FACULTY OF HEALTH AND MEDICAL SCIENCES
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An analysis of DNA repair mechanisms and mitochondrial function in accelerated aging disorders

Ph.D. thesis

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Preface & acknowledgements

This thesis is submitted as part of the Ph.D. program at University of Copenhagen. Along with coursework comprising 30 ECTS (half of one academic year), the research presented herein is the product of three years as a Ph.D. student, starting January 2010, where I have worked partly at the Center for Healthy Aging, University of Copenhagen, and partly at the Laboratory of Molecular Gerontology, National Institute on Aging, NIH. In this period I was supervised by Professor Lene Juel Rasmussen and Doctor Professor Vilhelm A. Bohr, and have been attached to the "Molecular Mechanisms of Disease" research school at the University of Copenhagen.

First and foremost, I would like to thank my advisors Vilhelm and Lene for giving me the opportunity to spend three years doing research on a topic that I would no doubt have pursued regardless. I am fairly confident that both my enjoyment and scientific output has been significantly higher than if I had been tackling the problem of aging in my back yard, and receiving a salary throughout this period can only be described as a luxury.
Though not my advisor in any real capacity, I feel fortunate that Mansour Akbari arrived at the Copenhagen lab not long after I did. Aside from always providing useful criticism, he has instilled in me both an admiration for doing science with great care and rigor, and a confidence that anyone who is astute and enthusiastic will always be in demand.
I have many things for which to thank Morten Scheibye-Knudsen: for teaching me how to dissect a mouse, keep the Seahorse XF24 from misbehaving, and for constantly offering feedback and criticism on papers, presentations and the like. But most of all I want to thank him for injecting a healthy dose of optimism into my scientific life; for encouraging me to pursue the big ideas, and for bouncing said ideas around in an open-minded and stimulating manner.
Ideas are all well and good, but at some point you have to get down to brass tackles. At that stage I have been extremely grateful to Peter Sykora and Tadokoro Takashi, who have always been ready to offer advice and assistance, and to call bullshit on my theories wherever possible.
But of course it's also easy to get caught up in nitty gritty details, and I would like to thank Kristian Moss Bendtsen both for bringing my focus back to the question of aging, and for many a provocative discussion on that topic.
When I started my Ph.D. I was not only ignorant about the biology of aging, but had next to no experience with biochemistry, cytology or indeed anything I've worked on these past three years. Without the help of Guido Keijzers, Tomasz Kulikowicz, Marie Rossi and Deborah Croteau to show me the ropes I would in all likelihood have ended

up in a terrible mess.
Naturally, things only work as they're supposed to half the time (at best). While failing experiments are for me to sort out, I want to thank both Claus Desler and the administrative staff in both Copenhagen and Baltimore for endless assistance with all manner of housekeeping, equipment malfunctions, and paperwork.
In this vein I would also like to thank my auxilliary supervisor Simon Bekker-Jensen, who has been a tremendous resource whenever I had concerns about my research and for keeping me on track throughout the Ph.D.
My parents have been indispensable in keeping me mentally sound through these three years, not to mention for getting me here in the first place. I say it every so often, but hardly often enough: I love you.
In a similar vein, I would not likely have made it through the homestretch of the Ph.D. without the support of my close friends Jerôme Baltzersen and Sisse Bøggild. Much gratitude is owed them for things not having to come crashing down at the very end!
I would be most remiss not to thank my many collaborators, who quite literally have made this thesis possible, as well as members of both the Copenhagen and Baltimore labs for making the research atmosphere so much more enjoyable. Finally, I am grateful to have received an Elite Research travel grant from the Danish Ministry of Science, which has allowed me to conduct a significant amount of research at the National Institute on Aging in Baltimore.

Abstract

The biology of aging is perhaps more challenging than the majority of scientific fields because the subject of research is so nebulous. Many theories and opinions exist on what exactly constitutes aging, and certainly on what the cause of it might be. One of the most prominent doctrines, embodied by the DNA Damage and Mitochondrial theories of aging, is that a major factor contributing to age-related decline is the accumulation of damage in our DNA which in turn interferes with cellular function.
Guided by this theoretical framework, I have worked to further our understanding of how basic cellular processes play into the accumulation of DNA damage. I have focused my study on proteins thought to be involved in repair and maintenance of DNA, as well as on mitochondria as a possible source of DNA damage. As a model system for how these processes function in aging I have mainly studied human segmental progerias, whose symptoms of accelerated aging provide some guidance as to which processes are most relevant for understanding the progressive dysfunction of the organism.
The RecQ family of helicases is particularly interesting in this regard, as mutations in three of its five human members are causative of such diseases. By investigating both the biochemistry and trafficking of these proteins I have provided additional information bearing on their roles in the cell: by implicating the non-essential helicase domain of RECQL4 in its associated diseases, I provide support for the idea of a secondary cellular role for this protein. Further, by describing the dynamics of the WRN and BLM helicases in different part of the nucleus it becomes clear that these proteins are engaged in constant scanning of DNA, and that their recruitment to DNA damage is dependent on the gradual creation of binding sites surrounding the site of damage.
Studying the physiology and gene expression profiles of cells lacking the two proteins implicated in Cockayne syndrome revealed a common pathway involving dysfunctional transcription. Through overstimulation of PARP signalling, this transcriptional defect likely ties in with mitochondrial function as cellular levels of ATP are depleted. Although the cells compensate with increased mitochondrial activity, this response may be insufficient to prevent cell death of neuronal cells in particular. This mechanism of pathogenesis provides a good fit for the symptoms of Cockayne syndrome, and could consolidate existing models for the disease's origin.
Combined with correlational work on the involvement of mitochondria in age-related decline, these findings emphasize aging as the result of concurrent dysfunction in multiple interconnected bodily systems. While a tremendous amount of further work will be needed to fully comprehend such a network of interactions, I hope that the results presented here constitute some progress on this topic.

Resumé

Biologien bag aldring kunne forestilles at udgøre en større udfordring end mange andre forskningsområder, i kraft af at selve emnet er så vagt. Der findes et hav af teorier og holdninger til hvad der præcist udgør aldring, og især hvad årsagen kunne være. En af de mest fremtrædende paradigmer, fx repræsenteret ved den DNA-skade baserede og den mitokondrielle aldringsteori, er at en hovedårsag til den aldersrelaterede svækkelse er ophobningen af skade i vores DNA, som hæmmer cellens funktioner.
Baseret på dette teoretiske fundament har jeg arbejdet på at forbedre vores forståelse af hvordan grundlæggende cellulære processer spiller ind i ophobningen af DNA skade. Jeg har fokuseret mine studier på proteiner der menes at være involveret i reparation og vedligeholdelse af DNA, samt på mitokondrier som en mulig kilde til DNA skade. Som modelsystem for hvordan disse processer fungerer i forhold til aldring har jeg primært beskæftiget mig med menneskelige sygdomme der involverer aspekter af fremskyndet aldring. Disse aldringsrelaterede symptomer kan hjælpe med at identificere hvilke cellulære processer der er mest relevante at studere med henblik på at forstå den gradvise kropslige funktionsforstyrrelse der sker med alderen.
Familien af RecQ helikaser er særskilt interessant i dette henseende, da mutationer i tre af dens fem humane medlemmer er årsag til sådanne sygdomme. Ved at undersøge både disse helikasers biokemi og bevægelser i cellen har jeg skabt ny viden vedrørende deres cellulære roller: ved at implikere helikase domænet af RECQL4 i dets tilknyttede sygdomme fremfører jeg yderligere opbakning for ideen om en sekundær rolle for dette protein. Ved at beskrive helikaserne WRN og BLMs bevægelser i forskellige dele af cellekernen bliver det klart at disse proteiner vedvarende skanner DNA, og at deres rekruttering til DNA skade er afhængig af at der gradvist dannes bindingssæder omkring skaden.
Ved at studere fysiologi og genudtrykkelse i celler der mangler de to proteiner der er involveret i Cockaynes syndrom, klarlægges fælles træk som for eksempel fejlagtig transkription. Denne defekt i transkription er koblet sammen med mitokondriel funktion, nok gennem overstimulering af PARP signalering hvorved cellen løber tør for ATP. Selvom cellerne kompenserer ved at øge deres mitokondrielle aktivitet er dette respons muligvis utilstrækkeligt til at forhindre celledød i især neuroner. Denne mekanisme for patogenese er i overensstemmelse med sygdommens symptomer og kan måske samle eksisterende modeller for sygdommens årsag.
Kombineret med arbejde der sammenholder mitokondriel funktion med aldersrelateret svækkelse fremhæver disse resultater aldring som produktet af sideløbende fejlprocesser i flere sammenkoblede kropslige systemer. Omend det vil kræve en enorm mængde arbejde før vi fuldt ud forstår et sådant netværk af interaktioner, håber jeg at de konklusioner der præsenteres her udgør et vist fremskridt inden for dette område.

Contents

Abbreviations

8-oxoG	7,8-dihydro-8-oxoguanine
AIF	Apoptosis-inducing factor
APE1	Apurinic/apyrimidinic endonuclease 1
ATR	Ataxia telangiectasia and Rad3-related protein
BER	Base excision repair
BGS	Baller-Gerold Syndrome
BLM	Bloom syndrome protein
CEN2	Centrin-2
CR	Caloric restriction
CRL4	Cullin4A-RING E3 ubiquitin ligase
CS	Cockayne syndrome
CSA	Cockayne syndrome protein A
CSB	Cockayne syndrome protein B
DDB1/2	DNA-damage-binding-1 and 2
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DSB	Double-strand break
ERCC1	DNA excision repair protein ERCC-1
ETC	Electron transport chain
EXO1	Exonuclease I
FEN1	Flap endonuclease 1
FIS1	Mitochondrial fission 1 protein
FRAP	Fluorescence recovery after photobleaching
IGF-1	Insulin growth-factor 1
LC3	Microtubule-associated proteins 1A/1B light chain 3A
MCM	DNA replication licensing factor MCM
MMR	Mismatch repair
MnSOD	Manganese superoxide dismutase
MRE11	Double-strand break repair protein MRE11
MSH	MutS homolog
mtDNA	Mitochondrial DNA
NAD^+	Nicotinamide adenine dinucleotide
NBS1	Nibrin
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
OGG1	8-oxoguanine DNA glycosylase

PAR	Poly(ADP-ribose)
PARP-1	Poly(ADP-ribose) polymerase 1
PCNA	Proliferating cell nuclear antigen
PGC-1	PPARγ co-activator-1
PINK1	PTEN-induced putative kinase 1
RAD50	DNA repair protein RAD50
RFC	Replication factor C
RPA	Replication protein A
RTS	Rothmund-Thomson Syndrome
ROS	Reactive oxygen species
Sld2	DNA replication regulator SLD2
TC-NER	Transcription-coupled nucleotide excision repair
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TFIIH	Transcription factor II H
TFIIS	RNA polymerase II elongation factor
TOR	Target of rapamycin
UCP2	Mitochondrial uncoupling protein 2
UNG	Uracil DNA glycosylase
WRN	Werner syndrome ATP-dependent helicase
WS	Werner syndrome
XP	Xeroderma pigmentosum
XPA	Xeroderma pigmentosum complementation group A
XPC	Xeroderma pigmentosum complementation group C
XPG	Xeroderma pigmentosum complementation group G
XRCC1	X-ray repair cross-complementing protein 1

Chapter 1

Introduction

The goal of this thesis has been a contribution to our understanding of the genomic and mitochondrial maintenance functions in the context of aging. The field of biogerontology is not blessed with as clearly defined objectives as say particle physics, and my work has therefore taken an expansive rather than a narrowly focused approach. I have tried to attack the problem of aging on several levels, including detailed characterization of proteins, mechanistic analysis of pathogenesis, and correlative exploration of potential aging factors. Because of this, the experimental platform for this thesis spans purification of recombinant proteins and their use in biochemical assays, miscellaneous scrutiny of overexpressing and knockdown cell lines, as well as work with human tissue samples and mouse models. A central theme has been the study of segmental progerias: human diseases where some (but not all) features of aging manifest at an accelerated rate. Because the symptoms of these diseases so closely resemble the deterioration that occurs in normal aging, one can hope that understanding the cellular processes that malfunction in these diseases, and how they do so, will offer insight into the driving force behind the aging process in general. In addition, a secondary focus has been the mitochondrion, an organelle whose broad role in aging has become increasingly clear over the past few decades.
As a result of these endeavours, I present two first-author publications in Chapter 2 (one accepted and one submitted) and one being prepared for submission in Chapter 3. Additionally, in Chapter 4, I present work contributed to two publications being prepared by other researchers.

This chapter provides, as best possible, an introduction to both the concept and study of aging. In Sections 1.1 and 1.2 I hope to portray the supreme relevance of this field of research, while also presenting some of the historical background that has lead up to the current state of affairs. For the sake of demonstrating the multifarious nature of aging, and also to give a balanced representation of current thinking in the field, Section 1.3 treats a number of theories on both *why* and *how* aging takes place. The end of that section pays special attention to two theories that are especially pertinent to the work presented in this thesis. Section 1.4 introduces the DNA repair pathways that are central to my work, while Section 1.5 presents the aims of this thesis and outlines the considerations behind my choice of projects.

Chapter 2 deals with a highly conserved family of proteins, the RecQ helicases, with

universally important roles in genomic maintenance. Three of the five human helicases have been identified as causative of diseases recognized as segmental progerias. In one of these, at best, do we fully understand what responsibilities these proteins hold in cellular function, and thereby what dysfunctions lead to the aging phenotype. With this in mind, the work presented in said chapter takes a highly detailed view of the RecQ helicases, focusing on interactions and activities at the protein level. In doing so the investigation becomes one step further removed from the study of aging per se, but only in the attempt to clarify the underlying mechanisms. Thus Section 2.1 describes a biochemical characterization of the RECQL4 helicase in the context of disease-causing mutations, while Section 2.2 gives treatment to the intra-nuclear trafficking of the WRN and BLM helicases.

Chapter 3 returns to a broader perspective, in an attempt to uncover the molecular underpinnings of the segmental progeria Cockayne's syndrome. The two proteins implicated in this syndrome are known, but despite some knowledge of their biological functions there is considerable disagreement as to the cause of pathology. Without preconceptions based on symptoms or protein functions we look for potential causes using microarray analysis of knockdown cell lines, which leads us to investigate the mitochondrial phenotype of Cockayne syndrome. Our findings suggest a common role for the two involved proteins in deactivating a signalling response to stalled polymerases in transcription.

Chapter 4 moves completely into the topic of mitochondria as a factor in aging. It is clear that these organelles are involved in age-related decline, but to what extent they are causative (either absolutely or proximally) has yet to be fully understood. Section 4.1 presents work contributed to a larger investigation of mitochondrial function in aging, aimed in particular at establishing whether the mitochondria of older humans produce more reactive oxygen species. Shortened telomeres in mice were recently shown to influence mitochondrial biogenesis, and Section 4.2 constitutes the background and project plan for a study elaborating on this by examining the autophagic degradation of mitochondria during telomeric dysfunction. While this project is not yet complete, initial progress and future plans are presented.

Finally, Chapter 5 summarizes the discussions and contributions presented in this thesis, and bring them into the greater context of understanding the aging process. Some speculation of a more general nature is offered, and future prospectives will be mentioned.

1.1 The inconvenient process of aging

Even though I haven't yet offered a definition of aging, I do not think that any reader of this thesis will feel left behind as I proceed directly to a discussion of the process and its consequences. We have all experienced that, in addition to greater wisdom and conservative political views, advancing years invariably bring about wrinkles, failing muscles, loss of hearing, and more. This decline does not appear to be a law of biology (if any such exist): hydras do not appear to display signs of aging [1], and lobsters continue to grow with minimal signs of aging upon reaching adulthood [2]. The hydrazoan *Turri-*

topsis nutricula can cycle between adult and immature states, possibly without limit [3], while the axolotl salamander matures without undergoing metamorphosis and thus retains the prodigious regenerative capacity seen in the embryonic state of many organisms [4]. Other organisms, such as the naked mole rat, show relatively normal progression of aging symptoms, but at a rate that is drastically lower than otherwise similar species [5, 6]. Nonetheless, senescence (the deteriorative processes of aging) is a fact of life for all humans, and indeed the vast majority of metazoans.

In recent years, this fact of life has repeatedly been highlighted as an imminent societal problem, particularly in the developed world [7, 8]. Outside of Africa, continuously improving treatment and prevention of leading causes of death produce a sustained increase in life expectancy beyond the burst caused by early 20th century advances in public health [9, 10]. Ironically, old age itself is the biggest risk factor for many of these causes: multiple changes in the cardiovascular system occur with aging, e.g. impaired heart rate modulation reducing cardiac output and arterial stiffening increasing blood pressure and the risk of stroke, and combine to make cardiovascular disease the leading cause of death in individuals aged 65 and above [11, 12]. Neurodegeneration, including diseases such as Alzheimer's and Parkinson's, is very strongly correlated with aging [13, 14], to the extent that there is debate as to whether old age directly (and 'naturally') causes neurodegeneration or merely increases the risk of neurodegenerative diseases [15]. Cancer risk also increases with age [16], probably both due to accumulated exposure to carcinogens and to decreased capacity to cope with oncogenic stress [17]. Delaying the onset of these diseases, whether for the sake of individual happiness or to reduce healthcare costs, would therefore appear to inherently produce diminishing returns unless combined with other measures. This is undoubtedly one reason why recent years have brought increased focus on research into the aging process itself.

