Is Nature our Best Assurance of Healthy Ageing?

Lessons from age-resilient animal species

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Abstract

Ageing is a complex and multifactorial phenomenon. The molecular mechanisms underlying ageing are characterized in nine distinct hallmarks of ageing. Although ageing is a common phenomenon in most species, not all species have the same lifespan and rate of ageing. There are species that are extraordinary long-living or extreme stress resilient. Do these species have different molecular mechanisms to resist the functional decline as described in the hallmarks of ageing? And could we explore the potential of these differences to extend human health and lifespan? Here I present two examples that show this might be the case by showing the unique capacity of the naked mole rat to maintain proteostasis and that of tardigrades to maintain genome stability. Both examples show that there are many adaptions that jointly increase the longevity and stress resilience of these species, making transfer of the improved traits complex. Nonetheless, the example of the tardigrade shows that a single tardigrade-specific gene can increase radiation resistance in human cells. This provides evidence that studying species with a natural longevity or extreme stress resilience might be a viable strategy to find mechanisms that, when transferred to humans, may increase human healthy lifespan.

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List of Abbreviations

Abbreviation	Description
DNA	Deoxyribonucleic acid
DSB	Double strand break
Dsup	Damage suppressor
HGT	Horizontal gene transfer
IGF-1	Insulin-like growth factor 1
IIS	Insulin and IGF-1 signaling
$k_{degradation}$	Degradation rate / protein turnover rate
MLS	Maximum lifespan
mTOR	Mechanistic target of rapamycin
NMR	Naked mole rat
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SSB	Single strand break
UPS	Ubiquitin-proteasome system

Introduction

"Perhaps nature is our best assurance of immortality." With this quote Elenore Roosevelt (My day, April 26, 1945) was referring to the beauty of nature, of flowers that are beginning to show and trees that are in full bloom. However, this quote also has some interesting aspects in the biology of ageing. Immortality or life extension are hot topics in biomedical research. Can we slow down or even prevent ageing? Can we find ways to tackle age-related pathologies, such as cancer and neurodegenerative diseases? To answer these questions, we first need to understand what happens to the body when we are ageing and what molecular mechanisms underlie these age-related changes.

Furthermore, how do other species deal with ageing? Basically, all mammals show some signs of ageing, however to different extend. There are species that can live twice as long as humans, such as the bowhead whale. Other species, like the naked mole rat show an incredible resistance to age and cancer, an age-related pathology. Additionally, some species expresses extreme stress resilience and can survive under severe environmental circumstances, for example thermophiles or tardigrades. This brings us to the other interesting part of Elenore Roosevelt's quote, *"Perhaps nature is our best assurance…"*. What can we learn from different species in nature that have evolved resistance to ageing and stress resilience? Could an understanding in the mechanisms underlying their age resistance help us slow down ageing and solve the problem of age-related pathologies? And is studying species with a natural longevity or extreme stress resilience a viable strategy to find mechanisms to increase human lifespan?

In this essay I will address these questions, by first explaining some molecular mechanisms underlying ageing and explain lifespan and stress resilience in more detail. Furthermore, I will elucidate the concept of age-resistancy and stress resilience in other species by two main examples, the naked mole rat and the tardigrade. Moreover, I will use these examples to demonstrate the potential value of studying evolutionary adaptions in age-resilient species and the potential for intervention to extend of human health and lifespan.

What is ageing?

In general, the underlying cause of ageing is the time-dependent accumulation of cellular damage[1]. In 2013, López-Otín *et al*. proposed nine hallmarks of ageing that ideally meets three criteria: it manifests during normal ageing; experimental aggravation of the hallmark accelerates ageing and experimental amelioration retards normal ageing process. According to

the authors there are four primary hallmarks of ageing; genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis. Furthermore, there are three antagonistic hallmarks, namely dysregulated nutrient sensing, mitochondrial dysfunction and cellular senescence. These hallmarks are originally a damage control response to the primary hallmarks, however, when exacerbated or chronically present, they become deleterious themselves. Finally, stem cell exhaustion and altered intercellular communication are defined as integrative hallmarks as they integrate all previous named hallmarks and are ultimately responsible for the age-related functional decline[2].

To comprehend the mechanisms underlying ageing and the molecular changes in the two example species (naked mole rat and tardigrade), I will elucidate aspects relevant to the examples of three hallmarks in more detail. I will first discuss genome stability, including DNA repair mechanisms, second the protein homeostasis and finally the cell metabolism.

Genome instability

During life your DNA is continuously challenged by numerous threats from the environment, like radiation and chemicals as well as endogenous hazards like mistakes made during DNA replication, repair errors, transpositions and damage due to reactive oxygen species (ROS)[3]. These threats could lead to divers lesions in the DNA, such as point mutations, deletions or single or double strand breaks in the DNA[3,4]. Fortunately, mechanisms have evolved to prevent and repair DNA damage. First, during DNA replication, there are mechanisms to prevent replication mistakes, such as proofreading and mismatch repair[5]. These processes combined ensure only 1-3 mistakes in 10 billion bases during DNA replication[6]. However, DNA can be damaged at later stages by both endogenous and exogenous factors. To repair this damage the

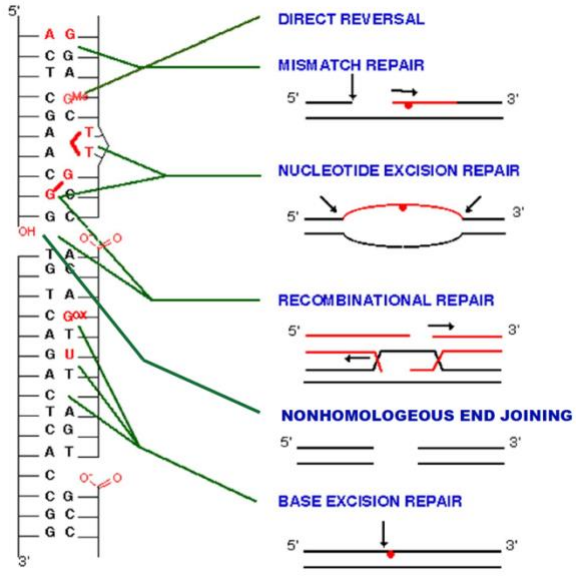

Figure 1. DNA repair mechanisms. Summary of common DNA repair mechanisms to prevent DNA replication mistakes or mend single or double strand breaks. Figure adapted from http://bbrp.llnl.gov/repair/html/overview.html

cell can use base excision repair, in which specific glycosylases recognize and remove 'unnatural' bases and exert a general repair of 'gaps' in the DNA[6]. Furthermore, to repair bigger lesions, nucleotide excision repair can be used. In this process the enzyme complex recognizes large disturbances in the helix and endonuclease cuts on both sides in the DNA backbone. Helicase removes fragment and the gap is resynthesized[6]. Double strand breaks (DSB) can be repaired using either nonhomologous end joining, the joining of two ends together without replacing the missing nucleotides, or homologous recombination, processing by nuclease and using the sister chromatid as a template (Figure 1)[6].

