–1806. https://doi.org/10.1038/nprot.2009.191.

De Figueiredo, L. F., Gossmann, T. I., Ziegler, M., & Schuster, S. (2011). Pathway analysis of NAD+ metabolism. *Biochem. J.*, 439, 341–348. Doi:10.1042/BJ20110320.

de Haan, J. B., Cristiano, F., Iannello, R., Bladier, C., Kelner, M. J., & Kola, I. (1996). Elevation in the ratio of Cu/Zn-superoxide dismutase to glutathione peroxidase activity induces features of cellular senescence and this effect is mediated by hydrogen peroxide. *Human Molecular Genetics,* 5(2), 283–292.

de Magalhaes, J. P. & Passos, J. F. (2018). Stress, cell senescence and organismal ageing. *Mechanisms of Ageing and Development*, 170, 2-9.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., & Campisi, J. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA,* 92, 9363-9367.

Di Stefano, M. & Conforti, L. (2013). Diversification of NAD biological role: the importance of location. *FEBS Journal*, 280, 4711–4728. doi:10.1111/febs.12433.
Doultsinos, D., Avril, T., Lhomond, S., Dejeans, N., Guedat, P., & Eric Chevet1 (2017). Control of the unfolded protein response in health and disease. *SLAS Discovery*, 22(7), 787–800. Doi: 10.1177/2472555217701685.

Druelle, C., Drullion, C., Desle, J., Martin, N., Saas, L., Cormenier, J., Malaquin, N., Huot, L., Slomianny, C., Bouali, F., Vercamer, C., Hot, D., Pourtier, A., Chevet, E., Abbadie, C., & Pluquet, O. (2016). ATF6α regulates morphological changes associated with senescence in human fibroblasts. *Oncotarget*, 7(42), 67699-67715.

Duan, J., Duan, J., Zhang, Z., & Tong, T. (2005). Irreversible cellular senescence induced by prolonged exposure to H2O2 involves DNA-damage-and-repair genes and telomere shortening. *The International Journal of Biochemistry & Cell Biology,* 37, 1407–1420.

Evans, C., Bogan, K. L., Song, P., Burant, C. F., Kennedy, R. T., & Brenner, C. (2010). NAD+ metabolite levels as a function of vitamins and calorie restriction: evidence for different mechanisms of longevity. *BMC Chemical Biology*, 10(2), 1-10.

Furukawa, A., Tada-Oikawa, S., Kawanishi, S., & Oikawa, S. (2007). H2O2 accelerates cellular senescence by accumulation of acetylated p53 via decrease in the function of SIRT1 by NAD+ depletion. *Cell physiol biochem,* 20, 45-54.

Gavilan, M. P., Pintado, C., Gavilan, E., Jimenez, S., Rios, R., M., Vitorica, J., Castano, A., & Ruano, D. (2009). Dysfunction of the unfolded protein response increases neurodegeneration in aged rat hippocampus following proteasome inhibition. *Aging Cell*, 8, 654–665. Doi: 10.1111/j.1474-9726.2009.00519.x.

Ghemrawi, R., Pooya, S., Lorentz, S., Gauchotte, G., Arnold, C., Gueant, J-L., & Battaglia-Hsu, S-F. (2013). Decreased vitamin B12 availability induces ER stress through impaired SIRT1-deacetylation of HSF1. *Cell death & disease,* 4, e553.

Ghosh, A. K., Garg, S. K., Mau, T., O'Brien, M., Liu, J., & Yung, R. (2015). Elevated endoplasmic reticulum stress response contributes to adipose tissue inflammation in aging. *Journals of Gerontology: Biological Sciences*, 70(11), 1320–1329. Doi:10.1093/gerona/glu186.

Greider, C. W. (1996). Telomere length regulation. *Anna Rev. Biochent,* 65, 337-365.

Gonzalez-Bermudez, L., Anglada, T., Genesca, A., Martin, M., & Terradas, M. (2019). Identification of reference genes for RT-qPCR data normalisation in aging studies. *Scientific Reports,* 9, 13970. https://doi.org/10.1038/s41598-019-50035-0.

Gorbunova, V. & Seluanov, A. (2009). Coevolution of telomerase activity and body mass in mammals: From mice to beavers. *Mechanisms of Ageing and Development,* 130, 3–9. Doi:10.1016/j.mad.2008.02.008.

Guo, Y-L., Chakraborty, S., Rajan, S. S., Wang, R., & Huang, F. (2010). Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal. *Stem Cells and Development*, 19(9), 1321-1332. Doi: 10.1089/scd.2009.0313.