While the societal burden of an aging population surely weighs heavily on political minds, it is by the individual that the progress of aging is most acutely felt. Whether in the exclusion from the 'young and attractive' club or the lack of recognition in the eyes of an elderly parent, the consequences of aging can *hurt* on a very personal level; the fact that one in four humans get cancer during their lives may be scary, but being diagnosed with it must be terrifying. And, rationalization aside, we all hope to avoid grey hair, senility and incontinence for as long as possible. Most people over the age of 30 have probably claimed to be "not as young as they used to" at one point or another. There is a multi-billion dollar market worldwide for cosmetics that (falsely) claim to reverse aging, as well as supplements claiming to postpone it [18]. In spite of (or perhaps because of) its universality, growing older is frequently on our minds.

At this point, it may be bothering some readers that I still have not offered a definition of aging. I am inclined to agree: it is vexing to study a biological process without an acknowledged definition to form the foundation of such research. This is not to say that no such definitions have been proposed, merely that there is not yet a definition that is universally accepted by gerontologists, or even by biogerontologists. Be that as it may, in the following sections I will attempt to shed some light on the current state of aging research, and on the more commonly accepted beliefs in the field.

1.2 Aging in the Modern Era

September 1951 saw the Second International Gerontological Congress held in St. Louis, which included a panel entitled 'What is Aging?' [19]. Here such pioneers as Vladimir Korenchevsky (71 at the time), Edmund Vincent Cowdry and Nathan W. Shock sat down to discuss that most central question in the nascent field of gerontology. At the time, the biological and physiological understanding of the aging process was distinctly incomplete: a telling query raised during the panel discussion was "is there functional decline of individual cells with increasing age?". Progeria was brought up as an interesting, but ultimately inexplicable, observation. Nonetheless, while the panel members did not presume that any such definition would be the final word on the matter, their intent was seemingly to try and formulate a description based on empirical evidence, in accord with scientific theory.

Gerontology at the time had a strong emphasis on social science, where the dedicated study of aging was relatively straightforward. In contrast, the field's biomedical branch was mainly the realm of medical doctors (including most of the panel members) and consequently focused heavily on the pathologies that develop with advanced age. But, as Dr. Lansing pointed out, there was no reliable way to distinguish between the progression of these diseases and the supposedly underlying process of aging. While such a distinction was of course crucial for classifying biomedical gerontology as a distinct field, it was dependent on some hitherto undiscovered measurable that was specific to the aging process. The matter of enzymatic assays was brought up during the discussion, with the conclusion that these would likely be important in future gerontological research, but by necessity such prospective studies could not factor in attempts to define aging.

In this regard the present situation is rather different: the purely biological study of aging is now a prominent aspect of gerontology, using a variety of model systems to overcome the temporal complications of studying the aging process. These include short-lived and/or genetically modified animal models, cross-sectional and longitudinal human studies, as well as studies on human diseases that display symptoms of accelerated aging. While Hayflick's discovery of limited cell division [20] and/or Kenyon's genetic lifespan-extension in *Caenorhabditis elegans* [21] are sometimes cited as catalysts for the rise of biogerontology, it is perhaps more plausible that the field gradually evolved to address the limitations in gross physiological analysis that were highlighted in the aforementioned panel discussion. In other words the need to answer basic questions on what actually happens to the aging body, coupled with the rapidly expanding toolbox available to biologists towards the end of the 20th century, made biogerontology the natural evolutionary step from the largely phenomenological early study of aging. Whether it crystallized around pioneering researchers or was the inevitable product of scientific progress, it is now a very active field spawning a steadily increasing number of publications in both top scientific journals and in several journals specializing in biogerontology. This is of course a desirable situation for a burgeoning biogerontologist, but an unfortunate side effect of the switch from top-down to bottom-up studies of aging has (perhaps unavoidably) been a certain degree of fragmentation in the theoretical foundations of the field. To put it more concretely, studies on the many biological processes that relate to aging have generated a multitude of theories seeking to explain the overall mechanism; one meta-study identified more than 300 interpretations, some of which were based more on evolutionary ideas about aging than on experimental evidence [22]. One would expect

this to cause some problems of communication between researchers in the field, and the situation is exacerbated by the fact that many of these interpretations do not necessarily invalidate each other. To quote Medvedev: "Many theories co-exist because they do not contradict each other, or because they try to explain different and independent forms of senescence" [22]. Some progress has certainly been made: for instance, it is now more or less accepted that aging results from some form of failure in maintenance and repair mechanisms rather than a preprogrammed biological process [23]. But despite this simplification, the present situation still involves a host of theories begging consolidation, where even such promising headlines as "Aging theories unified" merely refer to a connection between two theories [24].

Thus, the problem of aging is binate: it is inconvenient because it adversely affects us all, causing a loss of physical and cognitive capacity and bringing with it a plethora of other disabilities. It is furthermore inconvenient to the researcher because it involves numerous functions and dysfunctions at both the molecular, cellular and physiological level, and because the final product of all these has been presented to us *a priori* and thus given rise to a number of preconceptions about the process.

1.3 Theories of aging

While greater harmony in the aging field is a noble goal, the introduction of a Ph.D. thesis is hardly the place to set about achieving it. Instead I will present and briefly discuss the most prominent theories of aging, and delve a bit deeper into the discussion of those theories most relevant for the work presented in this thesis.
First off we should distinguish between two different categories of 'theories of aging', which I will refer to as explanatory and mechanistic theories of aging. The former attempt to explain *why* organisms age, and to reconcile this seemingly detrimental phenotype with Darwin's mechanism of evolution. To a certain extent, these theories can be divided into those positing that aging is supposed to happen, and those that present it as a breakdown of proper function. In either case these theories focus on forces directing aging in general, rather than the details of aging in specific organisms, and are thus more often the domain of theoretical biology. The theories I refer to as mechanistic, on the other hand, deal with the *how* of aging. These examine in detail the processes that (professedly) effectuate the physiological decline that comes with aging. The mechanistic theories therefore complement rather than compete with the explanatory theories, and varying degrees of linkage exist between the two groups. For instance, a theory that explains aging as a deliberate genetic program naturally begs synthesis with a theory that the functional decline of tissues occurs as telomeres shorten and limit cellular renewal.
Evidently I won't be able to cover all theories of aging, nor cover each one in sufficient detail. Thus I will limit my treatment to those theories that are, or have been, most widely recognized in the field, as well as those prominent theories that have put forth testable hypotheses.

1.3.1 Explanatory theories of aging

I see no reason to present these theories in any order other than chronological, and will therefore start by mentioning the **Theory of Programmed Death**, as suggested by

August Weissman in 1882 [25]. The central idea here is that the aging and eventual death of organisms is a trait evolved through natural selection in order to optimize the evolutionary fitness of a species/population; as younger generations would be marginally more evolved than older members, deliberate elimination of the latter would free up resources for the former and thereby confer a benefit to the group in spite of the disadvantage at the individual level. It should be noted that this assumes that, at least at a certain age, the continued existence of the less evolved members is actually detrimental to the survival of the group. In other words, competition for resources within the group would have to be more important than competition with other groups of organisms. Two main points of criticism for this theory have been raised: (1) that the trait that was supposedly selected for (programmed death of older generations) would only rarely manifest in wild populations where most organisms die from predation etc. long before their maximal lifespan, and (2) that according to Darwin's theory the individual fitness "cost" of aging would far outweigh the benefit of promoting the survival of marginally more fit young generations in terms of selection. As a corollary to the second point, it is not clear why programmed death would cause a gradual decline of fitness with increased age, rather than an abrupt death such as that evinced by female octopi after brooding [26]. On the other hand, the idea that aging is an evolved trait would explain the large variation in lifespan in different species, a fact that caused some trouble for later theories. Furthermore, the objection that age-related decline would not evolve could be countered by combining the programmed death theory with a theory proposing some form of wear-and-tear or accumulation of damage to be the cause of aging; thus age-related decline would not be an evolved trait, but rather a general problem of living organisms which would in turn reinforce the evolutionary pressure to eliminate older individuals for the benefit of the population as a whole.

This handily brings us to the **Mutation Accumulation Theory**, put forth by Peter Medawar at a 1951 lecture [27]. This theory is particularly noteworthy because it was one of the first to recognize that the force of selection would inherently weaken after an organism first begins to reproduce. In other words, traits that keep the organism alive until the first reproduction are more important than those keeping it alive beyond this point (barring an increase in reproductive rate with age). Medawar emphasized that this would be true even with no age-dependent loss of fitness: given a population with a constant risk of death per unit time and thereby an exponential decline in surviving members, and a number of progeny that increases linearly with time (i.e. constant reproductive rate) after a certain age, the resulting 'selective force' (chance of survival to age x times the number of progeny at x) would peak sharply soon after the age of first reproduction and then drop almost exponentially. This is illustrated in Figure 1.1, with both chance of survival, number of progeny and the resulting selective force plotted in the same graph.

The central idea of the Mutation Accumulation Theory is that because the force of selection acts so weakly at advanced ages, the genome could be "permitted" to accumulate genetic mutations with adverse effects that only manifest later in life. The combined effect of many such mutations would then be the gradual decline we know as aging. This can be likened to late-onset diseases like Huntington's, with the important difference that the mutations leading to aging would be common to all individuals in the population. The simplicity of Medawar's substantial observations is remarkable, although in retrospect the theory was probably too simple. Certainly it provided a better explanation

for why there is a long period of declining fitness preceding death by 'old age', without violating Darwin's evolutionary principles. But a major point of criticism is that the theory assumes that the mutations that cause aging have no negative effect on fitness (which would of course be selected against), while aging by any almost definition very clearly involves a decline in fitness. The force of natural selection may decline rapidly following sexual maturity, but it is present nonetheless and would (according to Darwin) act to gradually remove the mutations causing aging. Note that while it would seem that an age-related dip in reproductive rate could indeed eliminate the force of natural selection entirely beyond a certain age, this trait would itself be paradoxical in terms of evolution and thus require a similar explanation. While the theory accurately predicts a close correlation between age of sexual maturity and lifespan, explaining other inter-species trends and differences in longevity seem to require some elaboration. The Mutation Accumulation Theory to this day has both supporters and detractors (e.g. [28]).

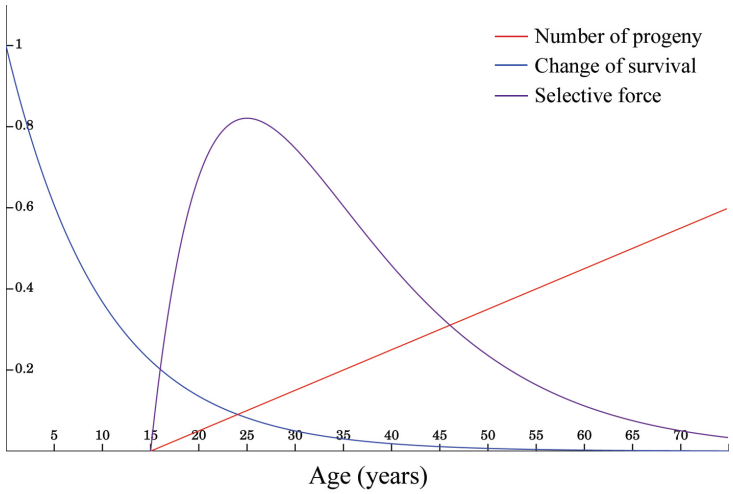

Figure 1.1: **Mutation Accumulation Theory**. For a population with a constant risk of death per year and thereby an exponential decline in surviving members over time (blue line), and a number of progeny that increases linearly with time after a certain age (red line), the resulting 'selective force' (chance of survival to age x times the number of progeny at x, purple line) would peak sharply soon after the age of first reproduction and then drop almost exponentially.

Another evolutionary theory, proposed by George C. Williams in 1957, is that of **Antagonistic Pleiotropy** [29]. This builds on the notion that selective pressure declines later in life, but differs from the Mutation Accumulation theory in that the negative impact on fitness is counteracted rather than discounted. Where Dewadar's theory uses the decreased selective pressure to explain how aging might have 'snuck past' evolution, the idea behind antagonistic pleiotropy is that for genes affecting more than one trait (pleiotropic genes) both the relative *timing* and *impact* of the effects influence whether a mutation is selected for or against. Specifically, where a mutation is beneficial in the early life of an organism but detrimental later on, the later effect would have to be much stronger to prevent selecting for the mutation for its positive effect at a time of greater force of selection. William's idea was that a large collection of such antagonistic effects

could result in a general decline later in life, which certainly does not seem impossible. First of all it conforms perfectly with Darwin, and also allows for different species to have vastly different lifespans simply through a different mosaic of antagonistic gene effects. In fact one could argue that it is surprising that different sets of pleiotropic genes produce such similar signs of aging in different organisms unless those effects are inevitably coupled to functions essential for life, and in that case the organisms that seem to buck the trend (e.g. the naked mole rat mentioned previously) are difficult to explain. Another objection that seemingly requires inevitability to refute is why evolution hasn't managed to achieve the same beneficial traits early in life without the adverse effect later, given the vast available timespan and that the evolutionary force of these traits is specifically sub-zero. This is especially odd since we now know that genes are activated and inactivated at different stages of development and maturity [30, 31]; why then would antagonistically pleiotropic genes not simply be switched off later in life? Despite these quandaries there is experimental evidence supporting testable predictions of the Antagonistics Pleiotropy theory [32] (though not conclusively: [33]), and it is still considered a potentially viable theory.

The **Disposable Soma Theory** of Thomas Kirkwood ([34, 35]) came later than the theories I've mentioned so far, and evidently builds on the foundation of existing thought. The core of the theory is that organisms operate with a limited supply of energy and that it may be evolutionarily advantageous to invest significant amounts of energy into accurate maintenance of only a restricted population of germline cells, while allowing somatic cells to undergo rapid expansion with reduced accuracy which ultimately leads to deterioration and death. Thus the theory is compatible with a variety of 'accumulation of damage' explanations, specifying only that whatever maintenance or repair mechanism prevent this damage in the germline function imperfectly in the soma. This is relevant for one particular argument of the theory, namely that any theory with no cause of aging until "late in life" (including all of the aforementioned, except certain instances of antagonistic pleiotropy) faces problems of potential circularity in defining when "late" occurs; if no changes happen in the first year, what causes them to start happening in the second? The disposable soma theory circumvents this by coupling a stochastic event of errors in macromolecular synthesis with a more general mechanism explaining the evolutionary cause of aging (the disposability of the soma). Two testable hypotheses are proposed: (1) that maintenance mechanisms would be superior in germline cells, and (2) that species longevity would be correlated with fidelity of macromolecular synthesis. The original theory did not specify which kind(s) of macromolecular synthesis was critical to aging, but at least in terms of DNA damage there is experimental support for these predictions: stem cells are indeed better at avoiding DNA damage, which may be caused by either more efficient repair mechanisms or slower cell cycles providing better opportunity for repair [36, 37]. The link between longevity and maintenance is less clear-cut, but experiments by Hart, Sacher and Setlow had at the time already shown some inter-species correlation between repair capacity and lifespan in both cell lines and organisms [38, 39]. The Disposable Soma Theory is not without critics, however. While the experimental data support an important role for maintenance mechanisms in aging, Goldsmith has argued that the link between inadequate maintenance and the reproductive advantage that is supposed to result from the trade-off in maintenance is not obvious [40]; In particular he questions why lower reproductive rates later in life would not free up resources for maintenance mechanisms. Whether or not it suffices to explain

how aging has evolved, the concept of a disposable soma is widely acknowledged, and the accommodation of stochastic events has kept the theory popular in recent years.

As I have mentioned, the major explanatory theories of aging all have their proponents, and paradigm shifts periodically occur as new data emerges that seems to fit one better than the others. These theories certainly see periods of obscurity, but more specific interpretations are proposed with some regularity by adherents seeking (sometimes successfully) to rekindle interest in a particular line of thinking while offering a more adequate explanation of aging. Examples include a disposable soma theory where time rather than energy is the limiting resource for replication versus maintenance [41], the reproductive-cell cycle theory of Atwood & Bowen that argues for antagonistic pleiotropy of hormones regulating the cell cycle [42], and repeated arguments by Blakosklonny for a TOR-driven quasi-program for aging [43].