With ageing, mutations accumulate and so do other forms of DNA damage, such as chromosomal aneuploidy and copy number variations[2]. Jointly these forms of genome instability can result in misfolded or dysfunctional proteins and protein complexes, since DNA provides the blueprint for protein synthesis. Various premature ageing diseases are linked to genome instability, such as Werner syndrome and Bloom syndrome[2]. Moreover, deficiencies in DNA repair mechanisms accelerate ageing in mice models and are associated with several human progeroid syndromes[2].

To summarize, genome stability is regulated by various mechanisms that prevent or repair DNA damage. However, with ageing, mutations and mistakes accumulate, affecting protein quality and contributing to the ageing phenotype and age-related pathologies.

Loss of proteostasis

Just like the loss of genome stability also, loss of proteostasis is one of the primary hallmarks of ageing[2]. This loss of protein homeostasis is not only related to forms of genome instabilities, such as accumulation of aneuploidy, replication errors, mutations or faulty mRNAs, but also processes involved in the folding and degradation of proteins are compromised[2]. The chaperone system is essential in the correct folding of larger protein and remaining protein stability[7]. This system is significantly impaired during ageing, leading to misfolding and dysfunction of proteins, hence contributing to the age phenotype and age-related pathologies[2].

In addition, there are two main proteolytic systems involved in the protein quality control and protein degradation; the autophagy-lysosomal system and the ubiquitin-proteasome system (UPS)[8]. In the autophagy-lysosomal system cellular components are encapsulated in a double-membraned vesicle, the autophagosome. Upon fusion of the autophagosome with lysosomes the content will be degraded and recycled[9]. The other protein degradation system is the ubiquitin-proteasome system. Proteins targeted for degradation are 'tagged' with a ubiquitin flag before being transported to and proteolyzed by the proteasome, a large enzyme

complex[10]. These quality control systems are essential for an efficient protein turnover, which is the balance between protein synthesis and protein degradation, and to maintain proteostasis.

Both endogenous and exogenous stressors could turn a folded protein into an unfolded protein. Normally, this unfolded protein will be either degraded via autophagy or UPS, or refolded by the chaperones. However, all these protein quality control systems are compromised during ageing, resulting in inefficient quality control and degradation and accumulation and aggregation of misfolded proteins[2] (Figure 2). Misfolded proteins and protein aggregates are molecular hallmarks of various neurodegenerative diseases, including Alzheimer's Disease, Parkinson's Disease and Huntington's Disease[9]. Previous studies showed that interventions to increase proteostasis network activity increase healthy lifespan in mammals[9].

In summary, genome instabilities could lead to faulty proteins and disrupted proteostasis. This proteostasis is maintained by three protein quality control mechanisms, the chaperone system, autophagy and UPS. These systems are affected during ageing, resulting in loss of proteostasis and protein aggregation. This contributes to the ageing phenotype and age-related pathologies, such as neurodegenerative diseases.

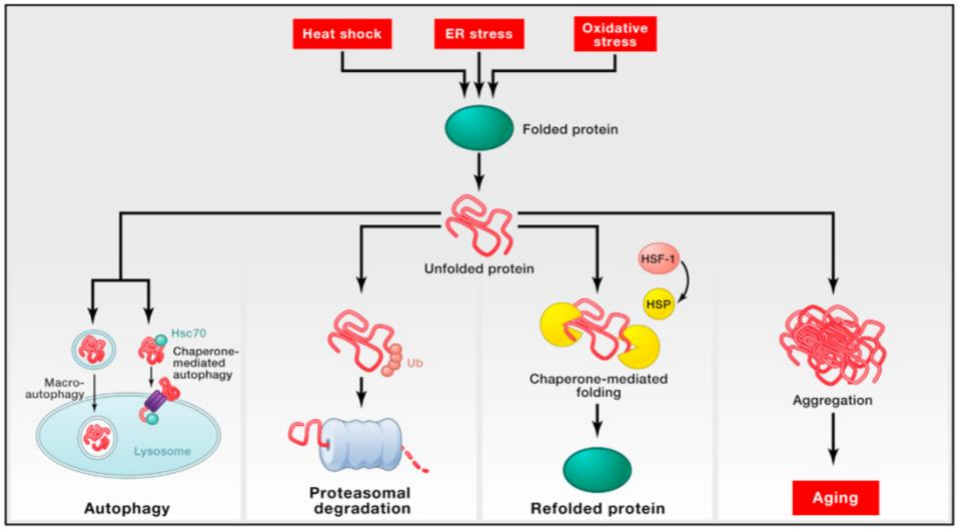

Figure 2. Loss of proteostasis. Endogenous and exogenous stressor lead to unfolding of proteins. These unfolded proteins are either refolded using chaperones, such as heat-shock proteins (HSP) or degraded via autophagy or the proteasome. Failure of this degradation or refolding results in protein aggregation, resulting in proteotoxic effect. Figure is adapted from López-Otín (2013).

Dysregulated cell metabolism

Proteostasis is partly regulated by cell metabolism, since mTOR activation inhibits autophagy. The mechanistic target of rapamycin or mTOR is part of an important pathway for cell metabolism and nutrient sensing; the insulin and insulin-like growth factor 1 (IGF-1) signaling pathway (IIS pathway). The IIS pathway is activated via the growth hormones (GH) and signals via phosphoinositide 3-kinase (PI3K), an enzyme involved in many cellular processes. PI3K activates the serine/threonine-specific protein kinase Akt, an inhibitor of the FOXO transcription factors (Figure 3)[11]. FOXO transcription factors activate various cellular pathways, such as DNA repair, glucose metabolism and cell cycle arrest. Interestingly, GH and IGF-1 levels are reduced during ageing[2]. In addition, the IIS pathway influences mTOR, which regulates autophagy and protein synthesis, hence expresses a central role in proteostasis. Growth factors can inhibit mTOR, as well as a shortage on ATP energy levels, whereas amino acids will activate mTOR. In mouse hypothalamic neurons, mTOR activity is increased during ageing, hence contributing to age-related obesity[12].