Hayflick, L. (1979). The Cell Biology of Aging. *The Journal of Investigative Dermatology*, 73 (1), 8- 14.

Hayflick, L. & Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Experimental cell research,* 25, 585-621.

Herbig, U., Jobling, W. A., Chen, B. P. C., Chen, D. J., & Sedivy, J. M. (2004). Telomere

shortening triggers senescence of human cells through a pathway involving ATM, p53, and $p21^{CIP1}$, but not $p16^{INK4a}$. *Molecular Cell*, 14, 501–513.

Hernandez-Segura, A., Nehme, J. & Demaria, M. (2018)[a]. Hallmarks of cellular senescence. *Trends in cell biology*, 28(6), 436-453. Doi:10.1016/j.tcb.2018.02.001.

Hernandez-Segura, A. Rubingh, R., & Demaria, M. (2018)[b]. Identification of stable senescence-associated reference genes. *Aging Cell*, 18:e12911. Doi:10.1111/acel.12911.

Herranz, N. & Gil, J. 2018. Mechanisms and functions of cellular senescence. *The Journal of Clinical Investigation*, 128(4), 1238-1246. https://doi.org/10.1172/JCI95148.

Hetz, C. & Papa, F. R. (2018). The unfolded protein response and cell fate control. *Molecular Cell*, 69, 169-181. https://doi.org/10.1016/j.molcel.2017.06.017.

Hewitt, G., Jurk, D., Marques, F. D. M., Correia-Melo, C., Hardy, T., Gackowska, A., Anderson, R., Taschuk, M., Mann, J., & Passos, J. (2012). Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nature communications,* 3, 708.

Ho, C., van der Veer, E., Akawi, O., & Pickering, J. G. (2009). SIRT1 markedly extends replicative lifespan if the NAD+ salvage pathway is enhanced. *FEBS Letters*, 583, 3081–3085. https://doi.org/10.1016/j.febslet.2009.08.031.

Hou, H., Ge, C., Sun, H., Li, H., Li, J. & Tian, H. (2018). Tunicamycin inhibits cell proliferation and migration in hepatocellular carcinoma through suppression of CD44s and the ERK1/2 pathway. *Cancer Science,* 1–13. Doi:10.1111/cas.13518.

Houtkooper, R. H., Canto, C., Wanders, R. J., & Auwerx, J. (2010). The secret life of NAD: an old metabolite controlling new metabolic signaling pathways. *Endocrine Reviews,* 31(2), 194–223.

Huschtscha, L. I. & Reddel, R. R. (1999). $p16^{INK4a}$ and the control of cellular proliferative life span. *Carcinogenesis,* 20(6), 921–926.

Hussain, S. G. & Ramaiah, K. V. A. (2007). Reduced eIF2a phosphorylation and increased proapoptotic proteins in aging. *Biochemical and Biophysical Research Communications,* 355, 365–370.

Imai, S. (2009). Nicotinamide phosphoribosyltransferase (Nampt): A link between NAD biology, metabolism, and diseases. *Curr Pharm Des*, 15(1), 20–28.

Imai, S. (2010). “Clocks” in the NAD world: NAD as a metabolic oscillator for the regulation of metabolism and aging. *Biochim Biophys Acta*, 1804(8), 1584–1590. Doi:10.1016/j.bbapap.2009.10.024.

Imai, S. & Yoshino, J. (2013). The importance of NAMPT/NAD/SIRT1 in the systemic regulation of metabolism and ageing. *Diabetes, Obesity and Metabolism,* 15 (Suppl. 3), 26–33.

Imai, S. & Guarente, L. (2014). NAD+ and sirtuins in aging and disease. *Trends in cell biology,* 24, 464-471.

Inoue, Y., Kawachi, S., Ohkubo, T., Nagasaka, M., Ito, S., Fukuura, K., Itoh, Y., Ohoka, N., Morishita, D., & Hayashi, H. (2017). The CDK inhibitor p21 is a novel target gene of ATF4 and contributes to cell survival under ER stress. *FEBS Letters,* 591, 3682–3691. Doi:10.1002/1873-3468.12869.

Itahana, K., Campisi, J. & Dimri, G. P. (2004). Mechanisms of cellular senescence in human and mouse cells. *Biogerontology*, 5, 1–10

Itahana, K., Itahana, Y., & Dimri, G. P. (2013). Colorimetric detection of senescence-associated β galactosidase. *Methods Mol Biol.*, 965, 143–156. Doi:10.1007/978-1-62703-239-1_8.

Iurlaro, R. & Munoz-Pinedo, C. (2015). Cell death induced by endoplasmic reticulum stress. *FEBS Journal*, 283, 2640–2652. Doi:10.1111/febs.13598.