1.3.2 Mechanistic theories of aging

Indeed, the analysis of ambiguous phenomena influencing aging, and by extension the interpretation of these in the mechanistic theories of aging, depends considerably on one's theoretical creed; the life-extending effect of caloric restriction (CR) has been shown to act via the IGF-1 [44, 45] and TOR [46, 47] pathways, but although this immediately smacks of programmed aging the regimen has also been shown to decrease the accumulation of DNA mutations [48] and could therefore just as well be seen as an induced defence against stochastic damage events. Thus, while the mechanistic theories could be grouped roughly into programmed and stochastic, such groupings inevitably involve exceptions and uncertainties and would therefore seem to have limited value [22]. Overall, I would agree with Kirkwood and others [23, 49] that it is unlikely for any one of these theories to suffice for explaining the mechanistic cause of aging, and I will therefore try to present the most popular theories without endorsing any one in particular. I will skip over the earliest theories of an 'aging gland' and simple mechanical wear-and-tear since these have been universally discarded, and focus first on theories not obviously involving an accumulation of damage. Proceeding to theories that are manifestly based on accumulating damage to some type of cellular component, I will examine most thoroughly the theories of DNA damage and in particular mitochondrial damage that underlie the practical work in this thesis.

The idea that cells have a limited capacity for replication is an especially long-lived explanation of aging, conceptually dating back to Weismann [25] but gaining serious momentum when Haylick and Moorhead showed that fibroblasts *in vitro* replicate a limited number of times before reaching cellular senescence [20]. In 1990 this 'Hayflick limit' was linked to **shortening of telomeres** with each passage of cultured cells [50] and eight years later it was shown that introduction of telomerase to counteract this shortening could immortalize cells [51]. At first glance this would seem to wrap up at least the mechanism behind the so-called "unsolved problem in biology", but alas the story turns out to be rather more complicated. Most demonstrably, it would be a fallacy to equate the cellular process of senescence with organismal aging; if programmed replicative senescence after ∼50 doublings was in fact the sole cause of aging, lifespan should be very predictable and limited by the decline of highly proliferative tissues. One would also expect mice, which have long telomeres and some telomerase activity throughout

life, to outlive humans by a good margin. Beyond this, the shortening of telomeres has more recently been shown to depend greatly on single-strand breaks induced (at least partially) by oxidative damage, owing in part to less efficient DNA repair compared to the rest of the genome [52, 53, 54, 55]. This may explain the observation of significant intra-clonal variation in replicative capacity for human fibroblasts *in vitro* [56], a fact that casts the Hayflick limit in a less obviously programmed light. This connection to oxidative stress has been proposed as a link between and telomeres in aging [57], while a more obvious association between telomeric dysfunction, PGC-1-induced mitochondrial biogenesis and cellular senescence was recently reported by the DePinho lab [58]. While the story may be more complicated than one could have hoped, there is strong evidence that telomeres and senescence are important in organismal aging. Several studies have shown that senescent cells accumulate in aging tissues of primates and humans [59, 60, 61], and a recent study by Baker *et al.* demonstrated that clearance of senescent cells can have a rejuvenating effect on the organism [62]. This finding is striking not least because it strongly supports the idea that senescent cells are not merely a product of aging but actively contribute to the process of decline, possibly through a distinct secretory phenotype [63]. An equally impressive result was obtained by Jaskelioff *et al.*, who showed that (re)activation of telomerase in knockout mice similarly led to a reversal of age-related symptoms [64]. Based on this it seems highly probable that telomeric dysfunction, rather than just senescent cells, is important for the aging process.

A more recent variation on the theme of limited cellular replication posits that **stem cell exhaustion** rather than general senescence is causative of aging. One inspiration for this is that while a somatic tissue would gradually run low on telomere length and thereby accumulate senescent cells while losing functional capacity, this behavior does not entirely fit with the phenotype of aging. Highly proliferating tissues (e.g. the gut) are not implicated in age-related dysfunction compared to tissues with limited replication (e.g. the heart). And indeed we know that many tissues rely on long-lived pools of stem cells to replenish cells damaged by both extrinsic and intrinsic events [65]. However, unlike embryonic and germline cells, these stem cells do not possess unlimited replicative capacity and display shortening of telomeres with successive replications (albeit more slowly than somatic cells) [66, 67]. Coupled with the increased exposure that accompanies such longevity, it seems straightforward to assume that these pools will eventually run out and thus induce declining tissue function. An elegant demonstration of this concept was achieved through temporary elimination of ATR in mice, which is highly toxic to replicating cells and promptly led to cellular depletion in proliferating tissues. These tissues were subsequently replenished (with ATR competent cells), but three months later the animals developed a progeroid phenotype [68]. While this could indicate that a replicative strain on stem cells is a cause of aging, clear-cut evidence for this hypothesis is still missing [69]. The situation is far from settled, but stem cells remain a hot topic, both for study and potential intervention.

Closely related to stem cell replication is immunosenescence: the fact that the ability to mount an appropriate immune response deteriorates with age, which is a major cause of age-related morbidity [70]. One possible cause of this is reduced production of immune cells, though other aspects of the inflammatory response as well as systemic changes are doubtlessly involved. On this basis Roy Walford proposed the **Immunologic Theory of Aging**, which envisions aging as a "...generalized, mild, but prolonged type of autoimmune phenomenon" arising from "...loss of recognition patterns between the body's

cells" [71]. Later adaptations have broadened their scope to include the failing immune response among other inadequate stress responses that together cause an adverse state of inflammation in the body [72, 73]. While immunosenescence is inarguably a very important part of the aging process, an immunologic explanation of aging seems better suited for humans than say *C. elegans*, and thus suffers as a general theory; furthermore, the broad scope of 'failing immune response and inflammation throughout the body' as the cause of aging is difficult to test directly [74].

Another role of the immune system that is relevant to aging is in the clearance of accumulated junk, such as protein aggregates [75] and at least some types of senescent cells [76]. This brings us to **Accumulative-Waste Theories of Aging**, where the common idea is that detrimental material accumulates with age and is causative of the accompanying decline in function. This has immediate appeal in the case of protein aggregation, which is implicated in age-associated neurodegenerative diseases like Alzheimer's, Parkinson's and Huntongton's, but other plausible candidates for age-inducing accumulation include damaged organelles (e.g. mitochondria [77]), damaged lipids [78] and the senescent cells mentioned above. In fact, one of the earliest identified biomarkers of aging was lipofuscin [79], a pigment granule that accumulates at a rate proportional to maximal lifespan in different species [80]. It can thus serve as a (weak) predictor of mortality, although the potential pathological role of lipofuscin remains uncertain [81]. As a theory of aging, waste accumulation is best supported by cellular work: in one study, autophagy was inhibited in human cells that were either confluent or allowed to divide (whereby accumulated waste is diluted). The latter scenario prevented the cell death by overload of undegraded autofluorescent material, suggesting that waste accumulation could be a significant cause of decline in post-mitotic tissues [82]. Another example involves cells from patients with Hutchinson-Gilford Progeria Syndrome (OMIM#176670), perhaps the most unambiguous accelerated aging disorder, which accumulate an abnormal form of Lamin A/C proteins. Treatment with the drug rapamycin (previously shown to induce autophagy and extend lifespan) enhanced degradation of the abnormal lamin and delayed cellular senescence [83]. This model is compatible with both antagonistic pleiotropy (accumulation of something beneficial becomes detrimental) and disposable soma (not-quite-adequate clearance in the soma). However, it faces considerable challenge from the difficulty in establishing causality between waste products and aging at the organismal level. Even if we believe that waste accumulation is causative rather than a symptom of functional decline, we cannot rule out that a more fundamental process is behind the accumulation; for example, misfolded proteins, dysfunctional mitochondria and senescent cells could all be caused by DNA mutations, which are seen to accumulate with age [84].

Even more generally, damage to DNA and other macromolecules can all be caused by oxidative damage, which is the premise of the **Free Radical Theory of Aging** [85]. Aerobic organisms rely on the electron transport chain (ETC) to produce energy through multiple electron transfers, with oxygen as the final acceptor. This leads to a significant endogenous production of reactive oxygen species (ROS) whose reactivity result in damaged DNA, protein and lipids, whose accumulation is proposed as the cause of aging. This theory is clearly complementary to stochastic explanations of aging, and to other mechanistic theories whose agents of decline could result from oxidative damage (senescence, waste accumulation etc.). There is a considerable amount of indirect evidence linking oxidative damage with aging: Supporters of the theory have estimated

the number of reactive oxygen attacks on DNA occur per day at 100,000 in a human cell [86] and that the level of protein with oxidative damage rises from ∼10% in young humans to as much as 50% in the aging brain [87, 88]. Production of ROS increases with age, while antioxidant defenses may decrease [89, 90]. More direct support was obtained in *Drosophila melanogaster* overexpressing the antioxidant enzymes Cu-Zn superoxide dismutase and catalase, which accumulated less oxidative damage with age and upon irradiation, and had improved walking speed, metabolism and lifespan [91]. On the other hand, similar overexpression in mice reduces oxidative damage but does not extend lifespan [92], and several other studies of both wild and genetically modified organisms have also failed to link antioxidant defenses to longevity [93]. Without questioning the validity of the theory, I would argue that its fundamental nature can potentially make it a trap: ROS are unquestionably an important source of damage to cellular components, but that does not necessarily mean that it is the principal cause of aging nor suggest what downstream phenomena could effectuate the decline. Because the Free Radical Theory does not conflict greatly with other hypotheses on aging and has a body of supporting evidence, it may be tempting to accept it regardless of its interpretive value in a specific scenario. I am obviously not the first to point this out, and the solution at hand is of course to invoke oxidative stress in models of aging only where a link can be drawn from free radicals to the aging phenotype. As a result this theory has produced a larger than usual number of more specific derivatives. Two of these are central for this thesis, and will be discussed in the following.

DNA Damage Theory of Aging

Various lesions in DNA disrupt the double helix and can induce mutations if not repaired properly, but can also block transcription (and thereby protein synthesis) until repaired [94]. DNA does not have the same turnover as the proteins and lipids that are also targeted by oxidative damage, and such lesions occur with a very high frequency (10^5 oxidative damage events estimated per cell per day in rats [84]). Because of this, DNA lesions have been suggested as the main cause of age-related decline induced by oxidative damage, by means of a reduction in levels of functional protein [94, 95]. This model was first proposed by Alexander in 1967 [96] and is distinct from earlier theories based on DNA damage, where the proposed deleterious events were accumulation of somatic mutations [97, 98]. These somatic mutation models were quickly criticized for requiring unrealistic levels of deactivated genes, notably by John Maynard Smith [49], and are less popular today than theories focused on DNA lesions.
There is substantial evidence consistent with the idea of DNA lesions being a factor in aging. Several types of DNA lesions have been shown to hinder both transcription [99, 100, 101] and replication [102, 103], and notably in the brain the synthesis of important proteins decreases with age (overview in [94]). There is extensive evidence that oxidative damage and single-strand breaks accumulate in neurons, as well as other tissues [95, 104, 105]. Higher levels of damage have been identified in promoters with reduced expression in older brains [106], alongside increased heterogeneity in gene expression in aged mouse hearts [107]. This accumulation correlates with declining capacity for repair by most of the pathways present in humans, at least in some cases due to lower expression levels of critical repair proteins ([108, 109, 110], and [111] for overview). In this regard the brain seems particularly susceptible, for several reasons: at least some

repair pathways are less active in the brain, possibly because genes involved in repair are turned off once the cells go post-mitotic [112, 113]. On the other hand, the longevity of post-mitotic neurons implies greater opportunity to accumulate damage and the inability to replace dying cells aggravates the risk of damage. This risk is further compounded by the fact that neuronal cells have more active transcription of DNA [114], and that deficient repair seems to increase the likelihood of apoptosis [115, 116].
While this is all circumstantial evidence, implications for DNA damage in aging are also found in studies of model organisms and humans with repair deficiencies. As previously mentioned there is some correlation between DNA repair capacity and species' lifespan [38, 39, 117], and indications of an inverse correlation between lifespan and propensity to form DNA damaging compounds [118]. This is further supported by the apparent importance of antioxidant enzymes described under the Free Radical Theory, though as mentioned these results are not conclusive across species. Beyond this, the three most marked accelerated aging disorders, Werner syndrome, Cockayne syndrome and Hutchinson-Gilford progeria, all show increased cellular sensitivity to oxidative stress and accumulation of DNA lesions [119, 120, 121].
Many different DNA lesions are produced constantly by a range of endogenous and exogenous sources (for overview see [122]). Though quantification of such events are of course wrought with significant uncertainty, it seems certain that different lesions occur at very different frequencies [123]. In all likelihood, many (or all) types of lesions will cause types of cellular dysfunction that could represent what we on the organismal level describe as aging: senescence and apoptosis, cell-cycle arrest, depletion of stem cell pools, as well as loss of proteostasis through reduced transcription, debilitating mutations and loss of genetic information. There is little doubt that double-strand breaks (DSBs), if left unrepaired, have the most severe consequences for the cell: during replication such breaks will lead to chromosomal rearrangements and aneuploidy, and of course DSBs will strictly arrest transcription. The presence of a pathway (non-homologous end joining, described in the next section) that imperfectly repairs double-strand breaks at the expense of mutagenesis emphasizes the extreme priority of repairing such lesions. However, DSBs occur very rarely compared to other types of lesions and are less likely to be left unrepaired since their detection triggers an extensive damage response that (among other things) causes cell-cycle arrest to allow sufficient time for repair. Conversely, lesions that lead to mismatched DNA, such as C-A conversion opposite oxidized guanine, are both much more common and more likely to escape detection and repair. During replication such mismatches will naturally lead to mutations, but in the context of the DNA damage theory they also appear to interfere with transcription either directly or as intermediates in the base excision repair process [124, 125]. It remains an open question of this theory which type(s) of damage are relevant for age-related decline during a normal lifespan, and whether this is consistent for all organisms and conditions.
It is evident that there is an abundance of potential links between DNA lesions and the aging process. But while the DNA damage theory of aging is widely recognized as both plausible and relevant in explaining aging, it should be noted that a clear causative relationship between DNA lesions and age-related decline has yet to be conclusively demonstrated. This does of course not invalidate the theory, merely highlight that it is a working model and precludes (so far) any experimental test of whether DNA damage is a contributing or an ultimate cause of age-related decline.

Mitochondrial Theory of Aging

This theory was put forth by Denham Harman in 1972 as an extension of his theory on free radicals [77, 126]. After he proposed the initial theory in 1956 it has become clear both that endogenous production of ROS exists and often outweighs exogenous sources [127], and that the majority of this production stems from the mitochondrial respiratory chain [128]. On this basis he suggested that the main victim of free radical damage was mitochondria, as evidenced primarily by a host of changes to mitochondrial lipids, enzymes and DNA with increasing age [126]. He noted that this mitochondrial "aging" was strongest in post-mitotic cells, and reasoned that mitochondria were somehow "rejuvenated" during cell division. Harman proceeded to discuss several aging phenomena in the light of this theory, including the inverse correlation between lifespan and metabolic rate, the effect of antioxidants and CR, and the apparent accelerated deterioration in late aging. As I will return to in Chapter 4, decades of further study have corroborated these observations and established that such changes occur more prominently to mitochondria than to other parts of the cell. One important development since the mitochondrial theory was originally proposed is an increased focus on damage to mitochondrial DNA (mtDNA) as compared to other components, under similar assumptions of importance as I presented above for nuclear DNA [129].

There are several reasons why mtDNA would be particularly susceptible to damage from endogenously produced reactive oxygen species. Compared to nuclear DNA especially, but also other cellular components, mtDNA is situated very close to the ROS-generating ETC and will therefore be both higher in the diffusion gradient for ROS and more likely to encounter highly reactive species before they are intercepted by antioxidant enzymes. Upon contact with reactive molecules, mtDNA also presents a greater target profile for damaging events: mtDNA has neither introns nor spacer regions between genes, such that any damaging event will strike a coding (and essential) gene [130]. Though many proteins are bound to mtDNA, it is not organized into nucleosomes and may therefore be more exposed than DNA in chromatin. Finally, once damaging events have taken place in mtDNA there appears to be a less extensive set of repair mechanisms to deal with the damage [131, 132]. Together these traits lead to the expectation of higher mutational rates in mitochondrial genes, which would of course affect the ETC by way of the mitochondrially encoded components. This situation forms the basis for the concept of a "vicious cycle", in which ROS from the ETC causes damage to mtDNA and thereby interferes with the function of the ETC. This feedback loop causes ROS production and mtDNA damage to spiral out of control, as illustrated in Figure 1.2, which leads to the destruction of the mitochondrion (and eventually the organism) [133].

The idea of such a vicious cycle is intuitively appealing, but its existence remains a point of contention in the field. Designing controlled experiments to test this is challenging because of the many varieties and pathways of ROS, but attempts have nonetheless been made. Some of these have measured accumulation of oxidative damage or relevant enzymatic activities [134, 135, 136], though perhaps the most telling are those that directly measure ROS production as a function of age [137, 138]. From these we have considerable evidence that ROS production does increase with age, which would appear to support the idea of a vicious cycle. However, while a study seeking to test the vicious cycle theory did find an inverse correlation between ROS production and lifespan in different wildtype strains of *D. melanogaster*, a mutant with reduced ROS production

did not show increased longevity, nor was lifespan reduced in a mutant with increased ROS production [139]. Going further, Sanz *et al.* saw no difference in lifespan when transplanting mtDNA from one strain to another. In like manner, a study on *C. elegans* did not find any autocatalytic effect of oxidative damage to mtDNA in either wildtype or antioxidant-deficient worms [140]. Another study exposed isolated mitochondria to different levels of extrinsic ROS and measured endogenous ROS production, thus simulating the initiation of a vicious cycle with a somewhat artificial setup [141]. They saw an equable increase in endogenous ROS production above a certain threshold of extrinsic ROS, but no exponential increase in ROS production that could represent a vicious cycle. All in all this concept, while appealing, is poorly supported by existing literature.