Both the IIS pathway and the mTOR pathway have been implicated to be accelerators of ageing[2]. In various model organisms the inhibition of the IIS or mTOR pathway, by either inhibiting one of the components or by caloric restriction, can extend life span. Treatment with rapamycin, an inhibitor of the mTOR pathway is considered the most robust chemical intervention to extend lifespan in many species including mammals[13].

In short, the IIS and mTOR metabolic pathway, is dysregulated during ageing. Inhibition of this pathway, by either chemical intervention or caloric restriction impacts lifespan in diverse organisms. mTOR takes part in the inhibition of autophagy and in protein synthesis, hence expresses a central role in protein homeostasis.

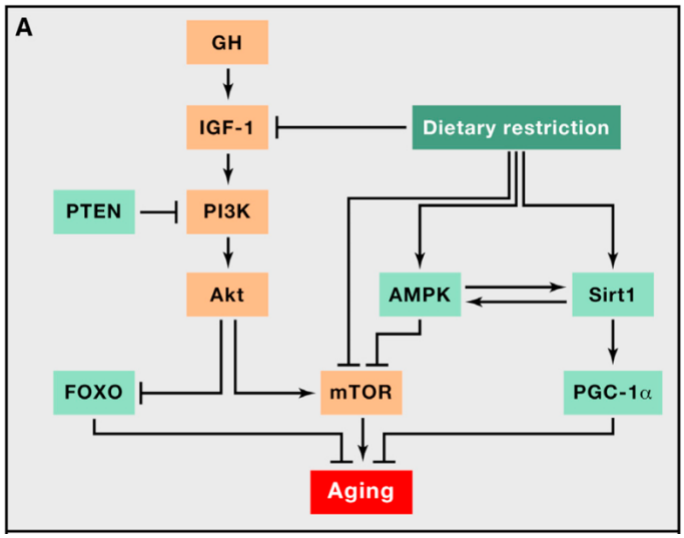

Figure 3. IGF-1 signaling pathway and ageing. Overview of the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway and its relation between dietary restriction and aging. Molecules that are pro-aging are shown in orange, and anti-ageing molecules are shown in light green. Figure adapted from López-Otín *et al.* (2013).

What is the potential studying of age- or stress resilient species in ageing research?

When we look around in nature, we see differences in the rate of ageing and in the maximum age of animals. Often, we look at lifespan differences between species, but what is the definition of a lifespan? According to the Cambridge Dictionary lifespan is characterized as '*the length of time for which a person, animal, or thing exists*'. In research, the term maximum life span (MLS) is often used as a measurement to compare lifespan between species. The MLS is highly variable between species; the mayfly only lives one day[14], while the bowhead whale that can live up to 200 years[15]. For humans the MLS is approximately 120 years, with a longevity record of 122 years by Jeanne Calment of France[16]. Most rodents live around 3-4 years, however there is one special exception: the naked mole rat (NMR). The NMR is great example of a species that is extremely long-living and resistant to ageing. Many of their anti-aging mechanisms are related to protein homeostasis. These mechanisms are something we might want to adapt for increasing proteome stability in humans during aging. In addition to the NMR, thermophiles have several adaptions that make them more resilient against high temperatures. They have differences in their amino acid sequences and protein binding properties, which increases protein stability[17,18]. Hence, the thermophiles may serve as another example of species that found a solution to protein instability and they are extremely resilient to harsh environments. However, the absolute champion in stress resilience is the tardigrade. This organism shows extreme genome stability and can withstand an environments as harsh as outer space[19]. Recently, a tardigrade specific gene (Dsup; discussed in more detail below) was discovered, that could increase radiation resistance in human cells.

The fact that organisms exist that are extreme stress resilient and natural long living, like the NMR and tardigrade raises questions about the molecular mechanisms behind this. Are there more genes like the tardigrade Dsup, that could increase lifespan and may be considered a 'supergene'? Or are there other mechanisms that can improve metabolism or proteostasis in humans? There is a huge variance in lifespan between species, which provides opportunities for research to discover new mechanisms that underlie ageing and the potential to increase lifespan.

When looking at different species and adaptions they have evolved, it makes sense to look into evolution in a bit more detail. Are there evolutionary arguments to support the development of

interventions that impact the rate of ageing? First an argument in favor will be discussed, followed by a counter argument with two examples.

Every organism is adapted to their specific environment. Humans do not need to survive at extreme cold or warm environments and therefore have more instable proteins, than for example the thermophiles. The thermophile proteins need to withstand extreme circumstances, therefore the evolutionary pressure on protein stability is higher is these organisms. However, this does not mean that we cannot adapt some tricks to increase human protein stability. In fact, it provides opportunities for research to study adaptions in other species to increase human health and/or lifespan.

However, evolution is full of trait-offs. This means that one adaption might not be as effective in one organism as it is in the other. One example of this is the addition of telomerase. During our lifetime the telomeres, the protective regions at the end of chromosomes, will shorten, resulting in cell cycle arrest. The enzyme telomerase increases the length of telomeres. In humans this enzyme is only expressed early in life, whereas most short-lived rodents express it their whole life. Researchers thought adding telomerase could prevent cell cycle arrest in humans and thereby extending lifespan. However, it turned out that telomere attrition has evolved to prevent cells from uncontrollable proliferation, in long-lived species. The addition of telomerase led to the formation of tumors in mammals[20]. Another example is rapamycin, an inhibitor of the mTOR pathway, leads to increased lifespan in mammals. However, rapamycin is also an immune suppressor and chronic treatment with this results in a less effective immune system[21]. Moreover, studies in healthy animals have shown that treatment with rapamycin disrupts glucose homeostasis and increases the incidence of type 2 diabetes[22].

In short, evolution shows that every organism is adapted to their specific environment, therefore species in harsh environments have increased selection pressure on e.g. protein stability. This provides opportunities for research to study adaptions in other species to increase human protein stability. However, evolutionary trait-off teach us that not every adaption is effective in humans and can even be detrimental.