Ji, Y., Luo, X., Yang, Y., Dai, Z., Wu, G., & Wu, Z. (2018). Endoplasmic reticulum stress-induced apoptosis in intestinal epithelial cells: a feed-back regulation by mechanistic target of rapamycin complex 1 (mTORC1). *Journal of Animal Science and Biotechnology*, 9(38). https://doi.org/10.1186/s40104-018-0253-1.

Kanemoto, S., Kondo, S., Ogata, M., Murakami, T., Urano, F., & Imaizumi, K. (2005). XBP1 activates the transcription of its target genes via an ACGT core sequence under ER stress. *Biochemical and Biophysical Research Communications*, 331, 1146–1153.

Kang, H. T., Lee, H. I., & Hwang, E. S. (2006). Nicotinamide extends replicative lifespan of human cells. *Aging Cell,* 5, 423–436. Doi:10.1111/j.1474-9726.2006.00234.x.

Kannan, S., Dawany, N., Kurupati, R., Showe, L.C., & Ertl, H.C. (2016) Age-related changes

in the transcriptome of antibody-secreting cells. *Oncotarget*, 7(12), 13340–13353. Doi:10.18632/oncotarget.7958.

Kennedy, A. L., Mc Bryan, T., Enders, G. H., Johnson, F. B., Zhang, R., & Adams, P. D. (2010). Senescence mouse cells fail to overtly regulate the HIRA histone chaperone and do not form robust Senescence Associated Heterochromatin Foci. *Cell Division,* 5:16. http://www.celldiv.com/content/5/1/16.

Khaidizar, F. D. (2017). The effect of *Nampt* overexpression on cellular senescence. Doctoral thesis. Graduate School of Biological Sciences. Nara Institute of Science and Technology. Japan. p 30.

Khaidizar, F. D., Nakahata, Y., Kume, A., Sumizawa, K., Kohno, K., Matsui, T., & Bessho, Y. (2017). Nicotinamide phosphoribosyltransferase delays cellular senescence by upregulating SIRT1 activity and antioxidant gene expression in mouse cells. *Genes to cells: devoted to molecular & cellular mechanisms,* 22, 982-992.

Kishino, A., Hayashi, K., Hidai, C., Masuda, T., Nomura, Y. & Oshima, T. (2017). XBP1-FoxO1 interaction regulates ER stress-induced autophagy in auditory cells. *Scientific Reports*, 7, 4442. Doi:10.1038/s41598-017-02960-1.

Kiyoshima, T., Enoki, N., Kobayashi, I., Sakai, T., Nagata, K., Wada, H., Fujiwara, H., Ookuma, Y., & Sakai, H. (2012). Oxidative stress caused by a low concentration of hydrogen peroxide induces senescence-like changes in mouse gingival fibroblasts. International Journal of Molecular Medicine, 30, 1007-1012. Doi:10.3892/ijmm.2012.1102

Koga, T., Suico, M. A., Shimasaki, S., Watanabe, E., Kai, Y., Koyama, K., Omachi, K., Morino-Koga, S., Sato, T., Shuto, T., Mori, K., Hino, S., Nakao, M., & Kai, H. (2015). Endoplasmic reticulum (ER) stress induces sirtuin 1 (SIRT1) expression via the PI3K-Akt-GSK3 signaling pathway and promotes hepatocellular injury. *The Journal of Biological Chemistry*, 290(51), 30366–30374. Doi:10.1074/jbc.M115.664169.

Krajarng, A., Imoto, M., Tashiro, E., Fujimaki, T., Shinjo, S., & Watanapokasin, R. (2015). Apoptosis induction associated with the ER stress response through up-regulation of JNK in HeLa cells by gambogic acid. *BMC Complementary and Alternative Medicine*, 15(26). Doi:10.1186/s12906-015-0544-4.

Lafleur, M., A., Stevens, J. L., & LawrenceE, J. W. (2013). Xenobiotic perturbation of ER stress and the unfolded protein response. *Toxicologic Pathology*, 41, 235-262.

Doi:10.1177/0192623312470764.

Le, T. M., Hashida, K., Ta, H. M., Takarada-Iemata, M., Kokame, K., Kitao, Y., & Hori, O. (2016). Deletion of Herpud1 enhances heme oxygenase-1 expression in a mouse model of Parkinson's disease. *Parkinson's Disease,* 2016, Article ID 6163934, 9 pages http://dx.doi.org/10.1155/2016/6163934.

Lennicke, C., Rahn, J., Lichtenfels, R., Wessjohann, L. A., & Seliger, B. (2015). Hydrogen peroxide – production, fate and role in redox signaling of tumor cells. *Cell Communication and Signaling,* 13:39. Doi:10.1186/s12964-015-0118-6.