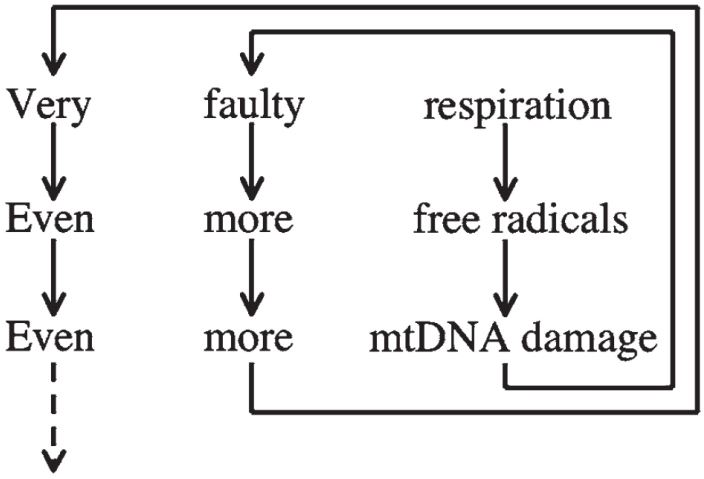

Figure 1.2: **A mitochondrial vicious cycle**. Mitochondrial respiration produces free radicals which in turn cause damage to mitochondrial DNA. This damage carries over into the proteins involved in respiration, which thus functions sub-optimally and produces a greater amount of ROS. The result is a vicious cycle of damage and impaired function which eventually leads to catastrophic failure. Adapted from [142]

Despite this, there is plenty of correlative evidence to support the mitochondrial theory of aging in general. As I described above and in the previous section, oxidative stress markers do accumulate with age, pertinently also in mitochondria, and this accumulation correlates with a loss of mitochondrial function [135]. Exposure to oxidative stress has been shown to cause loss of mtDNA (while persistent DNA alkylation did not) [143], and preservation of mtDNA was associated with successful aging in *C. elegans* [140]. However, it is not clear whether oxidative damage itself is the key driver in loss of mitochondrial function, or whether the effect is caused by mutations in and/or loss of mtDNA as a result of oxidative damage. This conundrum is reminiscent of the one described for the DNA damage theory, and resolving it would require detailed information about the mechanism of dysfunction that is simply not available at present. A powerful tool for this inquiry is the Polg-mutator mouse developed at the Karolinska Institute [144]. This mouse expresses a mutated version of the PolgA subunit of the mitochondrial polymerase, rendering the latter deficient in proofreading. This leads to a large increase in mtDNA mutations and deletions, and rapidly produces a pheno-

type closely resembling normal aging. While at first glance this would seem to almost prove the mitochondrial theory of aging, it should be pointed out that it only establishes causality between mutation/loss of mtDNA and an aging phenotype. That is, it does not actually link mitochondrial oxidative damage to aging, and in fact the Polg-mutator mouse does not display increased ROS production [145]. In line with this, mice heterozygous for manganese superoxide dismutase (MnSOD), and thus with reduced antioxidant defences, show an increase in oxidative damage and cancer susceptibility but no signs of accelerated aging [146]. This indicates that the mechanism by which mitochondria contribute to aging is more complex than simply producing damaging ROS. In support of this, it was recently reported that the Polg-mutator mouse, but not mice accumulating large-scale mtDNA deletions, has abnormal somatic stem cells from embryogenesis [147], suggesting a subtle interplay between mitochondrial function and stem cell pools.

It is apparent that the mitochondrial and DNA damage theories are an example of two aging theories that are easily compatible and may even complement each other: as I have outlined in the previous section, it is definitely possible to produce an aging phenotype through increased mitochondrial mutagenesis. Furthermore, many symptoms present in mitochondrial diseases, including neurodegeneration and loss of hearing/vision, resemble those we know from the very old [148]. But commonality between the phenotypes of aging and mitochondrial dysfunction is not all-inclusive, and changes in transcriptional profiles over time (whatever their cause) is one option for explaining the differentiating symptoms in aging. In turn, the DNA Damage Theory says little about the source of the blocking lesions that it ascribes the aging phenotype to; the prominence of oxidative lesions and origin of most endogenous ROS strongly implicate mitochondria in this regard. Perturbed transcription would expectedly affect mitochondrial function, and with $\sim$99% of mitochondrial proteins encoded in the nucleus this effect could be partially or completely independent of mtDNA. The idea of a vicious cycle therefore needn't be confined to mitochondria themselves, but could place them in a broader context of cellular function.

With such a network in mind, it seems natural to combine the study of DNA repair mechanisms with a consideration of mitochondrial function. DNA repair is crucial for preventing genetic instability by DNA damage, and a certain level of repair is strictly required for the survival of an organism [149]. Whether DNA damage itself is causative of aging or rather effectuates the decline of other functions, there is as mentioned a close correlation between its occurrence and organismal aging. Moreover, the fact that diseases showing accelerated aging often involve mutations in DNA repair proteins offers both motivation for studying repair processes in relation to aging, and a model system for doing so. While it is likely that most of the theories described in this section offer insight into age-related decline, DNA repair represents at the very least an excellent opportunity for examining the mechanistic underpinnings of the aging process and as such is central to the work presented in this thesis. Presumably because of their paramount importance, multiple pathways for repair of DNA damage are present as far back the evolutionary ladder as prokaryotes [150]. I will therefore use the following section to briefly outline these pathways and their potential roles in aging, before moving on to the concrete aims of my work.

1.4 DNA repair pathways

The cellular response to DNA damage is extensive, involving or affecting a diverse range of processes: to allow sufficient time for repair to take place without compromising cellular viability, activation of repair is closely linked to cell cycle checkpoints [151]. Other strategies for promoting survival in response to damage include global effects on transcription and dNTP biosynthesis [152, 153] and, just as DNA repair itself, these processes are mediated by signalling cascades involving myriad post-translational modifications [154]. Where repair fails the cell can utilize translesion synthesis to maintain replication at the cost of fidelity [155] or, if all else fails, initiate apoptosis [152]. But even within this extensive response, DNA repair is of prime importance for the actual resolution of damage. As mentioned in the previous section there are several types of DNA lesions, each affecting cellular function in a different manner and each repaired by a separate pathway. This section broadly describes the steps and main proteins involved in these, to ease interpretation of the work presented later.

Double-strand break repair

DSBs have the greatest potential to disrupt cellular function, and yet represent a significant challenge in terms of proper recognition because some cellular processes (e.g. V(D)J recombination) deliberately create DSBs but must not activate a damage response [156]. Additionally, the fact that both strands of DNA are broken means that there is no readily available template for repair. This loss of information is compounded in that undirected insults that are sufficiently energetic to cause breaks in both DNA strands also frequently damage or remove nucleotides surrounding the break. The coexistence of multiple DSBs thus represents a perilous situation where the open ends risk being incorrectly paired, resulting in often calamitous chromosomal rearrangements [156].
Two mechanisms of DSB repair exist to balance the critical importance of repairing DSBs with the need to maintain genomic stability through repair fidelity (Fig. 1.3). The more accurate mechanism is **homologous recombination**, where the DNA of a sister chromatid is used as a template for the damaged strands. This sub-pathway is fairly complicated, but begins with the MRE11-RAD50-NBS1 complex stimulating 5' to 3' resection of the ends flanking the DSB by either an exonuclease or a helicase-endonuclease pair [157]. Several possibilities exist at this step (for more details see [158]) but commonly strand invasion occurs by the sister chromatid region complementary to the single-stranded ends, thus providing a template for synthesis of a replacement strand. A complex system exists to resolve the single- and double Holliday junctions that result from this strand invasion (for details see [159]), wherein the RecQ helicase BLM (discussed in more detail in Chapter 2) may be required to prevent chromosomal rearrangements arising from strand crossover [160]. The presence of a template allows for repair with little or no loss of genetic information, but also principally limits homologous recombination to the S- and G2-phases due to the very low probability of localizing the homologous chromosomal sequence during G1-phase. In G1, DSBs are thus primarily repaired by the simpler mechanism of **non-homologous end joining** (NHEJ). Here, DSBs are recognized by first the Ku80/Ku70 heterodimer and then DNA-PKcs, which bring the loose ends together and form a foundation for the repair machinery. In classical NHEJ, blunt ends are created either through resecting of single-stranded overhangs by the protein Artemis, or by polymerases synthesizing new complementary DNA (see [161]

or [162] for a more detailed description). Finally, the resulting blunt ends are simply ligated to eliminate the DSB, and it is thus clear that repair by NHEJ very often results in deletions and frameshifts. That NHEJ is nevertheless readily utilized to repair DSBs bears witness to how important it is to avoid discontinuities in cellular DNA.

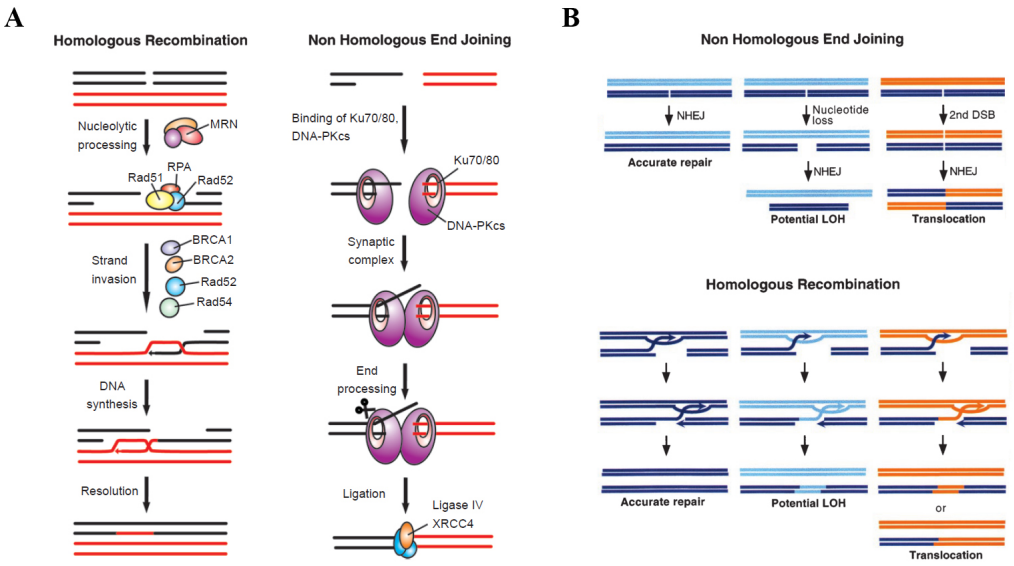

Figure 1.3: **Double-strand break repair**. (**A**) The steps of, and some proteins involved in, the two sub-pathways of DSB repair. When a sister chromatid is available as a template, homologous recombination occurs by resection around the DSB by the MRN complex, invasion by the complementary strand on the sister chromatid and synthesis of replacement DNA from this template. After this an elaborate system exists to resolve the resulting double Holliday junction. Non-homologous end joining is a simpler sub-pathway: breaks are sensed by the Ku heterodimer and DNA-PKcs, single-strand overhangs are either filled or resected, and the resulting blunt ends are ligated. (**B**) Potential results of DSB repair: creation of blunt ends in non-homologous end joining typically destroys genetic information, and can lead to loss of heterozygosity (LOH), while incorrect end-pairing leads to larger chromosomal rearrangements. LOH can also result from homologous recombination where a mutation exists in one allele, and despite protective mechanisms incorrect resolution of double Holliday junctions can lead to rearrangements. Adapted from [162] and [163]

While there is some discussion of DSBs as a cause of aging (see for example [164]), they are more commonly linked to the development of cancer by way of chromosomal rearrangements [156, 165]. However, the severity this type of lesion means that the cell is likely to commit suicide rather than allow unrepaired DSBs, and it is at present not clear how DSBs play into depletion of e.g. post-mitotic cells and stem cell pools. Indeed, Ku deficiency produces symptoms of accelerated aging in mice and cells [166, 167], suggesting at least a possible role for DSBs in the aging process.

Nucleotide excision repair

The nucleotide excision repair (NER) pathway exists to remove bulky lesions that distort the conformation of double-stranded DNA. These lesions are commonly caused by UV exposure and various chemicals, as well as by endogenous processes, and by blocking

polymerase progression lead to problems of both replication and transcription [122]. Because of this, NER comprises two methods for identifying lesions, which converge in a common machinery for repair of the lesion (Fig. 1.4). In the **global genome repair** sub-pathway, distorting lesions are sensed throughout the genome by either the complex of DDB1 and DDB2, or the complex XPC-RAD23B-CEN2. Due to the immense target area of such scanning, global genome repair is less efficient at recognizing lesions than **transcription-coupled nucleotide excision repair** (TC-NER). Here, the DNA damage response is initiated when an RNA polymerase stalls at blocking lesions. This leads to recruitment of first CSB (which I return to in Chapter 3), and subsequently CSA (part of an E3 ubiquitin ligase complex), XAB-2 (which binds XPA), the transcription elongation factor TFIIS, and other protein (see [168] for details). Together, these act to displace the RNA polymerase, open the DNA for access by repair proteins and possibly induce degradation of the polymerase [169]. At this point the two sub-pathways converge with the recruitment of TFIIH, XPA and XPG, which create a DNA bubble of ∼30 nucleotides at the site of damage. The damaged strand is then incised 5' of the lesion by the XPF-ERCC1 complex, and loading of DNA polymerase δ, η or κ is facilitated by RFC and PCNA; while new DNA is being synthesized, XPG makes a second incision 3' of the damaged site. The reaction is completed by removal of the damaged strand and ligation, performed either by FEN1 and ligase I, or the ligase III-XRCC1 complex [170]. I will examine the role of NER in aging more closely in Chapter 3, and will therefore simply point out here that mutations in NER proteins cause different diseases with varying levels of premature aging symptoms.

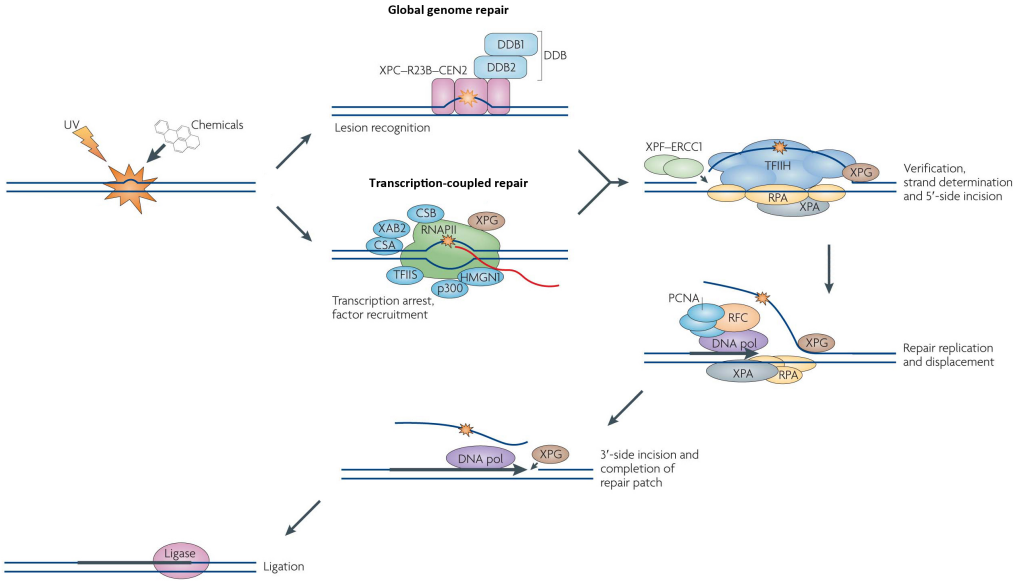

Figure 1.4: **Nucleotide excision repair**. Bulky lesions that distort the DNA double-helix are sensed throughout the genome (in global genome repair), or when they block transcription by RNA polymerases (in transcription coupled repair). Both sub-pathways mobilize the same repair machinery, opening a DNA bubble of ∼30 nucleotides around the lesion, which is removed by dual incision and replaced by nascent DNA. Adapted from [168]

Base excision repair

A number of single-strand DNA lesions that leave the overall structure of DNA intact are not covered by NER. These lesions include various chemical modifications and single-strand breaks, and are frequently the result of oxidative damage caused by endogenous and exogenous ROS. Such lesions are instead handled by base excision repair (BER), which is at its core a much simpler process than NER: in a simplified model, a damaged base is first excised by a glycosylase, the abasic site removed by an endonuclease and the resulting gap filled by a polymerase and a ligase (Fig. 1.5). However, the great range of possible base modifications are dealt with by at least 11 different glycosylases in humans, which simultaneous offer specificity and redundancy to the repair process [171]. A minority of these glycosylases are bifunctional in that they are able to both excise the damaged base and hydrolyze the DNA backbone; an example is OGG1, which is responsible for removing the commonly occurring oxidized guanine. Other glycosylases are monofunctional and only remove the damaged base, e.g. UNG which removes uracil from DNA, whereupon hydrolysis of the backbone is performed by an endonuclease (in most cases APE1). The simplest continuation of the repair process, termed **short-patch BER**, is that DNA polymerase β removes the 5' deoxyribosephosphate at the incision site and fills the gap. In some cases this lyase activity is prevented, which initiates **long-patch BER**. Here, recruitment of PCNA and RFC allow loading of DNA polymerase δ or η, which displace part of the damaged strand by synthesis of 2-7 nucleotides while the displaced strand is removed by FEN1. In both short- and long-patch BER, the final step is religation by ligase I or the ligase III-XRCC1 complex [149].