Value and drawbacks of traditional model organisms in ageing research

Ageing is a complex and multifactorial process. It takes multiple hallmarks to describe our current understanding of the molecular mechanisms underlying age-related decline and we are far from solutions to extend lifespan or cure age-related pathologies. Currently, the molecular mechanisms are mostly studied in traditional laboratory model organisms such as mice (*Mus*

musculus), baker's yeast (*Saccharomyces Exiguus*), worm (*Caenorhabditis elegans)* and flies (*Drosophila melanogaster)*. Although studying ageing mechanisms in these model organisms has taught us a lot about ageing and age-related pathologies, there are some arguments why they may be problematic. Firstly, there are some translational difficulties. Most of these organisms are short living, which is convenient for ageing research, however might not be representative for human ageing. Furthermore, there is often a lack in genetic variability in laboratory animals, due to inbred strains. This could provide translational problem, since humans express huge genetic diversity. Moreover, model organisms are not humans and are often less complex. Therefore, molecular mechanisms might differ between the organism and the human situation. Although, researches try to mimic human ageing in these models, translation can be tricky. A good example of this is the telomerase knock-out mouse. Humans and larger animals stop expressing telomerase at some point in life. However, small rodents, such as mice, continuously express telomerase during their lifespan. The natural expression profile of telomerase differs between the species, therefore completely different molecular mechanisms could be involved.

Secondly, the traditional model organisms are often kept in a laboratory environment, unrepresentative for human ageing (e.g. constant temperature, food availability, pathogen free). A study found that the lifespan advantage of the long-living mutants from traditional model organisms (yeast, mice, nematode and fruit fly) was diminished in more challenging conditions (Figure 4)[35]. This suggests that information on ageing mechanisms obtained from these traditional models might only apply in very specific environmental conditions. The discovered

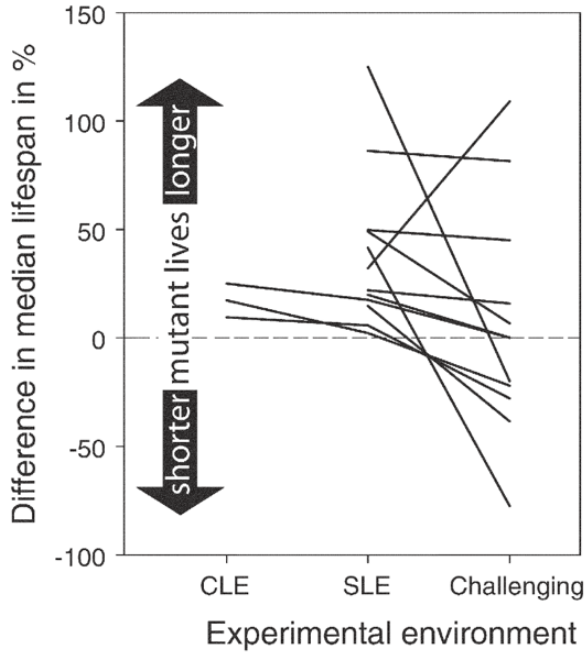

Figure 4. Environmental effect on lifespan of long-lived mutants. Lines connect environmental manipulations carried out within one study. CLE: cafeteria style laboratory environment, SLE: standardized laboratory environment, and challenging: environment was made more challenging in various ways. Figure adapted from Briga & Verhulst (2015).

life-extending genes might not extend life in more natural environments. How does this relate to humans and human ageing research? Do humans in de Western world relate more to animals living in the wild or laboratory held organisms? This is an essential question, since environment appears to affect the effects of longevity genes. In my opinion, humans are somewhere in between. We do not live completely in the wild, with our healthcare systems, solid houses and food availably. However, we are exposed to pathogens and changes in environments, such as natural diseases. Therefore, results of studies in the traditional model organisms in laboratory conditions might not be fully translatable to the human situation. This does not mean that traditional models are useless. Much of present-day medicine is inspired by studying these traditional model organisms and they have proven they value also in ageing research. However, it's wise to keep the translational shortcomings in mind, when studying aspects of human ageing in these models. Furthermore, it opens the door to look further than traditional model organisms and see what value other species can bring us in ageing research.

Which molecular mechanisms underlie age resilience in other species?

In this next paragraph the two example species will be discussed. Firstly, protein homeostasis and metabolism in the naked mole rat and secondly the increased genome stability in the tardigrade. An overview of studies elucidating the molecular mechanisms underlying the age- and stress resilience in these species will be given. For each species one key article will be highlighted and these articles are descripted in more detail in appendix 1 and 2.

What molecular changes underlie age resistance in the naked mole rat?

The NMR or *Heterocephalus glaber*, is a small burrowing rodent from the north east of Africa that can live up to 30 years[23,24]. The NMR not only has an extraordinary long lifespan, but it has a long health span since it does not develop cancer or signs of ageing[23]. This makes the NMR an interesting model organism for studying ageing and cancer. How come that these animals basically do not age and can we find molecular causes for this ageing resilience?

In the past years researchers found that the NMR has an increased protein stability[25], increased resistance to oxidative stress[25] and high levels of autophagy[26] which contribute to the longevity of this animal. Moreover, both mTOR and IGF-1, two important molecules in cell metabolism, are downregulated during NMR ageing. Since mTOR inhibits autophagy, its downregulation contributes to increased autophagy[27]. In addition, the protein turnover kinetics, discussed in more detail below, is low compared to shorter lived rodents[28]. Furthermore, the

NMR has evolved an extra tumor suppressive system to prevent uncontrollable proliferation of NMR fibroblasts compared to mouse or human cells. This system is called early contact inhibition and induces cell division arrest when cells come in contact with either each other or with the extracellular matrix[29]. All of the adaptions, except early contact inhibition, relate to protein homeostasis and stability. Therefore, the NMR is taken as an example species with increased proteostasis in this essay.

A recent study of Swovick *et al.* (2018)[28] studied the proteome turnover kinetics of different rodents. The eight chosen rodent species represent a range of organismal properties, such as body mass, metabolic rate, lifespan and evolutionary distance. The authors show that there are significant differences in $k_{degradation}$ distribution (= turnover rate) between the different rodent species, with those of the long live NMR being especially slow. Furthermore, Swovick *et al.* (2018) show a strong negative correlation between lifespan and $k_{degradation}$ (Figure 5). This result indicates that long living species have a slower protein turnover rate compared to shorter living species. The authors imply that these differences are due to variation in the relative activities of the autophagy and UPS pathways between cells from the different species. This suggests that the systematic shifts in protein turnover among species is driven by differences in activities of cellular degradation pathways, rather than differences in protein target sequences. Furthermore, they showed that the NMR has the slowest protein turnover rate of the studied rodents (Figure 5). Suggesting that the NMR evolved a more efficient proteostasis, which might contribute to their longevity. This study is described in more detail in Appendix 1: Slow protein turnover in the NMR.