Lenox, A., R., Bhootada, Y., Gorbatyuk, O., Fullard, R., & Gorbatyuk, M. (2015). Unfolded protein response is activated in aged retinas. *Neuroscience Letters,* 609, 30-35. http://dx.doi.org/10.1016/j.neulet.2015.10.019.

Li, M., Zhao, L., Liu, J., Liu, A-L.,Zeng, W-S., Luo, S-Q., & Bai, X-C. (2009). Hydrogen peroxide induces G2 cell cycle arrest and inhibits cell proliferation in osteoblasts. *The Anatomical Record*, 292, 1107–1113. Doi:10.1002/ar.20925.

Li, D-Y., Yu, J-C., Xiao, L., Miao, W., Ji, K., Wang, S-C., & Geng, Y-X. (2017). Autophagy attenuates the oxidative stress-induced apoptosis of Mc3T3-E1 osteoblasts. *European Review for Medical and Pharmacological Sciences*, 21, 5548-5556.

Lin, S-J. & Guarente, L. (2003). Nicotinamide adenine dinucleotide, a metabolic regulator of transcription, longevity and disease. *Current Opinion in Cell Biology*, 15, 241–246.

Lin, W-C., Chuang, Y-C., Chang, Y-S., Lai, M-D., Teng, Y-N., Su, I-J., Wang, C. C. C., Lee, K-H., Hung, J-H. (2012). Endoplasmic Reticulum Stress Stimulates p53 Expression through NF-kB Activation. *PLoS One*, 7(7), e39120. Doi:10.1371/journal.pone.0039120.

Liochev, S. I. (2013). Reactive oxygen species and the free radical theory of aging. *Free Radic Biol Med.*, 60, 1-4. https://doi.org/10.1016/j.freeradbiomed.2013.02.011.

Liu, J., Wang, L., Wang, Z., & Liu, J-P. (2019). Roles of telomere biology in cell senescence, replicative and chronological ageing. *Cells*, 8(54). Doi:10.3390/cells8010054.

Liu-Bryan, R. (2015). Inflammation and intracellular metabolism: new targets in OA. *Osteoarthritis and Cartilage,* 23, 1835-1842. http://dx.doi.org/10.1016/j.joca.2014.12.016

Ma C., Pi C., Yang Y., Lin L., Shi Y., Li Y., Li Y., & He X. (2017). Nampt expression decreases age-related senescence in rat bone marrow mesenchymal stem cells by targeting Sirt1. *PLoS ONE,* 12(1), e0170930. Doi:10.1371/journal.pone.0170930.

Martindale, J. J., Fernandez, R., Thuerauf, D., Whittaker, R., Gude, N., Sussman, M. A., & Glembotski, C. C. (2006). Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6. *Circulation research,* 98, 1186-1193.

Martinez, G., Duran-Aniotz, C., Cabral-Miranda, F., Vivar, J. P., & Hetz, C. (2017). Endoplasmic reticulum proteostasis impairment in aging. *Aging Cell*, 16, 615–623. Doi:10.1111/acel.12599.

Massudi H, Grant R, Braidy N, Guest J, Farnsworth B, Guillemin, G. J. (2012). Age-associated changes in oxidative stress and NAD^+ metabolism in human tissue. *PLoS ONE,* 7(7), e42357. Doi:10.1371/journal.pone.0042357.

Matjusaitis, M., Chin, G., Sarnoski, E. A., Stolzing, A. (2016). Biomarkers to identify and isolate senescent cells. *Ageing Research Reviews,* 29, 1–12.

Matos, L., Gouveia, A. M., & Almeida, H. (2015). ER stress response in human cellular models of senescence. *Journals of Gerontology: Biological Sciences*, 924–935. Doi:10.1093/gerona/glu129.

Mirzayans, R., Andrais, B., Hansen, G., & Murray, D. (2012). Role of $p16^{INK4A}$ in replicative senescence and DNA damage-induced premature senescence in p53-deficient human cells. *Biochemistry Research International*, Volume 2012, Article ID 951574, 8 pages. Doi:10.1155/2012/951574.

Munoz-Espin, D. & Serrano, M. (2014). Cellular senescence: from physiology to pathology. *Molecular Cell Biology*, 15, 482-496.

Naidoo, N. (2009). ER and aging-protein folding and the ER stress response. *Ageing Research Reviews,* 8, 150-159. Doi:10.1016/j.arr.2009.03.001.