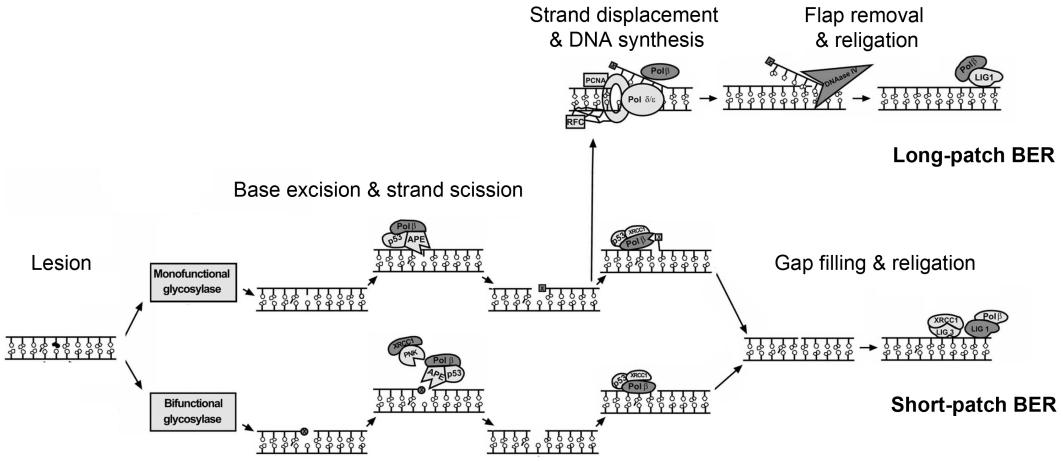

Figure 1.5: **Base excision repair**. In short-patch base excision repair a damaged base is removed by one of several (mono- or bifunctional) glycoylases, followed by hydrolysis of the DNA backbone by either an endonuclease like APE1 or a bifunctional glycosylase. The resulting gap is then filled and religated. Long-patch BER deviates at the gap filling step, where 2-7 nucleotides are synthesized and displace part of the damaged strand, which is then removed by FEN1. Adapted from [172]

Despite the apparent simplicity of the BER process, a great deal of research have revealed a net of interactions both between proteins active in BER and with various protein partners (for overview see [173]). The latter include proteins implicated in pre-

mature aging (for instance WRN, which I will return to in Chapter 2), and numerous mouse models lacking proteins involved in or associated with BER also show premature aging [108, 174]. Moreover, as described in the previous section, oxidative DNA damage has been correlated with normal aging and is increased in segmental progerias [175, 176]. Of all the DNA repair pathways, BER is thus most strongly implicated in aging.

Mismatch repair

As the name suggests, mismatch repair (MMR) resolves mismatched bases in double-stranded DNA. The primary target of MMR is base mismatches, and improperly annealed DNA created by errors in DNA replication or faulty strand pairing during recombination processes [177]. It should be noted that many MMR proteins also have other biological roles, but I will refer to [178] for a discussion of these. In essence, the MMR process follows the same principles as NER, in that a stretch of nucleotides surrounding a lesion is removed and replaced by newly synthesized DNA. However, the challenge of identifying which strand carries the incorrect base necessitates a specialized machinery for MMR. The basis for this recognition is the presence of single-strand breaks in the vicinity of the mismatch; in *Escherichia coli* these are created by the endonuclease MutH at GATC sites left unprotected for a short while after synthesis, while in humans strand breaks are inherently present between Okazaki fragments [179].

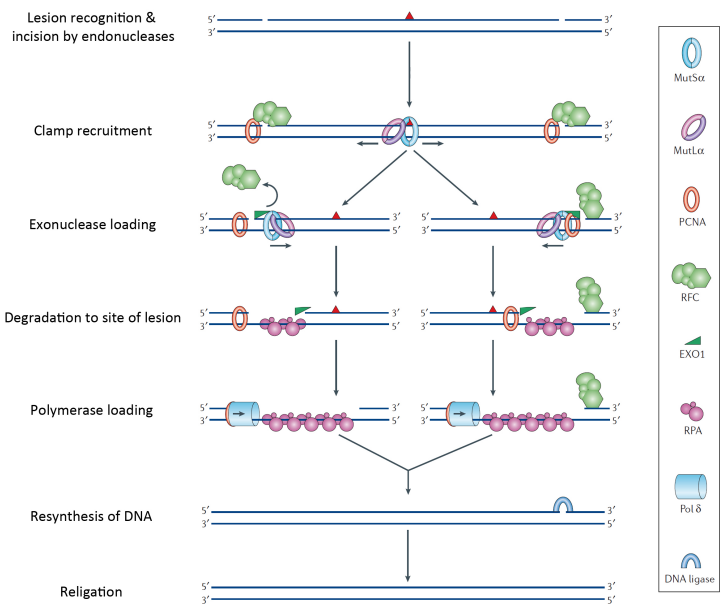

Figure 1.6: **Mismatch repair**. Proper recognition of the strand to repair at DNA mismatches rests on surrounding breaks originating from the replication process. These recruit PCNA and RFC, which act as stops for the sliding of mismatch-recognizing heterocomplexes and thereby define the region of strand degradation mediated by EXO1. The resulting single-stranded DNA is protected by RPA until resynthesis can occur, and the repair process is concluded by religation of the replacement DNA. Adapted from [179]

Although specific target recognition is achieved through different heterocomplexes formed from the five human MSH proteins, a simplified version of the MMR system can be presented as in Fig. 1.6: the mismatch is recognized by a MutSα heterocomplex, which recruits MutLα. This clamp moves away from the mismatch until it encounters PCNA and RFC at the flanking single-strand breaks, removes the latter protein and recruits EXO1. This exonuclease is activated by MutSα, leading to degradation of the strand towards the mismatched site (possibly through repeated EXO1 loading when upstream of the mismatch) while RPA stabilizes the resulting single-stranded region. Removal of the mismatch stops EXO1 activation by MutSα, and DNA polymerase δ is recruited by the PCNA molecule at the start of the single-stranded region. Finally, DNA ligase I completes the repair process.
While defects in MMR have been linked to several types of human cancer and can lead to extremely high mutational rates, there is little evidence to suggest that accelerated aging occurs in either animal models or human patients. As such, MMR will not be examined further in this thesis.

1.5 Aims & Rationale

The work presented in this thesis is based on the theoretical foundation of the DNA damage and mitochondrial theories of aging, making the following assumptions: (**1**) DNA damage is closely linked to, and possibly causative of, age-related decline. (**2**) DNA repair is essential to prevent such damage, and its malfunction can lead to accelerated aging. (**3**) Mitochondrial function is both important in aging and interconnected with DNA repair. The research questions that I wish to address are as follows:

- **Is the accelerated aging observed in DNA repair disorders caused by DNA damage innately, or by secondary cellular effects?**

- **Can mitochondrial dysfunction be placed either upstream or downstream of deficient DNA repair in the aging process?**

Even focusing on two closely related mechanistic theories of aging we are confronted with a throng of potentially important elements at the cellular level of detail in which these theories operate. Even if one was to leave the sources of DNA damage to environmental toxicologists, the four repair pathways described in the previous section each involve a number of different proteins and in many cases show some level of crosstalk with other repair pathways [180]. Mitochondria constitute a vast area of research on their own, and their involvement complicates things tremendously. Beyond the inevitable reliance of these organelles on cellular protein synthesis & trafficking pathways for their biogenesis and proteostasis [181], Ca^{2+} and ROS-based signalling by mitochondria lead to an interdependent network of communication [182]. Mitochondrial function further depends heavily on fission/fusion events and continuous recycling, and excessive ROS production is tightly linked to mitochondrial function [183, 184]. Confronting such an extensive subject matter necessitates either taking a broad perspective, taking into consideration various relevant topics at the risk of disjointedness, or alternatively a more focused study that is liable to lose sight of its extended significance (or lack of same). The work presented in is thesis is based on the former approach.

Furthermore, the study of aging in particular requires a choice between a wide range of model systems, each offering both advantages and drawbacks. Examining aging directly in human subjects requires prohibitively long studies and involves great expense, regulation and confounding variables. At the opposite end of the spectrum, *in vitro* studies often provide convincing results, but it can be difficult to relate these results to the aging of an organism. Between these extremes are a gamut of model organisms that provide ease of husbandry and genetic manipulation in inverse proportion to their relevance for human aging. A few systems fall outside this sliding scale, for various reasons. One example is cultured human cells, which are highly relevant in terms of genetics but nevertheless takes aging out of its crucial systemic context. In contrast to this stands the study of segmental progerias, many of which I have mentioned previously. These diseases seldomly allow for direct examination of patients due to their extreme rarity, but patient cell lines and animal models nonetheless allow for simulations of normal aging accelerated by genetic differences. These cases naturally offer a tantalizing opportunity to explore pathways that apparently participate in the aging process, even though they as a rule only represent rapid development of a subset of aging traits [185, 186]. I would be remiss not to mention mutant animals that conversely appear to age at a slower-than-normal rate, e.g. the Ames/Snell dwarf mice [187] and the *daf-16 C. elegans* mutant [21]. These certainly also provide exciting and complementary opportunities for studying the mechanisms of aging, though at least when dealing with organisms the rapidly aging mutants offer the clear advantage of changing faster rather than slower. To be sure, each model system can be an optimal choice for certain studies, and the fact that my work mainly deals with segmental progerias should be seen as a question of opportunity rather than of doctrine.
Thus Chapters 2 & 3 both deal with segmental progerias, those associated with RecQ helicases and Cockayne Syndrome respectively, while Chapter 4 presents work focused on the role of mitochondria in aging. In all three chapters I study proteins involved in DNA repair and genomic instability, in the hope of clarifying the mechanistic relationship between DNA damage and aging.

Chapter 2

RecQ Helicases

The five human RecQ helicases (RECQL, WRN, BLM, RECQL4 and RECQL5) shown in Fig. 2.1 belong to a highly conserved protein family that is generally recognized to be important in genomic maintenance [188, 189, 190]. They have received a great deal of attention because three of the five (WRN, BLM and RECQL4) have been linked to human diseases showing signs of accelerated aging: Werner syndrome (WS, OMIM#277700) produces a phenotype strongly resembling normal aging. This includes both physical features (grey hair, altered facial features, wrinkled skin, short stature) and early onset of aging-related diseases (e.g. cataracts, osteoporosis and diabetes) [188]. Indeed, microarray analysis of cells from WS patients revealed a gene-expression profile very closely matching that of normal aged cells [191]. WS patients also show increased incidence of sarcomas. Bloom syndrome (BS, OMIM#210900) shares some of these age-related features (e.g. short stature, distinct facial features and early onset of some diseases) and is therefore considered a segmental progeria. However, the most striking phenotype of BS is greatly increased cancer susceptibility (with a normal distribution of type), most likely arising from increased chromosomal breakage and sister chromatid exchanges [192]. Meanwhile, mutations in the *RECQL4* gene are associated with three partially overlapping disorders: Rothmund-Thomson syndrome (RTS, OMIM#268400), RAPADILINO syndrome (OMIM#266280) and Baller-Gerold syndrome (BGS, OMIM#218600). Like WS and BS, growth retardation is seen in RAPADILINO, many cases of RTS and some cases of BGS. RTS also shares some symptoms of premature aging seen in WS, including hair loss and early development of cataracts, as well as a predisposition for osteosarcomas. Radial ray defects are seen in all three syndromes, while patellar defects occurs in RAPADILINO and a few cases of RTS and craniosynostosis is unique to BGS. Poikiloderma is common to RTS and BGS, but not RAPADILINO [193, 194, 195].
While I will not go into details regarding the functions of each RecQ helicase, the broad picture is that these helicases play roles in maintenance of genomic integrity [188]. BLM is best characterized and acts with topoisomerase IIIα to resolve double Holliday junctions during homologous recombination and thereby prevent crossover recombination of DNA [160]. WRN seems to be involved in various aspects of DNA processing, though with the possible exception of resolving G4-quadruplexes at telomeres its concrete role in these processes is not well described [119]. As described in Sections 2.1 and Paper I, RECQL4 has a definite role in initiation of replication and quite possibly also in DNA repair. RECQL and RECQL5 are poorly understood, but *in vitro* and interaction studies suggest roles in replication and repair [188, 190].

In summary, we have but limited understanding of what roles the human RecQ helicases play in the cell. At the same time, dysfunction in these proteins leads to phenotypes that are in several cases reminiscent of aging. This combination marks the RecQ helicase family as a prime target for attempts to uncover events at the cellular level that could underlie the aging process in humans. Specifically, determining whether the symptoms of accelerated aging are connected to DNA-protective functions of these proteins could clarify the role of DNA damage in aging. On this basis I have completed two projects examining the cellular functions of different RecQ helicases, which will be described in the following.

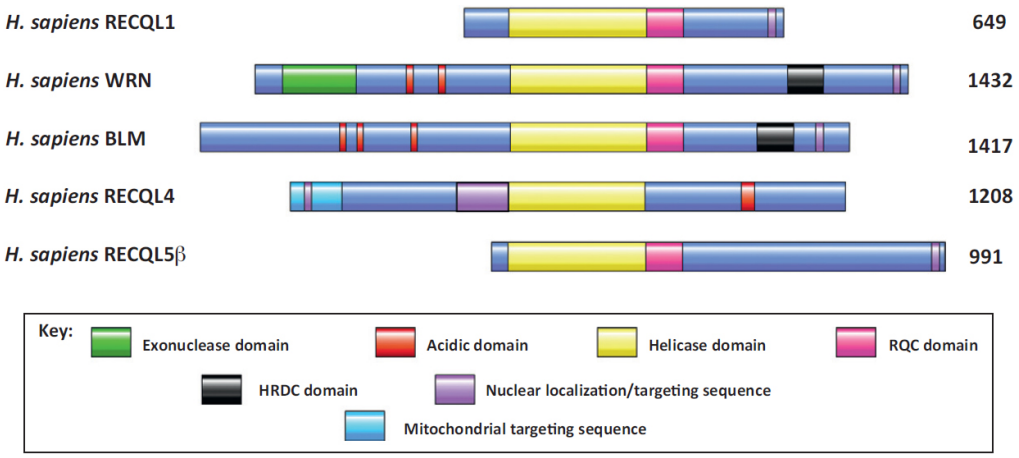

Figure 2.1: **The human RecQ helicases**. The five members of the human RecQ helicase family share a central helicase domain, which for all but RECQL4 is followed by a RecQ family C-terminal (RQC) domain. WRN and BLM share a helicase RNase D C-terminal (HRDC) domain, while WRN uniquely possesses an N-terminal exonuclease domain. Adapted from [196].

2.1 Characterizing patient mutations in RECQL4

The first of these projects deals with RECQL4 and its associated syndromes. The goal here was both an attempt to correlate the biochemical effect of point mutations with the resulting disease phenotype, and in the process hopefully gain further clues about the normal cellular functions of the protein. In this section I will begin by providing some background information on RECQL4, and proceed to describe the approach and findings of the paper presented in Section 2.1.1.

While mutations in *RECQL4* have been implicated in disease for more than a decade [197, 198], it was not until 2005 that the protein was reported to be essential for DNA replication [199]. In this role RECQL4 interacts with MCM10 through an N-terminal domain homologous with the *Saccharomyces cerevisiae* protein Sld2, and thereby recruits the MCM2-7 helicase complex [200, 201]. In line with this, the N-terminal region of RECQL4 was sufficient to restore viability in chicken DT40 cells [202], while in *Xenopus laevis* egg extracts the N-terminal region of RecQ4 was required for binding of DNA polymerase α and initiation of replication [203]. The role of the conserved helicase do-

main in RECQL4 is not nearly as clear. In contrast to the reports described above, the helicase domain was required for viability in *Drosophila* [204], and a helicase dead mutant was unable to restore replication in *Xenopus* [199]. RECQL4-deficient cells are sensitive to a number of genotoxic agents [205, 206], and the helicase/C-terminal domains seem to be important for RECQL4's role in recovery after radiation [207, 208, 209]. Indeed, RECQL4 has been shown to colocalize with genomic maintenance proteins [210], and has been implicated in several DNA repair pathways [211, 212, 213]. Based on this sum of data it is tempting to suggest that while the N-terminus of RECQL4 exerts its crucial role in replication, the helicase domain mediates a secondary role in genomic maintenance that may be compromised in RECQL4-associated diseases. In terms of biochemistry RECQL4 shares most of its activity with the other RecQ helicases: it was initially shown to possess ATPase activity, stimulated by DNA binding, and the ability to anneal single-stranded DNA [214]. Helicase activity was not seen at first, but eventually a weak activity was reported [215, 216, 217].