In summary, the NMR has various mechanisms that contribute to its longevity, mostly involving protein homeostasis. NMRs show increased autophagy, regulated through a downregulation of the mTOR metabolic pathway, resistance to oxidative stress, increased protein stability and a low protein turnover rate.

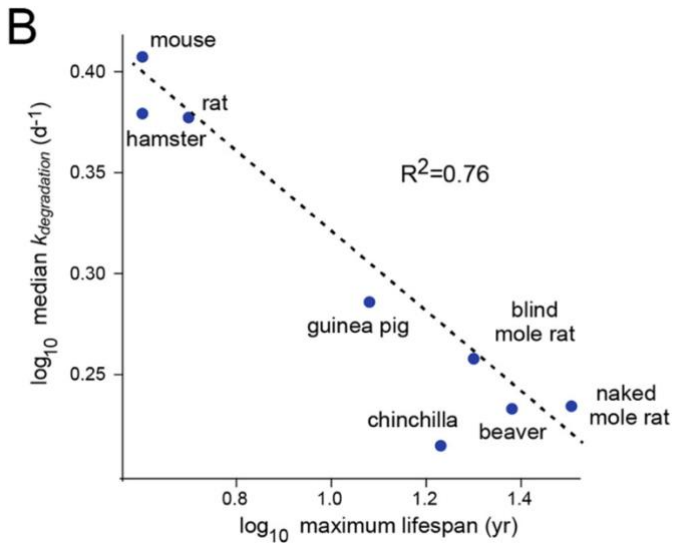

Figure 5. Protein turnover rate negatively correlates with lifespan. Correlation of protein turnover rate (median $k_{degradation}$) with maximal lifespan of rodent species. Figure adapted from Swovick *et al*. (2018).

Why is the tardigrade extremely stress resilient?

As indicated before, the tardigrade is the absolute champion when it comes to stress resilience and is therefore a valuable species to study increased genome stability. What can we learn from the metabolism and genome stability of this species that may help us improve stress resilience (and hence ageing) in humans? What makes that these organisms are so well adapted to a stressful environment?

The tardigrade, or water bear, is a tiny aquatic animal with four pairs of legs[30]. All tardigrades need require surrounding water to grow and reproduce, however some species have the ability to handle near complete dehydration. These tardigrade species can adopt a metabolically dehydrated active state in which they are radiation resistant. Moreover, they can endure temperatures between -273 ºC until 100 ºC[31,32], high hydrostatic pressure[33] and they can survive in outer space for 10 days[30].

A recent study by Hashimoto *et al.* (2016)[34] studied the underlying molecular mechanisms of stress resilience in the tardigrades (*R. varieornatus*) and showed four main distinct adaptions (Study in more detail in Appendix 2 - Lessons from the tardigrade in stress resilience). Firstly, tardigrades have more copies of SOD and MERII genes, which are involved in the clearance of ROS and repair of double strand DNA breaks, respectively. This suggests that the tardigrade has more resources to prevent DNA damage and to repair DNA damage. Secondly, the tardigrade genome shows a significant gene loss in the β-oxidation peroxisomal pathway. This is a major catabolic pathway of fatty acids. Lack of this pathway could result in less production of hydrogen peroxide, one of the major oxidative stressors. This suggests that

the tardigrade might have additional strategies to adapt to oxidative stress compared to humans. Thirdly, the authors show that the tardigrade lacks signaling component to connect various stressors, like oxidative stress, genotoxic stress and hypoxia, suggesting that tardigrades are less sensitive to detecting stressors. Moreover, the tardigrades are missing inhibitors of the mTOR pathway, therefore decreasing autophagy activity. The authors speculate with this reduced autophagy, the tardigrade avoids excessive destruction of cell components to reuse them after rehydration. Furthermore, Hashimoto *et al.* identified a new tardigrade-unique DNA-associated protein, named Dsup. Dsup is a highly basic protein and has physical affinity for DNA *in vitro*. The C-terminal of Dsup is responsible for the co-localization of Dsup with nuclear DNA. Most interesting, when transfecting human HEK293 cells with Dsup, they are more resistant to X-ray-induced DNA damage (Figure 6; Supplementary Figure 2; Supplementary Figure 3). This is a meaningful finding, since it suggests that the molecular adaptions in other species, can be implemented in human cells and even more importantly has protective effect. This implies that it could indeed be possible use these 'supergenes' to increase human genome stability and maybe also other processes, like proteostasis or cell metabolism. The potential of implementing such 'supergenes' in humans is discussed more detailed in the next paragraph.

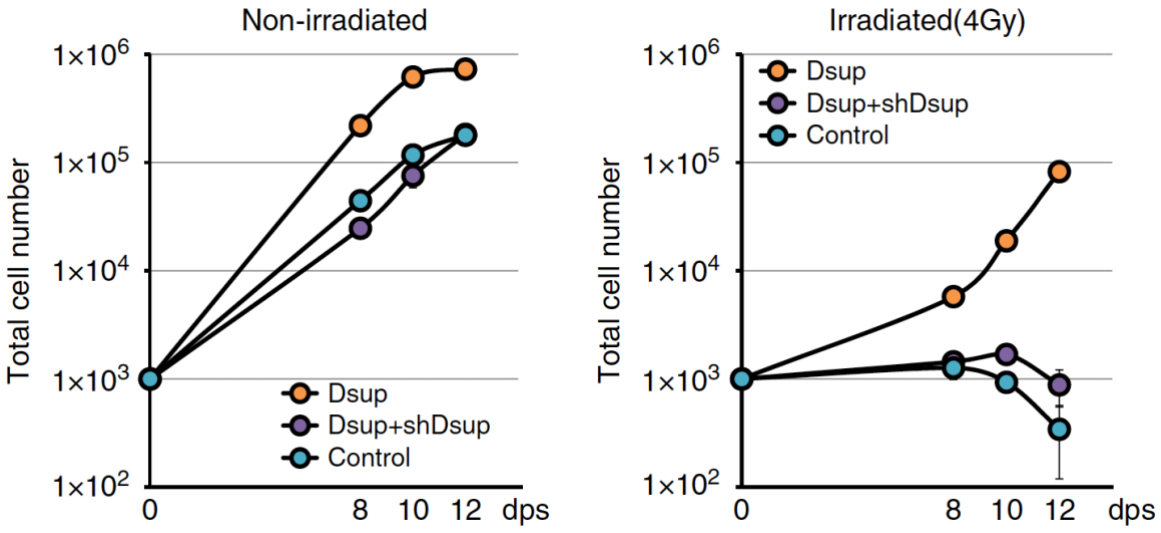

Figure 6. Effect of Dsup on radiation tolerance of HEK293 cells. Comparison of growth curves of untransfected cells (Control), Dsup-expressing cells (Dsup) and Dsup-knockdown cells (Dsup+shDsup) in non-irradiated and irradiated conditions. Values represent mean ± sd. Figure adapted from Hashimoto *et al.* (2016).