Nakahata, Y., Sahar, S., Astarita, G., Kaluzova, M., & Sassone-Corsi, P. (2009). Circadian control of the NAD^+ salvage pathway by CLOCK-SIRT1. *Science*. 324, 654-656.

Nakahata, Y., Yasukawa, S., Khaidizar, F. D., Shimba, S., Matsui, T., and Bessho, Y. (2018).

Bmal1-deficient mouse fibroblast cells do not provide premature cellular senescence in vitro. *Chronobiol Int,* 35, 730-738.

Narita, M. (2007). Cellular senescence and chromatin organization. *British Journal of Cancer*, 96, 686-691. Doi:10.1038/sj.bjc.6603636.

Nikiforov, A., Kulikova, V. & Ziegler, M. (2015) The human NAD metabolome: Functions, metabolism and compartmentalization. *Critical Reviews in Biochemistry and Molecular Biology,* 50(4), 284-297. Doi:10.3109/10409238.2015.1028612.

Nuriliani, A., Nakahata, Y., Ahmed, R., Khaidizar, F. D., Matsui, T., & Bessho, Y. (2020). Overexpression of nicotinamide phosphoribosyltransferase in mouse cells confers protective effect against oxidative and ER stress induced premature senescence. *Genes to Cells,* 25(6), 1-10. Doi:10.1111/gtc/12794.

Oslowski, C. M. & Urano, F. (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods in enzymology,* 490, 71-92.

Paez-Ribes, M., Gonzalez-Gualda, E., Doherty, G. J., & Munoz-Espin, D. (2019). Targeting senescent cells in translational medicine. *EMBO Mol Med,* 11, e10234.

Pawlikowski, J. S., Adams, P.D., & Nelson, D. M. (2013). Senescence at a glance. *Journal of Cell Science*, 126, 4061-4067.

Paz Gavilan, M., Vela, J., Castano, A., Ramos, B., del Rio, J.C., Vitorica, J., & Ruano, D. (2006). Cellular environment facilitates protein accumulation in aged rat hippocampus. *Neurobiology of Aging,* 27, 973–982. Doi:10.1016/j.neurobiolaging.2005.05.010.

Peek, C., B., Affinati, A. H., Ramsey, K. M., Kuo, H-Y., Yu, W., Sena, L. A., Ilkayeva, O., Marcheva, B., Kobayashi, Y., Omura, C., Levine, D.C., Bacsik, D. J., Gius, D., Newgard, C. B., Goetzman, E., Chandel, N. S., Denu, J. M., Mrksich, M., & Bass, J. (2013). Circadian clock NAD^+ cycle drives mitochondrial oxidative metabolism in mice. *Science*, 342, 1243417. Doi:10.1126/science.1243417.

Petrova, N. V., Velichko, A. K., Razin, S. V., & Kantidze, O. L. (2016). Small molecule compounds that induce cellular senescence. *Aging Cell*, 15, 999–1017. Doi:10.1111/acel.12518.
Pienkowska, N., Bartosz, G., Pichla, M., Grzesik-Pietrasiewicz, M., Gruchala, M., Pizarro, J. G., Folch, J., De La Torre, A. V., Verdaguer, E., Junyent, F., Jordan, J., Pallas, M., & Camins, A. (2009). Oxidative stress-induced DNA damage and cell cycle regulation in B65

dopaminergic cell line. Free Radical Research, 43(10), 985-994.

Pizarro, J. G., Folch, J., De La Torre, A. V., Verdageuer, E., Junyent, F., Jordan, J., Pallas, M., & Camins, A. (2009). Oxidative stress-induced DNA damage and cell cycle regulation in B65 dopaminergic cell line. *Free Radical Research*, 43(10), 985-994. Doi:10.1080/10715760903159188.

Pluquet, O., Pourtier, A., & Abbadie, C. (2015). The unfolded protein response and cellular senescence. A review in the theme: cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. *American journal of physiology. Cell physiology,* 308, C415-425.

Prola, A., Da Silva, J. P., Guilbert, A., Lecru, L., Piquereau, J., Ribeiro, M., Mateo, P., Gressette, M., Fortin, D., Boursier, C., Gallerne, C., Caillard, A., Samuel, J-L., Francois, H., Sinclair, D. A., Eid, P., Ventura-Clapier, R., Garnier, A., & Lemaire, C. (2017). SIRT1 protects the heart from ER stress-induced cell death through eIF2α deacetylation. *Cell Death and Differentiation,* 24, 343–356. Doi:10.1038/cdd.2016.138.

Rajesh, K., Papadakis, A. I., Kazimierczak, U., Peidis, P., Wang, S., Ferbeyre, G., Kaufman, R. J., & Koromilas, A. E. (2013). eIF2α phosphorylation bypasses premature senescence caused by oxidative stress and pro-oxidant antitumor therapies. *Aging*, 5(12), 884-901.