To accomplish the goals stated above, we opted to perform a (mainly) biochemical characterization of RECQL4 mutants previously reported in patients with the different RECQL4-associated syndromes. The idea was to attempt to link specific biochemical deficiencies to different types and/or severities of disease, and we initially chose six point mutations from RAPADILINO and RTS cases with and without cancer, as well as SNPs associated with these diseases [195]. We created plasmids containing *RECQL4* harboring these mutations and proceeded with two independent, *E. coli*-based protein expression and purification schemes. Three of the selected mutants turned out to be unstable during purification, which narrowed our investigation to the remaining three. We performed a simple evaluation of structural integrity, as protein yields were insufficient for e.g. circular dichroism, and ran isotope-based assays for DNA binding capacity, helicase activity, ATPase activity and strand annealing activity. We further created human expression vectors harboring YFP-fusions of the mutant and wild type RECQL4 proteins, which were expressed in cultured cells to quantify recruitment to and retention at laser-induced DSBs. While the loss of three mutants curbs our ability to correlate biochemical traits with disease phenotypes, we nevertheless uncovered a consistent pattern of activity: all three examined mutants had impaired helicase and ATPase activities, while we did not observe significant differences in DNA binding, strand annealing or dynamics and DNA damage sites. Though our structural assay only allows tentative conclusions, the mutation F637S seemed to affect the structure of the protein and was correspondingly the most strongly affected in terms of helicase and ATPase activities. This is consistent with a previous report showing that the c.1390+2delT mutation common in RAPADILINO leads to impaired helicase and ATPase activities [196].

A full understanding of RECQL4's cellular functions remains elusive, and we cannot explain how mutations manifest as different syndromes nor why a third of RTS patients do not have mutations in RECQL4 [218]. Nonetheless, our work here further supports that the function of the helicase domain underlies the pathology of diseases, which could suggest that the RECQL4-independent cases may involve protein partners in this function.

2.1.1 Paper I

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Research Paper

The helicase and ATPase activities of RECQL4 are compromised by mutations reported in three human patients

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Key words: *RecQ helicase, RECQL4, Rothmund-Thomson Syndrome, RAPADILINO Syndrome*
Abbreviations: *BGS, Baller-Gerold syndrome; BLM, Bloom syndrome protein; HRDC, Helicase/RNAse D C-terminal; RQC, RecQ C-terminal; RTS, Rothmund-Thomson syndrome; WRN, Werner syndrome ATP-dependent helicase*
Received: *11/1/12; Accepted: 12/1/12; Published: 12/4/12*
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Abstract: RECQL4 is one of five members of the human RecQ helicase family, and is implicated in three syndromes displaying accelerating aging, developmental abnormalities and a predisposition to cancer. In this study, we purified three variants of RECQL4 carrying previously reported patient mutations. These three mutant proteins were analyzed for the known biochemical activities of RECQL4: DNA binding, unwinding of duplex DNA, ATP hydrolysis and annealing of simplex DNA. Further, the mutant proteins were evaluated for stability and recruitment to sites of laser-induced DNA damage. One mutant was helicase-dead, had marginal ATPase activity and may be structurally compromised, while the other two showed greatly reduced helicase and ATPase activities. The remaining biochemical activities and ability to recruit to damage sites were not significantly impaired for any of the mutants. Our findings demonstrate a consistent pattern of functional deficiency and provide further support for a helicase-dependent cellular function of RECQL4 in addition to its N-terminus-dependent role in initiation of replication, a function that may underlie the phenotype of RECQL4-linked disease.

INTRODUCTION

The RecQ family of helicases is conserved across multiple species and has been firmly linked to genomic maintenance [1–3]. Five RecQ helicases are present in humans: RECQL1, Bloom (BLM), Werner (WRN), RECQL4 and RECQL5. A central helicase domain, which allows for 3' to 5' unwinding of DNA, is conserved across the family [1,4]. In addition, RECQL4 possesses an N-terminal domain homologous to the *S. cerevisiae* protein Sld2 [5], the RecQ C-terminal (RQC) domains thought to affect protein interactions of the other members of the RecQ helicase family (Fig. 1C) [1,4,6,7]. Of the five human RecQ helicases, three (WRN, BLM and RECQL4) are associated with diseases involving segmental premature aging and cancer predisposition [8–11]. While the mechanisms behind Werner (OMIM 277700) and Bloom Syndromes (OMIM 210900) are not yet fully understood, the responsible proteins are at this stage relatively well-described as being required to resolve DNA secondary structures, and prevent inadvertent homologous recombination, respectively [12–19]. In contrast, the biological role of RECQL4 is not nearly as well understood [5,20–25].

Although RECQL4 has the same conserved helicase domain as the other RecQ helicases, *in vitro* experiments did not initially reveal any DNA helicase activity [24,26]. Weak ATP-dependent unwinding was eventually demonstrated, first in the presence of single-stranded competitor DNA [27] and later in the absence

of the competitor [28,29]. Like the other RecQ helicases, RECQL4 exhibits strong strand annealing activity [26], which masks the helicase activity when longer duplex substrates are used. The extent to which the helicase activity of RECQL4 is critical for its biological role is not clear. A well-established role of RECQL4 is in the initiation of replication, where it interacts with the MCM10 protein and plays an essential part in assembling the CDC45-MCM2-7-GINS replication complex [20,30]. While the interaction with MCM10 was reported to inhibit the helicase activity of RECQL4 [20], mutants of *Drosophila* and *Xenopus* RECQL4 lacking functional helicase domain were unable to restore viability of knockout cells [5,21,27]. On the other hand, the helicase domain was not required to restore viability of knockout chicken DT40 cells [31], and reported human patients with mutations/deletions in the helicase domain obviously demonstrate viable replication [10,32].

RECQL4 has been implicated in several DNA repair pathways, either by being required for the repair of certain types of DNA damage [33–35], or through interaction with known DNA repair factors [22,25,36,37]. It has been suggested that the helicase domain plays an important role in this function [21,31,38], but a specific mechanism has not been identified. Mutations in RECQL4 occur in three human diseases, Rothmund-Thomson syndrome (RTS, OMIM 268400), RAPADILINO syndrome (OMIM 266280) and Baller-Gerold syndrome (BGS, OMIM 218600). These syndromes have partially overlapping phenotypes, with bone defects common to all three but for instance poikiloderma/sparse hair common to RTS and BGS, and osteosarcomas common to RTS and RAPADILINO [39–41]. One might speculate that such a variety of phenotypes arising from mutations in a single protein indicate multiple functions for RECQL4, and that the similarities to Werner and Bloom syndromes suggest dysfunctional genomic maintenance [1,8]. Patient mutations generally lie outside of the Sld2-like domain, which appears to be crucial for RECQL4's role in initiation of replication. Also, several of these mutations create premature stop codons that may prevent expression of the protein entirely [39,41]. While it is difficult to predict exactly how a replication-impaired phenotype might manifest, the survival of human patients with mutations in RECQL4 stands in contrast to studies on *Drosophila*, where deletion of the Sld2-like domain eliminated viability [21,38,42,43].

In the present study we examined three RECQL4 mutations previously reported in human patients [41]. Each mutation represents a single amino acid substitution in a highly conserved residue of the helicase domain of RECQL4. After expressing and purifying the mutant proteins we evaluated their thermal stability, recruitment to DNA double-strand breaks, ability to bind, anneal and unwind DNA, as well as hydrolyze ATP. From these analyses we uncovered a consistent pattern of functional deficiency, which may serve as an initial step in uncovering the cellular origin of RECQL4 disease phenotypes.

RESULTS

Overview of patient mutations

There are only twelve described patient mutations in *RECQL4* amenable to biochemical characterization, as the majority of mutations are either splicing errors, or frameshifts which introduce premature stop codons [41]. Further, most patients have compound heterozygous mutations, which inevitably but unfortunately hampers attempts to link molecular studies to patient phenotypes. We elected to study the three patient mutations P466L, F637S, and F697L where the affected amino acids lie within the highly conserved helicase domain of RECQL4. These are highlighted in Fig. 1A, which also shows the conserved helicase motifs in yellow. The crystal structure of RECQL4 is not yet available, but to get an impression of where the affected residues are located we threaded the aligned sequence of RECQL4 onto the crystal structure of human RECQL1 (PDB ID: 2WWY), omitting the RQC domain, which is not present in RECQL4. Human RECQL4 shares 41% identity and 56% similarity with the helicase domain of human RECQL1 and the model structure is shown in Fig. 1B. Because the mutations lie so close to highly conserved motifs (Fig. 1), we expect their structural position in this map to resemble that of RECQL4, though we cannot say for certain which differences exist in the structures of RECQL1 and RECQL4. The three mutants (P466L, F637S and F697L) were successfully purified (Supp. Fig. 1), and are highlighted in magenta in Fig. 1.

Overall structure of mutants appears to be conserved

In order to evaluate the structural stability of our mutants compared to WT, we analyzed their unfolding as a function of temperature by measuring the fluorescent signal of a SYPRO® Orange protein binding dye. In this assay, melting curves can be extracted from an increase in fluorescent signal, which is observed as the protein unfolds to reveal additional dye binding sites [44] (see Supp. Fig. 2 for melting curves and raw data). While F637S showed clean single-step unfolding, both WT and the remaining mutants appeared to demonstrate more complex unfolding. The higher initial fluo-

rescence observed from F637S may indicate partial structural destabilization by this mutation. While we were not able to accurately describe the unfolding of WT and the other mutants based on our data, it appears consistent with two-step unfolding. If that is the case, P466L may show an effect on the second unfolding step, though as mentioned our data cannot establish this conclusively. Nonetheless we estimated apparent melting points from all the melting curves by approximating single-step unfolding, and these results are summarized in Supp. Table 2. By this analysis, we did not see significant differences in melting temperature for any of the mutants as compared to the WT.

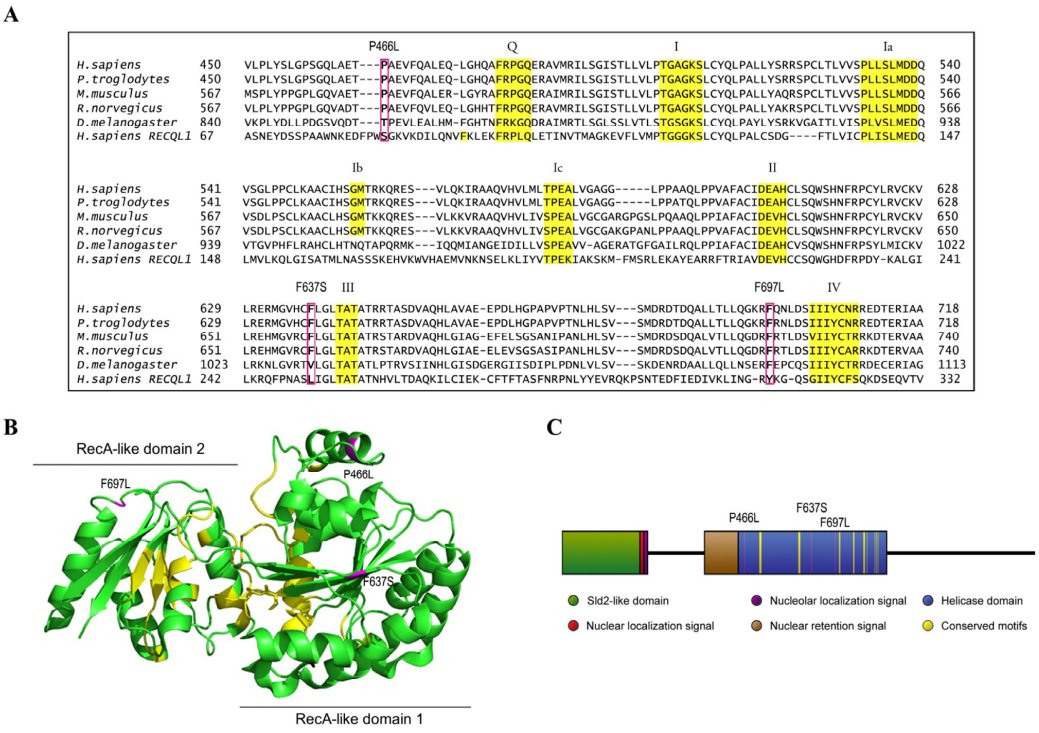

Figure 1. Homology map and model structure of RECQL4. (**A**) RECQL4 homologues across several species, illustrating the highly conserved nature of the amino acids examined in this study. Important helicase motifs are highlighted in yellow [50], while mutations are boxed in magenta. The sequence of RECQL1 is included to show the alignment used to create the model structure in **B**. (**B**) The crystal structure of RECQL1 without the RQC domain that is not present in RECQL4 (PDB ID: 2WWY), and with the amino acids homologous to the examined mutations in RECQL4 highlighted in magenta. Labels indicate the mutations in RECQL4. Important helicase motifs are highlighted in yellow, and the RecA-like domains 1 and 2 are indicated. (**C**) Domain map of RECQL4, with the examined mutations highlighted in magenta, and with important helicase motifs highlighted in yellow.

All mutants are still able to bind DNA

Being prerequisite for other types of activity, our first object of investigation was to test the ability to bind DNA. We chose to use single-stranded DNA as the most realistic model for RECQL4's DNA binding *in vivo*, and proceeded to test the binding of each mutant to a single-stranded 37-mer oligonucleotide at a range of protein concentrations. Fig. 2A shows a decrease in intensity of the lower band (free DNA), signifying protein binding and retardation of the DNA in the gel, leading to the appearance of a corresponding upper band representing protein-bound DNA. The upper band may smear considerably when the DNA-protein complex dissociates in the gel, and we therefore used the intensity of the lower band to quantify the level of unbound DNA. Although all three mutants showed a trend of slightly decreased binding (approximately 80% of WT at 100 nM protein, Fig. 2B), this difference was not statistically significant. We thus conclude that the mutants all show proficient DNA binding.

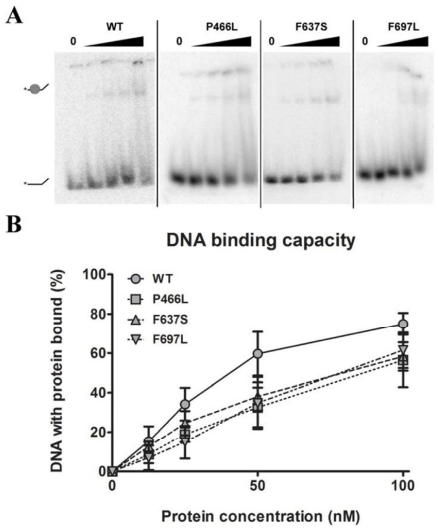

Figure 2. DNA binding is not significantly affected by mutations. (**A**) Amalgamated gel from representative experiments showing DNA binding of WT and mutants at 0, 12.5, 25, 50 and 100 nM protein. (**B**) Binding data compiled from triplicate experiments. Mutants universally appear to have slightly reduced binding compared to wild-type, but the difference is not significant. Error bars represent standard error of mean from three experiments.

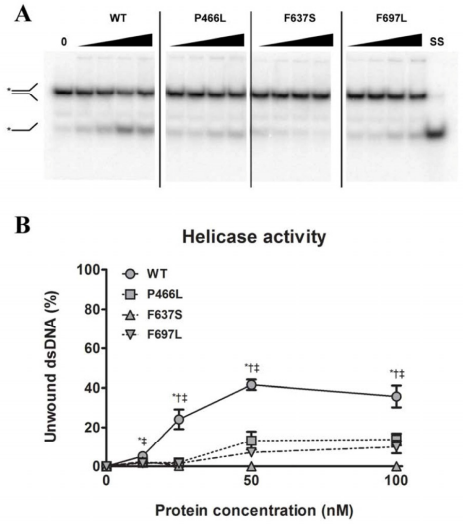

Figure 3. All mutants have decreased helicase activity. (**A**) Amalgamated gel from representative experiments showing helicase activity of WT and mutants at 0, 12.5, 25, 50 and 100 nM protein, as well as a single-stranded control. (**B**) Unwinding data compiled from triplicate experiments. The P466L and F697L mutants show significantly reduced helicase activity compared to wild-type (38% and 28% of WT respectively at 100 nM protein), while F637S has no detectable activity. *, †, and ‡ denote $p < 0.05$ between WT and P466L, F637S and F697L, respectively. Error bars represent standard error of mean from three experiments.