How could studying age-resilient species increase human health and lifespan?

There is a whole world of species with adaptions to extreme environments and variabilities in lifespan. This creates a gigantic pool of potential in variables in coping strategies against ageing and age-related pathologies. With the current advanced molecular techniques, it has become easier to sequence and compare new genomes. This makes it possible to study the genetics of more species, to search for adaptations or 'supergenes', like the tardigrade specific Dsup gene. We could not only have a look at eukaryotes but have a greater look at the full width of the evolutionary tree. There might be lessons or mechanisms we can adapt from bacteria or plants to extend lifespan.

By studying the molecular mechanisms underlying stress resilience and longevity in species like the tardigrade and NMR could be implemented in human ageing research in various ways. Potentially, we could use gene therapy to administer genes with a protective role or 'supergenes', such as Dsup. Additionally, drugs could be developed to mimic the effect of molecular processes or genes found in these species. Furthermore, cells could be genetically engineered with the DNA from these animals to produce therapeutic molecules or provide treatments (e.g. stem cells treatment)[36].

Of course, before this becomes reality some ethical and practical issues need to be resolved. There are ethical considerations, whether we should genetically manipulate humans. There are risks bound to therapies. Rapamycin, for example, is the most robust chemical life-extending molecule in mammals, however chronic treatments could lead to immunodeficiency and dysregulation of the metabolic system. In addition to the risks, there are some practical issues. For example, some of the longest-living species, such as the bowhead whale, are huge and very difficult to keep in a laboratory or experiment on. However, with the modern techniques, previously discussed, we don't need to breed all animals in the lab to study their genome. As long as a sample from a species is present, we can uncover significant detail about the genetic background and differences in genomes related to ageing. Finally, there are many factors influencing ageing and the question remains whether a single therapy or gene would have a significant effect on an organism so complex as a human being. Looking at the tardigrade and NMR, they have adopted different, and sometimes controversial methods to increase protein stability. An example of this is the autophagy process, which is enhanced in the NMR by downregulation of mTOR, but decreased in the tardigrade, by the absence of mTOR inhibitors. This suggest that there might not be a 'one shoe fits all'-principle in place,

but it does show us that there are various ways to increase protein stability, hence potential for implication in humans. Moreover, the fact that the tardigrade specific gene Dsup has such a positive effect on human cells, raises hope that these strategies indeed could contribute to the extension of human lifespan.

Where to look next?

Where could we look next? The given examples mainly involved genome and protein stability. However, in both examples the cell metabolism seems to be important. There are some interesting candidates that show increased metabolism or can survive without nutrition for long period of time. Snakes, for example, can reduce their metabolic rate up to 70% and survive for months without any food[37]. Moreover, the West-African lungfish can go three to four years without food intake[38]. When food is scarce it buries itself in the mud and goes into hibernation-like state until more favorable conditions present themselves[38]. In general, hibernating animals are interesting to study metabolic mechanism. All these species can spend a long period of time without nutrient consumption. A stunning example are bears that wake up and just runs out of the cave, full of energy and without any muscle wasting[39,40]. This would be impossible in humans, where muscles work via the principle 'use it or lose it'. What makes that the muscles of these bears remain completely preserved and functional even though they don't use them for months?

Moreover, there are some organisms thought to be immortal because of their tremendous regenerative capabilities. The *Hydra* and the planarian flatworm are examples of such immortal, hyper regenerative species. Even from a tiny piece of the flatworm, it can regenerate itself completely. With the modern techniques it has become easier to take samples from these species and find out differences in the genome related to cell metabolism, regeneration and ageing.

"Perhaps nature is our best assurance of immortality" (Elenore Roosevelt, 1945). This quote describes the potential of other species in nature to help increase human lifespan perfectly. Ageing is multifactorial and very complex and long-living species have many adaptions that contribute to their longevity and stress resilience. Therefore, we could learn a lot from studying the underlying molecular mechanisms of the huge variance nature has to offer. The example of the Dsup gene in the tardigrade shows us that one single gene can already have a significant positive effect. Even if we might not be able to extend human lifespan, we

might be able to use adaptations from these species to develop treatments for the age-related pathologies and extend human health span.

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Appendix 1: Slow protein turnover in the NMR

Introduction

The NMR is the longest living rodent and studies suggest that its longevity is caused by protein stability, high autophagy and resistance to oxidative stress[25,26]. Swovick *et al.*[28] conducted a study this year, in which they compared the proteome turnover kinetics of different rodent species. Protein turnover is important to ensure damaged proteins are replaced by functional proteins and contributes to the regulation of protein expression levels[41]. In this study Swovick *et al.* (2018) globally quantified protein turnover kinetics in the primary dermal fibroblast of eight different rodent species (Table 1), using dynamic isotope labeling and quantitative proteomics. The eight chosen rodent species represent a range of organismal properties, such as body mass, metabolic rate, lifespan and evolutionary distance. The rodents belong to three distinct sub orders of the *Rodentia*, namely *Myomorpha*, *Castorimorpha* and *Hystricomorpha* and are separated by ±73 million years of evolution.