Ren, M-T., Gu, M-L., Zhou, X-X., Yu, M-S., Pan, H-H., Ji, F., & Ding, C-Y. (2019). Sirtuin 1 alleviates endoplasmic reticulum stress-mediated apoptosis of intestinal epithelial cells in ulcerative colitis. *World J Gastroenterol,* 25(38), 5800-5813. Doi:10.3748/wjg.v25.i38.5800.

Rhinn, M., Ritschka, B., & Keyes, W. M. (2019). Cellular senescence in development, regeneration and disease. *Development,* 146, dev151837. Doi:10.1242/dev.151837.

Rufini, A., Tucci, P., Celardo, I., & Melin, G. (2013). Senescence and aging: the critical roles of p53. *Oncogene,* 32, 5129-5143.

Rutkowski, D. T., Arnold, S. M., Miller, C. N., Wu, J., Li, J., Gunnison, K. M., Mori, K., Akha, A. A. S., Raden, D., Kaufman, R. J. (2006). Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS biology,* 4, e374.

Rutkowski, D. T. & Kaufman, R. J. (2007). That which does not kill me makes me stronger: adapting to chronic ER stress. *TRENDS in Biochemical Sciences,* 32(10), 469-476.

Salama, R., Sadaie, M., Hoare, M., & Narita, M. (2014). Cellular senescence and its effector programs. Genes & Development, 28, 99–114. http://www.genesdev.org/cgi/doi/10.1101/gad.235184.113.

Schosserer, M., Grillari, J., & Breitenbach, M. (2017). The dual role of cellular senescence in developing tumors and their response to cancer therapy. *Front. Oncol.*, 7(278). Doi: 10.3389/fonc.2017.00278.

Schroder, M. & Kaufman, R. J. (2005). ER stress and the unfolded protein response. *Mutation Research,* 569, 29-63. Doi:10.1016/j.mrfmmm.2004.06.056.

Serrano-Negron, J. E., Zhang, Z., Rivera-Ruiz, A. P., Banerjee, A., Romero-Nutz, E. C., Sanchez-Torres, N., Baksi, K., & Banerjee, D. K. (2018). Tunicamycin-induced ER stress in breast cancer cells neither expresses GRP78 on the surface nor secretes it into the media. *Glycobiology*, 28(2), 61–68. Doi:10.1093/glycob/cwx098.

Shen, M., Wang, L., Guo, X., Xue, Q., Huo, C., Li, X., Fan, L., & Wang, X. (2015). A novel endoplasmic reticulum stress-induced apoptosis model using tunicamycin in primary cultured neonatal rat cardiomyocytes. *Molecular Medicine Reports*, 12, 5149-5154, Doi:10.3892/mmr.2015.4040.

Shin, J., He, M., Liu, Y., Paredes, S., Villanova, L., Brown, K., Qiu, X., Nabavi, N., Mohrin, M., Wojnoonski, K., Li, P., Cheng, H-L., Murphy, A. J., Valenzuela, D. M., Luo, H., Kapahi, P., Krauss, R., Mostoslavsky, R., Yancopoulos, G. D., Alt, F. W., Chua, K. F., & Chen, D. (2013). SIRT7 represses Myc activity to suppress ER stress and prevent fatty liver disease. *Cell reports,* 5, 654-665.

Shoulders, M. D., Ryno, L. M., Genereux, J. C., Moresco, J. J., Tu, P. G., Wu, C., Yates, J. R., Su, A. I., Kelly, J. W., & Wiseman, R., L. (2013). Stress-independent activation of XBP1s and/or ATF6 reveals three functionally diverse ER proteostasis environments. *Cell Reports,* 3, 1279-1292. http://dx.doi.org/10.1016/j.celrep.2013.03.024.

Sies, H. (2017). Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox Biology*, 11, 613–619. http://dx.doi.org/10.1016/j.redox.2016.12.035.

Song, J., Ke, S-F., Zhou, C-C., Zhang, S-L., Guan, Y-F., Xu, T-Y., Sheng, C-Q., Wang, P., & Miao, C-Y. (2013). Nicotinamide phosphoribosyltransferase is required for the calorie restriction–mediated improvements in oxidative stress, mitochondrial biogenesis, and

metabolic adaptation. *Journals of Gerontology: Biological Sciences,* 69(1), 44–57. doi:10.1093/gerona/glt122.

Soto-Gamez, A., Quax, W. J., & Demaria, M. (2019). Regulation of survival networks in senescent cells: from mechanisms to interventions. *Journal of Molecular Biology*, 431, 2629-2643. https://doi.org/10.1016/j.jmb.2019.05.036.