Each mutation adversely affects helicase activity

Since substrate binding is a prerequisite for the 3'-5' DNA helicase activity of RECQL4, we next examined whether the mutant proteins were able to unwind a short DNA fork substrate. The substrate has a 22 bp duplex region followed by a 15 bp non-complementary region, and has previously been used to demonstrate helicase activity of RECQL4 in the absence of single-stranded competitor DNA [28]. For gels like those shown in Fig. 3A we calculated the relative intensity of the bottom (single-stranded) band versus the top (double-stranded) band, and plotted the results graphically in Fig. 3B. In contrast to the binding data, all three mutants showed significantly reduced ability to unwind this substrate, as compared to WT. For P466L and F697L we observed negligible activity below 50 nM protein, and activities ranging from 20 to 37% of WT at 50 and 100 nM protein. Meanwhile, F637S showed no detectable helicase activity. To confirm that the difference in

activity was not an artifact of the purification we repeated the experiments using independent protein preparations based on an alternate purification protocol (described in the supplementary information), and observed the same pattern of activities (data not shown).

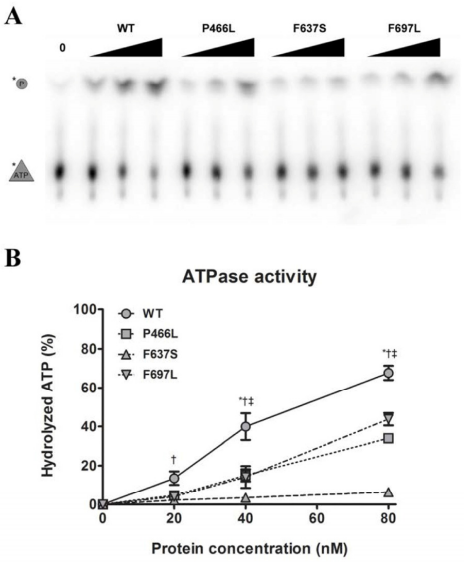

Figure 4. Decreased helicase activity correlates with lower ATPase activity. (**A**) Representative gel showing ATPase activity of WT and mutants, at 0, 20, 40, and 80 nM protein. (**B**) ATPase data compiled from triplicate experiments. The activities of P466L, F697L and in particular F637S are significantly reduced compared to wild-type (at 50%, 65% and 9% of WT at 80 nM protein, respectively). *, †, and ‡ denote $p < 0.05$ between WT and P466L, F637S and F697L, respectively. Error bars represent standard error of mean from three experiments.

Deficient helicase activity correlates with inability to hydrolyze ATP

The helicase activity of RECQL4 is dependent on hydrolysis of ATP [26], which in turn requires the presence of DNA. Our next step was therefore to examine the ability of the mutants to hydrolyze ATP in the presence of DNA. We observed the cleavage of labeled phosphate (top band) from ATP (bottom band) during incubation with a fixed concentration of DNA and varying concentrations of protein (Fig. 4A). Plotting the data revealed a pattern similar to that observed for helicase activity. P466L and F697L showed significantly reduced activity as compared to WT, with ~40% of WT activity at 20 and 40 nM and peaking at ~50 and 65% at 80 nM protein for P466L and F697L, respectively (Fig. 4B). As we saw for helicase activity, the F637S mutant was even more strongly affected, peaking at approximately 10% of WT activity for 80 nM protein. This pattern was confirmed using the alternate protein preparation, though F637S was not quite as severely impacted.

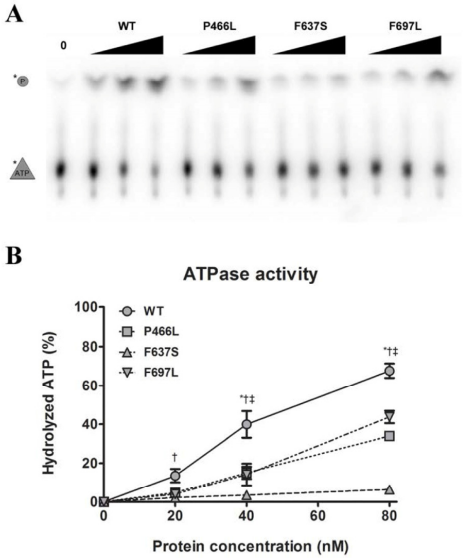

Figure 5. Strand annealing activity is not correspondingly reduced: Amalgamated gel from representative experiments showing annealing activity of WT and mutants at 0, 2.5, 5, 10 and 20 nM protein, as well as double- and single-stranded controls. (**B**) Annealing data compiled from triplicate experiments. At 2.5 and 5 nM protein, WT shows significantly higher activity than F637S, but at higher concentrations this difference is not seen. ‡ denote $p < 0.05$ between WT and F697L. Error bars represent standard error of mean from three experiments.

Strand annealing activity is not correspondingly affected by mutations

Like the other RecQ helicases, RECQL4 can pair single-stranded DNA to double-stranded DNA. This annealing activity is in direct opposition to the DNA unwinding (helicase) activity, such that a variation in annealing activity could change the amount of unwound substrate in helicase assays and thereby lead to an

apparent difference in helicase activity. To test this, we performed strand annealing assays utilizing a 5'-labeled single-stranded DNA substrate and a complimentary strand in the absence of ATP. We observed the reduction of the lower band (single-stranded DNA) and the corresponding appearance of the higher band (double-stranded DNA) with increasing protein concentration. Note that since the annealing activity of RECQL4 is stronger than the helicase activity, a much lower range of protein concentrations was used here. As shown in Fig. 5B, F697L showed significantly lower activity than WT, at 20 and 40% for 2.5 and 5 nM protein, respectively, while the other two mutants show an apparent but not significant decrease. At higher concentrations, corresponding to the lowest concentrations used in the helicase assays, there is no significant difference between WT and mutants (also confirmed with the alternate protein preparation).

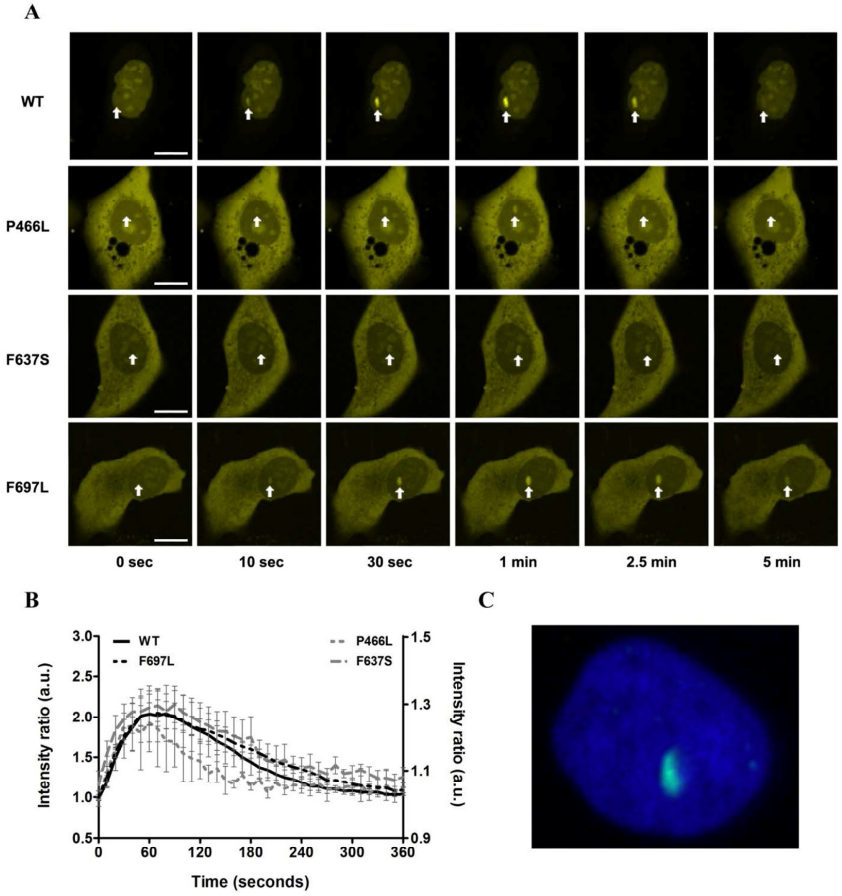

Figure 6. Recruitment to DNA double-strand breaks is not affected. (**A**) The recruitment of either WT or mutant RECQL4 to DNA double-strand breaks induced by microirradiation is shown for six time points (0, 10, 30, 60, 150 and 300 seconds). Damage sites are indicated by white arrows. In all cases recruitment occurs rapidly, peaks after about one minute and has largely abated after five minutes. Scale bars are 10 μm. (**B**) Protein recruitment quantified as the ratio of intensities at the damage site compared to the rest of the nucleus. The absolute signal from five examined cells is plotted with standard error of mean. While there is variation in absolute signal, the recruitment and retention dynamics of WT and mutants are remarkably similar. Only P466L shows a trend of accelerated release from the damage site. WT and F697L are plotted on the left y-axis, P466L and F637S on the right. (**C**) Immunohistochemical staining of γH2AX (green) following laser irradiation of the nucleus (DAPI, blue) to confirm the creation of double-strand breaks.

All mutants recruit to DNA double-strand breaks with equivalent dynamics

To evaluate whether the mutant proteins are stable in human cells we expressed YFP-RECQL4 fusion proteins (WT and mutants) in U2OS cells, and as a functional test monitored recruitment to sites of double-strand breaks induced by micropoint laser irradiation [25]. We observed recruitment of both WT and mutant RECQL4 to the damaged site within ten seconds (Fig. 6A). Fig. 6B shows the time course of recruitment, with recruitment level represented as the ratio of signal at the damaged site vs. signal level of the rest of the nucleus. For both WT and mutants, accumulation saturated after about one minute and had mostly faded after five minutes. While there is variation in the absolute signal, the recruitment and retention dynamics of WT and mutants are very similar. Only P466L shows a trend of accelerated release from the damage site.

Trafficking of RECQL4 between cytoplasm and nucleus has been reported previously [45], and we observed varying relative distribution of RECQL4 in these two compartments. Although we did not examine cellular localization in detail, the WT protein showed predominantly nuclear localization more frequently than the mutants. Since the examined mutations do not fall within nuclear localization or retention signals (Fig. 1**C**) we speculate that a potential difference in localization might arise from the presence of a greater amount of non-fusion GFP in the cytoplasm, perhaps originating from partially degraded fusion protein (Supp. Fig. 3). Simply, we conclude that the mutant proteins localize to DNA damage sites with largely unaltered dynamics, albeit with the caveat that these mutants may show a different nuclear-cytoplasm distribution than WT RECQL4.

DISCUSSION

Our data shows that the helicase and ATPase activities are strongly affected for all three mutants, similar to what was recently found for the c.1390+2delT mutation found in other RAPADILINO patients that causes a 44 amino acid deletion just prior to the helicase domain [46]. In view of the ATP-dependent nature of the helicase activity, this could be interpreted in two ways: either a given mutation prevents ATP hydrolysis, which in turn inactivates the helicase function, or the mutation disrupts helicase activity, which results in ATP not being hydrolyzed regardless of the capacity for doing so. Given that ATPase activity was measured in the presence of single-stranded DNA that would not provoke unwinding, we find the first option most likely. While our sample size is limited, it is noteworthy that all three mutations seem to target the same enzymatic capacity; it might be edifying to examine additional patient mutations in this manner to see how broadly the pattern fits.

It must be noted that at low protein concentrations WT showed higher strand annealing activity than the mutants. While this may be of consequence on its own, it is unlikely that it would influence our observations regarding helicase activity: the difference in annealing activity was evident only at protein concentrations low enough that effectively no helicase activity was observed, while at concentrations comparable to those used in the helicase assay the annealing activity of WT and mutants had converged. From our data we cannot say whether annealing activity is in itself important for the biological role of RECQL4, nor whether this function is affected in some human patients.

Although the examined mutants display very similar patterns of activity, minor differences do emerge in the various assays. These presumably arise from the differences in position and physiochemical properties of the mutated residues. Based on the model structure of shown in Fig. 1B, we can speculate on the observed changes in activity and the location of each mutation examined in this study. In the model structure, P466 is located at the N-terminus of an α-helix, relatively close to the ATP binding region. Proline residues at α-helix N-termini are known to stabilize proteins [47], and are also well known helix-breakers. Therefore, the P466L substitution may produce an aberrant elongation of the α-helix which could disrupt local structure enough to impair activity. Alternatively, the P466L substitution may loosen the α-helix, and this decrease in local rigidity may affect the ability to hydrolyze ATP. F637S occurs within a β-sheet that is fully buried in the helicase core (RecA-like domain 1). Thus, the substitution from aromatic to hydrophilic residue may destabilize the helicase core, and thereby affect the catalytic activity (indeed, F637S exhibited the least helicase and ATPase activity). F697 is located in a loop between an α-helix and a β-sheet in conserved motif IV (Fig. 1A). Since this β-sheet also forms the core of the RecA-like domain 2, the elimination of an aromatic side chain may again alter the local conformation of this region. These hypotheses are consistent with the structural data presented in Supp. Fig. 2 and Supp. Table 2, which suggests the possibility that F637S may be partially destabilized, while P466L and F697L appear more intact structurally.

In addition to the implicit relevance of human disease-associated mutations, the syndromes involving RecQ helicases are of interest because they display segmental

premature aging and can arguably be used to study normal human aging. This is most obvious in Werner syndrome, where most mutations lead to truncation of the protein; Bloom syndrome is also most commonly the result of truncation, although loss-of-function missense mutations have been reported [7]. While some mutations in *RECQL4* also lead to loss of protein, both missense mutations and the deletion of exon 7 common in RAPADILINO syndrome [41] stand in contrast to the truncations of WRN and BLM. It is therefore noteworthy that the missense mutations examined here result in loss of function, in the same vein as mutations of WRN and BLM.

With this in mind, what can our results impart about the origin of disease in these patients? Two lines of reasoning present themselves. It could be argued that despite the relatively weak helicase activity displayed by RECQL4, this activity is crucial for the proper functioning of the protein. Because RECQL4's function in replication depends on the N-terminal region, and not on the helicase domain [31,42], this interpretation presupposes that RECQL4 serves more than one role in the cell, and that the observed phenotype stems from dysfunction in a role other than initiation of replication. This hypothesis is strongly supported by a recent study on cells expressing RECQL4 without the helicase and C-terminal domains; these cells replicate normally when unstressed, but are sensitive to ionizing radiation, which induces S-phase arrest [48]. This would suggest that at least the active helicase function is not required for RECQL4's role in replication, consistent with earlier observations [31]. It also does not rule out the possibility that RECQL4 could function as a passive helicase alongside MCM2-7, as has previously been suggested [21]. One argument to support this interpretation is that RECQL4 retains the (functional) helicase domain that defines the family; if this domain did not serve any cellular function, one would not expect it to have been evolutionarily conserved. Further, the fact that these mutations did not significantly reduce the capacity to bind DNA substrates could mean that the mutants bind their normal cellular substrate, but are unable to process it and thereby block the relevant pathway. This is particularly plausible in light of the opposed enzymatic activities for unwinding and annealing of DNA. Since the mutations did not reduce the annealing activity as they did the helicase, the mutant proteins might actively counteract the unwinding of a substrate.

Another interpretation is that while the helicase activity is affected in these mutants, this is a consequence of the deficient ATPase activity, and the helicase activity not important in itself. Rather, the ability to hydrolyze ATP is required for an independent function of the protein, and its deficiency produces the observed phenotype. This function could involve interactions with other proteins, possibly in a recruitment role. While it is impossible to draw extensive conclusions regarding this hypothesis based on the data presented here, the fact remains that only about two-thirds of patients with Rothmund-Thomson syndrome have mutations in *RECQL4*. Given that the disease phenotype can arise independently of *RECQL4* mutations, it should be reconciled whether the affected function of RECQL4 depends on another protein that is also impaired by a mutation causing the remaining third of RTS cases. Identifying interaction partners of RECQL4 could help to further test this hypothesis.

In conclusion, our findings reveal that mutations of RECQL4 from three RAPADILINO patients all reduce the helicase and the ATPase activities of the protein. These observations are consistent with the hypothesis that RECQL4 has one or more cellular functions in addition to its role in initiation of replication, functions which are dependent on the a functional helicase domain. Further study of RECQL4 patient mutations could resolve whether such a secondary function underlies the disease phenotypes.

METHODS

Protein expression and purification. The wild-type RECQL4 (WT) and the three mutants (P466L, F637S, and F697L) were expressed and purified identically. WT RECQL4 with a cleavable N-terminal glutathione S-transferase (GST) tag and a C-terminal 9-histidine tag was inserted into the pGEX6p1 vector (GE Healthcare), and mutants were generated by site-directed mutagenesis using PCR amplification, as described previously [28]. Plasmids were transformed into *E. coli* Rosetta2 (DE3) (Novagen), and cultures grown at 37°C until the OD_{600} reached 0.6. Protein production was induced by adding 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and cultures were further incubated at 16°C for 16 hours. The transformants were then harvested by centrifugation and stored at -80°C.

Purification was carried out as described previously [28], with the following modifications: the first column used was 140 mL MonoQ to accommodate a larger volume of lysate, and elution from the SP sepharose column was done with a 250-500 mM KCl gradient. Protein concentrations were determined by gel analysis combined with bicinchoninic acid (BCA) assays.