Rodents analyzed in this study. The lifespan and body mass data were obtained from AnAge database (45)

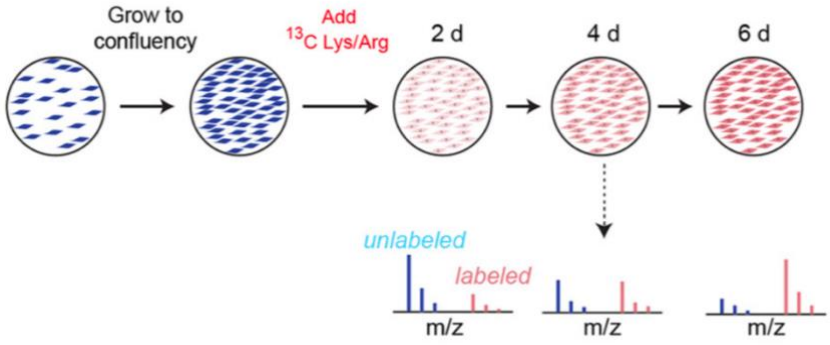
		adult body mass (g)	maximum lifespan (yr)
Myomorpha	mouse *(Mus musculus)*	30	4
	rat *(Rattus norvegicus)*	400	5
	hamster *(Mesocricetus auratus)*	105	4
	blind mole rat *(Nannospalax ehrenbergi)*	160	20
Castorimorpha	beaver *(Castor canadensis)*	20,000	24
Hystricomorpha	chinchilla *(Chincilla lanigera)*	642	17
	guinea pig *(Cavia porcellus)*	728	12
	naked mole rat *(Hetercephalus glaber)*	35	32

Table 1. Overview of rodents used in the study. Suborder, name, adult body mass and maximum lifespan of rodent species analyzed in the study by Swovick *et al.* (2018).

Methods

The different rodent cell lines were cultured until 100% confluence, with exception of the NMR fibroblast. These cells reached only 70% confluence because of a phenomenon called early contact inhibition. This phenomenon is a topic of interest in cancer research[29]. After the cells reached confluency, they were kept in quiescent state for 4 four days before isotopic labeling with ^{13}C Lysine/Arginine for various timepoints (0, 2, 4, 6 days). After labeling the m/z values were measured using Mass Spectrometry (Supplementary Figure 1). Degradation rates ($k_{degradation}$) were determined using a previous validated kinetic model[42–44], following three assumptions. Firstly, protein synthesis is a zero-order process with respect to protein concentration. Secondly, protein degradation occurs are a constant fractional rate that is consistent for the whole protein pool. And thirdly, the total protein concentration of each

individual cell does not change during the experiment and the system is at a steady-state condition. Based on these assumptions a single exponential equation was composed. The MS/MS data of mouse, rat, hamster, guinea pig and NMR were compared to specie-specific uniprot databases, since these species have a well-annotated proteome. The other species, blind-mole rat, beaver and chinchilla were compared to mouse uniprot databases.



Supplementary Figure 1. Experimental design of Swovick *et al.* (2018)

Results

The authors show that there are significant differences in $k_{degradation}$ distribution (= turnover rate) between the different rodent species and propose two general mechanisms to explain inter-species variability in protein turnover kinetics. Firstly, during the course of evolution, protein targets might have diverged in sequence and thereby altered their relative susceptibility to cellular degradation pathways. Secondly, the relative activities of the autophagy and UPS pathways might vary among cells from different species. However, they found that the inter-species variation is also evident in fully conserved proteins, implying that the second explanation is more viable. This suggests that the systematic shifts in protein turnover among species is driven by differences in activities of cellular degradation pathways, rather than differences in protein target sequences. Furthermore, Swovick *et al*. (2018) show a strong negative correlation between lifespan and $k_{degradation}$. This result indicates that long living species have a slower protein turnover rate compared to shorter living species (Figure 5).

Discussion

This study was the first study to use one cell type to compare different species, which is one of its strengths. However, dermal fibroblast are cells that consistently proliferate[6] in their natural situation. During this study they are kept in an unnatural quiescent state before isotopic labeling. This raises the question whether the data is representative for the situation in a living

organism. The authors chose this quiescent state for cells to ensure that cellular proliferation was not contributing to the kinetics of isotopic labeling and to enable the analysis of turnover kinetics of protein is with longer half-lives than the rate of cellular proliferation.

Appendix 2 - Lessons from the tardigrade in stress resilience

Introduction

When looking for organisms with a resistance to extreme environmental conditions, the tardigrade is a great example. This organism can adopt a state of dehydration in which it is resistant to extreme temperatures, both low and high, resistant to radiation, hydrostolic pressure and can survive in outer space for ten days[30]. Recently, Hashimoto *et al.* (2016)[34] conducted experiments to unravel the molecular mechanisms behind this resilience of the tardigrade.