Suo, R., Zhao, Z-Z., Tang, Z-H., Ren, Z., Liu, X., Liu, L-S., Wang, Z., Tang, C-K., Wei, D-H., & Jiang, Z-S. (2013). Hydrogen sulfide prevents H2O2-induced senescence in human umbilical vein endothelial cells through SIRT1 activation. Molecular Medicine Reports, 7, 1865-1870. Doi:10.3892/mmr.2013.1417.

Tristan, C., Shahani, N., Sedlak, T. W., & Sawa, A. (2011). The diverse functions of GAPDH: views from different subcellular compartments. *Cell Signal*, 23(2), 317–323. Doi:10.1016/j.cellsig.2010.08.003.

Toussaint, O., Royer, V., Salmon, M., & Remacle, J. (2002) Stress-induced premature senescence and tissue ageing. *Biochemical Pharmacology,* 64, 1007-1009.

Tsuru, A., Imai, Y., Saito, M., & Kohno, K. (2016). Novel mechanism of enhancing IRE1alpha-XBP1 signalling via the PERK-ATF4 pathway. *Scientific reports,* 6, 24217.

van der Veer, E., Ho, C., O'Neil, C., Barbosa, N., Scott, R., Cregan, S. P., & Pickering, J. G. (2007). Extension of human cell lifespan by nicotinamide phosphoribosyltransferase. *The Journal of biological chemistry,* 282, 10841-10845.

van Deursen, J. M. (2014). The role of senescent cells in ageing. *Nature,* 509, 439-446.

Vilema-Enriquez, G., Arroyo, A., Grijalva, M., Amador-Zafra, R. I., & Camacho, J. (2016). Molecular and cellular effects of hydrogen peroxide on human lung cancer cells: potential therapeutic implications. *Oxidative Medicine and Cellular Longevity*, Volume 2016, Article ID 1908164, 12 pages. http://dx.doi.org/10.1155/2016/1908164.

Walter, P. & Ron, D. (2011). The unfolded protein response: from stress pathway to homeostatic regulation. *Science,* 334, 1081-1086.

Walters, S. N., Luzuriaga, J., Chan, J. Y., Grey, S. T., & Laybutt, D. R. (2013). Influence of chronic hyperglycemia on the loss of the unfolded protein response in transplanted islets. *J Mol Endocrinol,* 51, 225-232.

Whittemore, K., Vera, E., Martínez-Nevado, E., Sanpera, C., and Blasco, M. A. (2019). Telomere shortening rate predicts species life span. *PNAS*, 116 (30), 15122-15127. https://doi.org/10.1073/pnas.1902452116.

Wiley, C. D. & Campisi, J. (2016). From ancient pathways to aging cells-connecting metabolism and cellular senescence. *Cell Metabolism*, 23, 1013-1021. http://dx.doi.org/10.1016/j.cmet.2016.05.010.

Wu, J., Chen, S., Liu, H., Zhang, Z., Ni, Z., Chen, J., Yang, Z., Nie, Y., & Fan, D. (2018). Tunicamycin specifically aggravates ER stress and overcomes chemoresistance in multidrug-resistant gastric cancer cells by inhibiting N-glycosylation. *Journal of Experimental & Clinical Cancer Research*, 37(272). https://doi.org/10.1186/s13046-018-0935-8.

Yaku, K., Okabe, K., & Nakagawa, T. (2018). NAD metabolism: Implications in aging and longevity. *Ageing Research Reviews,* 47, 1-17.

Yan, Z., Gao, J., Lv, X., Yang, W., Wen, S., Tong, H., & Tang, C. (2019). Quantitative Evaluation and selection of reference genes for quantitative RT-PCR in mouse acute pancreatitis. *BioMed Research International,* Volume 2016, Article ID 8367063, 11 pages. http://dx.doi.org/10.1155/2016/8367063.

Yang, H. & Fogo, A. B. (2010). Cell senescence in the aging kidney. *J Am Soc Nephrol,* 21, 1436–1439. Doi:10.1681/ASN.2010020205.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., & Mori, K. (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell,* 107, 881-891.

Yoshizaki, K., Fujiki, T., Tsunematsu, T., Yamashita, M., Udono, M., Shirahata, S., & Katakura, Y. (2009). Pro-senescent effect of hydrogen peroxide on cancer cells and its possible application to tumor suppression. *Bioscience, Biotechnology, and Biochemistry,* 73(2), 311-315. Doi:10.1271/bbb.80517.