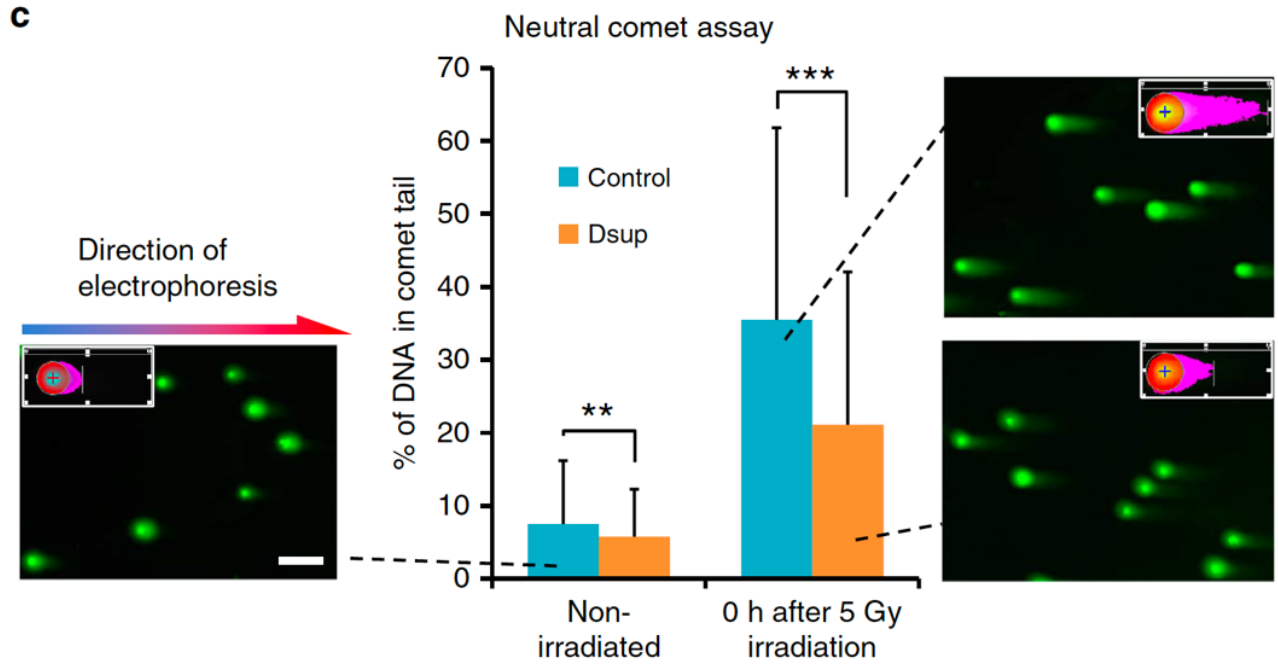
Methods

In this study the authors used high-quality genome sequencing techniques to precisely analyze the gene repertoire, such as the proportion of horizonal gene transfer (HGT), and characteristic gene expansion or deletion of *R. varieornatus*, an extremotolerant tardigrade species, in dehydrated state. They performed BLAST search against the non-redundant database of National Centre for Biotechnology Information to evaluate HGT, the non-sexual transfer of genetic material between genomes[45]. Furthermore, Hashimoto *et al*. analyzed gene expression profiles during dehydration and rehydration and focused on abundantly expressed tardigrade-unique genes. Furthermore, they assessed the effects of Dsub, a tardigrade specific gene on SSB using the alkaline comet assay. The effect of DSB was examined using the neutral comet assay. A comet assay or single cell gen electrophoresis is a technique to quantify and analyze DNA damage in individual cells. DNA unwinds under either alkaline or neutral conditions, after which it undergoes electrophoresis. This allows broken DNA fragments to migrate away from the nucleus, which can be made visible with a fluorescent dye. The extent of DNA moving away from the nucleus is proportional to the amount of DNA damage[46]. In this study they irradiated HEK293 transfected with Dsup with 5 or 10 Gy radiation. Moreover, they quantified the γ-H2AX foci per nucleus. γ-H2AX is a validated biomarker for double strand breaks[47].

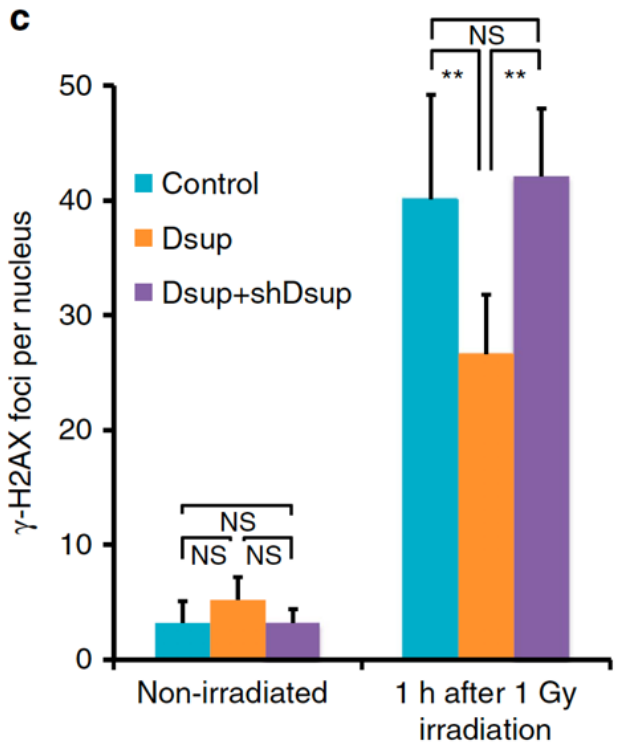
Results

Firstly, this study showed no extensive HGT in this tardigrade species. Secondly, in comparison with other metazoans the tardigrade has a characteristic expansion of both superoxide dismutase (SOD) genes and MRE11 genes. Most multicellular organisms express less than ten SODs, however in the tardigrade sixteen SODs were found. SODs are important for the detoxification of superoxide radicals, a specific type of ROS[48]. Additionally, the tardigrade contained four MRE11 genes, whereas most metazoans only contain one gene.

MRE11 is an important player in the repairment of DNA DSBs[49]. Suggesting that the tardigrade has more resources to prevent DNA damage and to repair DNA damage. Thirdly, the tardigrade genome shows a significant gene loss in the β-oxidation peroxisomal pathway. This is a major catabolic pathway of fatty acids. This could result in less production of hydrogen peroxide, an oxidative stressor, suggesting that the tardigrade has different methods to deal with oxidative stress. Fourthly, a selective loss of stress-responsive signaling pathways was observed. Showing that the tardigrade lacks signaling component to connect various stressors, like oxidative stress, genotoxic stress and hypoxia, to the downregulation of the mTOR pathway. Furthermore, some tardigrade-unique genes were abundantly expressed, including previous identified heat-soluble proteins, CAHS and SAHS. These genes are suggested to be involved in the protection of biomolecules during desiccation[50] and therefore involved in the tolerability to extreme environments of the tardigrade. Moreover, Hashimoto *et al.* identified a new tardigrade-unique DNA-associated protein, named Dsup. Dsup is a highly basic protein and has physical affinity for DNA *in vitro*. The C-terminal of Dsup is responsible for the co-localization of Dsup with nuclear DNA. Most interesting, when transfecting human HEK293 cells with Dsup, they are more resistant to X-ray-induced DNA damage (Figure 6; Supplementary Figure 2; Supplementary Figure 3).



Supplementary Figure 2. The effects of Dsup on DSBs by X-ray irradiation (5 Gy) in a neutral comet assay. Three hundred comets were analyzed for each condition. ** P > 0.01 and *** P > 0.001 (Welch's t-test). Values represent mean ± sd; scale bars = 100 mm.



Supplementary Figure 3. Quantitative comparison of γ-H2AX foci number among untransfected HEK293 cells (Control), Dsup-expressing cells (Dsup) and Dsup-knockdown cells (Dsup+shDsup) under non-irradiated and X-ray irradiated (1 Gy) conditions. At least 70 cells were analysed for each condition. Values represent mean ± sd; ** P > 0.01; NS = not significant (Tukey–Kramer's test).

Discussion

The results from this study seem very promising. The effect of Dsup on the radiation tolerance of HEK293 is miraculous. However, is the comet assay the best way to assess DSB and DNA damage? Are there other methods we can use to determine this, such as single cell sequencing?