Zainuddin, A., Chua, K. H., Rahim, N. A., & Makpol, S. (2010). Effect of experimental treatment on GAPDH mRNA expression as a housekeeping gene in human diploid fibroblasts. *BMC Molecular Biology,* 11(59). http://www.biomedcentral.com/1471-2199/11/59.

Zhang, H-H., Ma, X-J., Wu, L-N., Zhao, Y-Y., Zhang, P-Y., Zhang, Y-H., Shao, M-W., Liu, F., Li, F., & Qin, G-J. (2016). Sirtuin-3 (SIRT3) protects pancreatic b-cells from endoplasmic

reticulum (ER) stress-induced apoptosis and dysfunction. *Mol Cell Biochem*, 420,95–106. Doi:10.1007/s11010-016-2771-5.

Zhong, J-T., Yu, J., Wang, H-J., Shi, Y., Zhao, T-S., He, B-X., Qiao, B., & Feng, Z-W. (2017). Effects of endoplasmic reticulum stress on the autophagy, apoptosis, and chemotherapy resistance of human breast cancer cells by regulating the PI3K/AKT/mTOR signaling pathway. *Tumor Biology*, 1–9. Doi:10.1177/1010428317697562.

Ziegler, D. V., Wiley, C. D., & Velarde, M. C. (2015). Mitochondrial effectors of cellular senescence: beyond the free radical theory of aging. *Aging cell,* 14, 1-7.

8.0 Supplementary

Supplement 1.

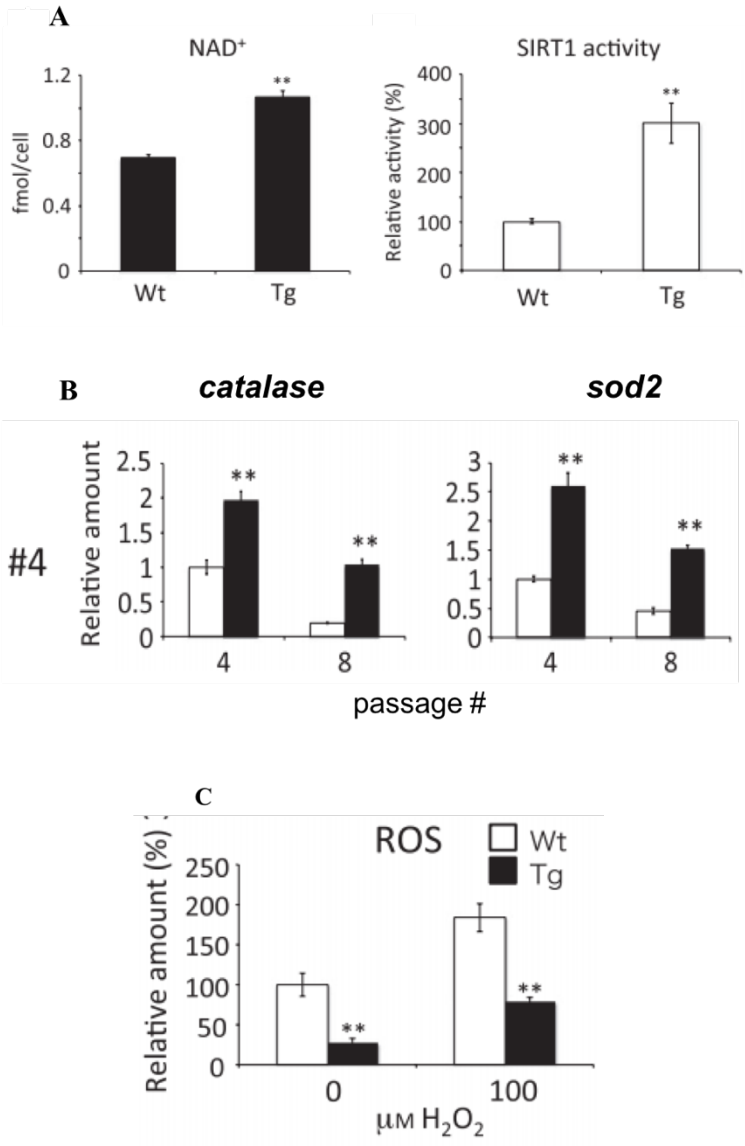

Figure 18. The mechanism of TG-MEFs in response to oxidative stress. Resistance against H_2O_2 was regulated by the increasing of NAD^+ level accompanied with the increasing of SIRT1 activity (A.), which increase the antioxidant enzymes, *catalase* and *sod2* (B.). Further, the increasing of *catalase* and *sod2* scavenge more ROS followed H_2O_2 treatment (C.) (Khaidizar et al., 2017).