An alternate protocol for expression and purification was used to verify that any difference in activity within

independent protein preparations was not an artifact of the purification. The alternate protocol is described in the supplementary information. We attempted purification of three additional RECQL4 mutants (R522C, R522H and L678P), but these turned out to be unstable using both the original and alternate purification protocols (see Supp. Fig. 1) and were not subjected to further analysis.

Oligonucleotide substrates. Oligonucleotides were synthesized and PAGE purified by Integrated DNA Technologies (Coralville, IA), with sequences listed in Supp. Table 1. Indicated strands were 5' radiolabeled with [γ-^{32}P] ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs). Unincorporated [γ-^{32}P] ATP was removed using MicroSpin G-25 columns (GE Healthcare). To create fork and full duplex substrates, corresponding oligonucleotides were combined in annealing buffer (50 mM Tris–HCl pH 7.0 and 25 mM KCl) in a 1:2 ratio of labeled to unlabeled oligonucleotide, heated to 90 °C for 10 minutes and cooled slowly to room temperature.

Enzymatic assays. For DNA binding assays, RECQL4 (amount indicated in figure legends) was incubated in reaction buffer (25 mM Tris HCl, pH 7.4, 50 mM KCl, 0.1 mg/mL bovine serum albumin (BSA), 1 mM DTT) with 0.5 nM labeled Fork-Top DNA for 15 minutes at room temperature. Reactions received glycerol to 17% for loading and were run at 4°C, 100V for 80 minutes on a 5% 29:1 acrylamide:bis-acrylamide gel in 0.5x Tris/Borate/EDTA buffer.

For helicase assays, RECQL4 (amount indicated in figure legends) was incubated in reaction buffer (30 mM Tris HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 11% glycerol, 0.1 mg/mL bovine serum albumin, 1 mM DTT, 5 mM adenosine triphosphate (ATP)) with 0.5 nM labeled Fork-Top/Fork-Bottom for 30 minutes at 37°C . Stop dye (10 mM Tris HCl, pH 8.0, 10 mM EDTA, 10% glycerol, 0.3% sodium dodecyl sulphate (SDS)) was added to reactions before running at 125V for 120 minutes on a 12% 19:1 acrylamide:bis-acrylamide native gel in 1x Tris/Borate/EDTA buffer.

For ATPase assays, RECQL4 (amount indicated in figure legends) was incubated in reaction buffer (30 mM Tris HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 11% glycerol, 0.1 mg/mL bovine serum albumin, 1 mM DTT, 50 µM (cold) ATP, 10 µM 10 µCi/µL [γ-^{32}P] ATP) with 0.2 µM M13mp18 single stranded DNA (New England Biolabs) for 1 hour at 37°C. 167 mM EDTA was added to stop reactions before separating samples by thin-layer chromatography on Baker-flex Cellulose PEI sheets (J.T. Baker) for 45 minutes in 0.8 M LiCl, 1 M formic acid.

For strand annealing assays, RECQL4 (amount indicated in figure legends) was incubated in reaction buffer (30 mM Tris HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 11% glycerol, 0.1 mg/mL bovine serum albumin, 1 mM DTT) with 0.5 nM labeled Fork-Top oligo and 0.5 nM unlabeled Fork-Bottom for 20 minutes at 37°C temperature. Stop dye (10 mM Tris HCl, pH 8.0, 10 mM EDTA, 10% glycerol, 0.3% SDS) was added to reactions before running at 125V for 120 minutes on a 16% acrylamide native gel in1x Tris/Borate/EDTA buffer.

All gels were exposed on storage phosphor screens (GE Healthcare) and scanned using a Typhoon 9400 imager (GE Healthcare). Resulting images were analyzed using ImageQuant 5.2 (GE Healthcare). All assays were performed in triplicate, and error bars plotted as standard error of mean. All graphs are normalized to the negative control as zero activity.

Thermostability testing. Evaluation of the thermostability of WT and mutants was performed by measuring the increase in signal from SYPRO® Orange protein stain (Life Technologies) upon temperature-induced protein unfolding [44]. 2 µg protein in 20 µl buffer containing 20 mM KH_2PO_4 pH 7.5, 178 mM KCl, 6% glycerol, 1 mM DTT and 5x SYPRO® Orange were incubated in a MyIQ quantitative polymerase chain reaction (PCR) machine (BioRad), and subjected to 141 steps of 0.5°C increase in temperature every 15 seconds, for a total range of 20-90°C. Fluorescent signal was measured throughout, with peaks describing unfolding of the protein and the resulting increase in SYPRO® Orange binding. Melting curves were calculated from these peaks as described previously [44].

Preparation of plasmids for fluorescence microscopy. Vector YFPc2 (Clontech) was digested with EcoRI and SalI, followed by insertion of either WT or a mutant gene (P466L, F637S or F697L) in an EcoRI and XhoI fragment from the pGEX6p1 vector described in section 2.1. The resulting vectors were verified by sequencing, and used to express YFP-RECQL4 (WT or mutant) fusion protein for microscopy, as described in section 2.6.

MicroPoint laser irradiation and microscopy. U2OS cells were maintained in DMEM (Gibco), supplemented with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 g/ml) (Gibco, Life technologies), and grown at 37°C in a humidified atmosphere containing 5% CO_2. One day prior to transfection approximately 10^5 cells were seeded in 15 mm dishes with thin glass

bottoms (Mat-Tek). Cells were transfected with Lipofectamine LTX (Life Technologies Inc.) according to manufacturer's instructions using 1 μg of the relevant vectors. Targeted DNA damage was introduced using the MicroPoint® Ablation Laser System from Photonic Instruments at 14% laser power (3.3 μW), and fluorescent protein recruitment and retention monitored as described previously [49]. Images were acquired every 10 seconds for at least 5 minutes. Immuno-histochemical staining to confirm the presence of double-strand breaks was performed as described previously [49].

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Conflict of Interest Statement

The authors of this manuscript have no conflict of interests to declare.

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SUPPLEMENTAL DATA

Alternate protein expression and purification

Human RecQL4 with N-terminal GST and C-terminal 9-His tags was expressed using the pGEX6p1 vector (GE Healthcare) in the E. coli strain Rosetta2 (DE3) (Novagen) as described in section 2.1. Frozen cell pellet from 8 L culture (~30 g) was resuspended in 150 mL of lysis buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, 25 mM Imidazole, 1 mM PMSF, 1 tablet/50 ml Complete EDTA-free Protease Inhibitor Cocktail (Roche)), disrupted by sonication, spun 30 min. at 37,000g, and filtered through 45 µm membrane. All purification steps were carried out at 4°C using ÄKTApurifier instrument (GE Healthcare). Clarified lysate was loaded onto HisTrap FF 5 mL column equilibrated in Buffer A (50 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol) supplemented with 25 mM Imidazole. The column was washed with 20 column volumes (CV) of Buffer A + 50 mM Imidazole, and the protein eluted with 10 CV of Buffer A + 250 mM Imidazole. Fractions containing RecQL4 were pooled, and loaded onto GSTrap 4B 5 mL column equilibrated in Buffer A. The sample was recirculated through the column for 1 hour to maximize binding. Next, the column was washed with 10 CV of Buffer A and developed with Buffer A supplemented with 50 mM reduced Glutathione. Peak fractions with RecQL4 protein were pooled, adjusted with DTT to 1 mM final concentration, and subjected to cleavage with 120 units of PreScission protease (GE Healthcare) for 15 hrs. at 4°C. Cleaved protein was diluted 1:6 (v/v) with Buffer A + 25 mM Imidazole and loaded onto HisTrap FF 5 mL column equilibrated in the same buffer. The column was washed with 30 CV of Buffer A + 75 mM Imidazole and eluted with 10 CV of Buffer A + 250 mM Imidazole. Fractions with purified RecQL4 protein were pooled and concentrated using Amicon® Ultra-4 centrifugal filter unit with 50 kDa cutoff (Millipore). Concentrated protein was diluted 1:1 with cold 100% glycerol, aliquoted and stored at -80 °C.

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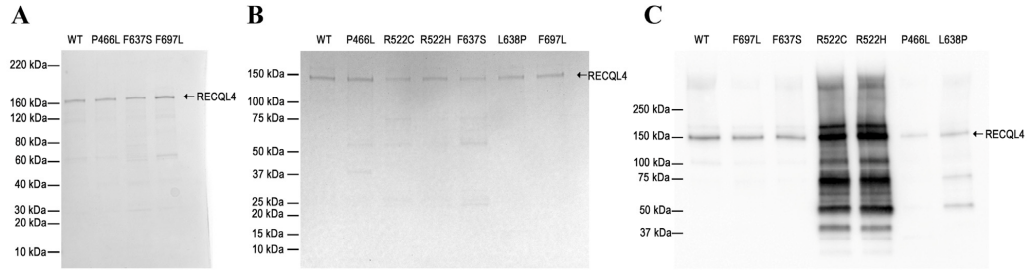
Supplementary Table 1. Oligonucleotide

Oligo	Sequence (5'-3')
Fork-Top	GTAGTGCATGTACACCACACTCTTTTTTTTTTTTTT
Fork-Bottom	TTTTTTTTTTTTTTGAGTGTGGTGTACATGCACTAC

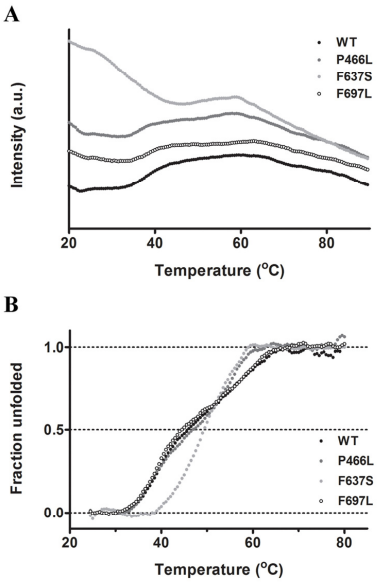
Supplementary Table 2. Estimated melting points of WT and mutants. The melting temperatures of WT and mutants from the data in Supp. Fig. 2 using approximated single-step unfolding. Except for F637S no significant differences are apparent, suggesting that the mutant proteins retain the same overall structure. The change in Tm (ΔTm^{obs}) was calculated as follows: $\Delta Tm^{obs} = Tm^{obs}$ (mutant) $- Tm^{obs}$ (wild type). Experimental error, ± 2.5 °C, n=2.

Protein	T_m^{obs} (˚C)	ΔT_m^{obs} (˚C)
WT RECQL4	45.8	-
P466L	47.0	+1.2
F637S	49.3	+3.5
F697L	44.5	-1.3

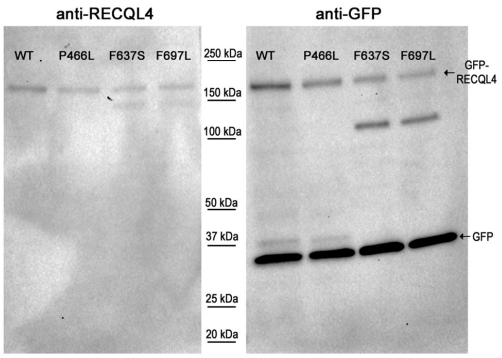
$\Delta T_m^{obs} = T_m^{obs}$ (mutant) - T_m^{obs} (wild type)
Experimental error = ±2.5 ˚C, n = 2



Supplementary Figure 1. Expression and purity of proteins. (**A**) Protein gel from the primary purification used for biochemical assays, showing WT RECQL4 and the three examined mutants. Lanes are loaded with approximately 0.3 µg protein. (**B**) Protein gel from the alternate purification, showing WT and all mutants. Lanes are loaded with approximately 1 µg protein. (**C**) Western blot for RECQL4 using in-house antibody (described in (1)), showing degradation of R522C, R522H and L638P. Lanes are loaded with approximately 0.1 µg protein.



Supplementary Figure 2. Protein unfolding data. (**A**) Raw fluorescence data from the SYPRO® Orange-based thermostability assay, offset for clarity. Protein unfolding manifests as an increase in fluorescence superimposed on the steady decrease inherent to the assay. While F637S shows clean single-step unfolding (a single rise in intensity), both WT and the remaining mutants appear to demonstrate more complex unfolding. (**B**) Melting curves for WT and mutants are generated by quantifying the relative increase in fluorescence upon unfolding, and melting temperatures extracted at the point of 50% unfolding.



Supplementary Figure 3. Expression of GFP-RECQL4. Lysates from cells used in microscopy experiments (Fig. 6), expressing (WT or mutant) GFP-RECQL4 were examined by Western blotting against RECQL4 (left) and GFP (right). All samples show a band at the expected size of GFP-RECQL4, though free GFP is also present in all samples.

2.2 Dynamics of WRN & BLM localization

The second project examines the WRN and BLM helicases, albeit with a view to draw more general conclusions about the trafficking and localization of DNA repair proteins between subcompartments of the nucleus. Because of this, the specifics of these proteins (aside from their nucleolar localization and recruitment to sites of DNA damage) are of lesser importance, and I will therefore proceed directly to a discussion of our research.
This project was a collaboration with the Center for Models of Life at the Niels Bohr Institute, University of Copenhagen, and a central feature was to use mathematical modelling in combination with experimental data in order to describe a biological system. The system in question was the dynamic localization of DNA repair proteins, and based on the expertise and materials available in our lab WRN and BLM were chosen as specific exemplars. Both proteins localize to the nucleus, where they in unstressed cells upconcentrate in nucleoli by an unknown mechanism [219, 220]; upon induction of DNA damage they gradually accumulate in foci with γH2AX and other markers of DNA damage. We wanted to examine whether this redistribution was purely limited by the proteins diffusing around the nucleus, or whether active trafficking could be involved in their recruitment to damage. To this end we set up a system combining micropoint laser irradiation to induce DSBs with fluorescence recovery after photobleaching (FRAP) to measure the mobility of fluorescently tagged proteins. By specifically targeting regions in the nucleoplasm, nucleoli and sites of DNA damage (where we first induced damage with a high laser intensity and then bleached using a lower intensity) we obtained data that could be fitted with different mathematical models to determine limiting factors for fluorescence recovery.
Our modelling was based on reaction-diffusion systems and presented three related ways to fit the data: a complete reaction-diffusion model (hereafter referred to as the full model) that has previously been shown to provide a good description of protein dynamics measured by FRAP [221], a reaction-limited model and a diffusion-limited model (for full derivations see Section 2.2.1). The latter two are simplified versions of the full model that assume either that the protein does not undergo binding reactions/that the binding affinity is very low (diffusion model), or that binding occurs with strong affinity such that diffusion ceases to be a limiting factor (reaction model). By fitting all three models to our FRAP data we can determine whether either of these assumptions is true based on whether either of the simplified models fit the data as well as the full model. In such cases we can use the relevant model to reveal additional information about the system: When the system is described by diffusion we can calculate an effective diffusion coefficient, which may be lower than what would be expected from the size of the protein if low-affinity binding events take place. We can then calculate the fraction of protein that is bound at any given time. Conversely, if the dynamics are reaction limited we can calculate the limiting factor, namely the unbinding rate of the protein.
With this approach we were able to describe distinct dynamics for WRN and BLM at each location in the nucleus: in the nucleoplasm both proteins are described by diffusion, but with a much slower effective diffusion rate than expected from their size. This is caused by very frequent binding and unbinding events, whereby the vast majority of protein is in the bound state at any given time. On the other hand, in nucleoli both proteins are limited by slow unbinding events, which establishes that their higher concentration in this subcompartment is not exclusively caused by a higher number of

binding sites. Finally, both proteins display more complex dynamics at sites of DNA damage: first, both a mobile and an immobile fraction exist, where the latter may represent proteins involved in DNA repair. Second, while the FRAP data was described by effective diffusion, the accumulation at sites of damage occurred on a much slower time scale that could not be limited by diffusion. This accumulation was thus limited by the gradual creation of binding sites, which may represent the recruitment of other DNA repair proteins and/or histone modifications surrounding the damaged site.
In addition to these primary observations, we applied our modelling to a range of previously published FRAP studies on different DNA repair proteins. We discovered that these proteins were uniformly described by effective diffusion (in the nucleoplasm), but that they grouped into two distinct families. The first of these has effective diffusion coefficients close to the predicted values, indicating little interaction with DNA in unstressed conditions. Based on this we term this family 'Responders', on the assumption that their recruitment to sites of DNA damage is dependent on signalling from other proteins. Members of the second family have manifold lower effective diffusion coefficients than predicted, indicating frequent binding and unbinding events that may represent scanning of DNA for damage (though not necessarily for breaks themselves). We term this family 'Scanners', and speculate that its members include early participants in DNA damage responses.

2.2.1 Paper II

Distinct dynamics for DNA repair proteins in the nucleoplasm, nucleoli and at sites of DNA damage.

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Abstract

DNA damage is contained by DNA repair proteins and failure to find and repair damage leads to devastating diseases such as cancer and premature aging. While extensive research has been done on the functions of DNA repair proteins, it is not well understood whether proteins find DNA damage in a processive manner by "scanning the DNA", or in a distributive fashion by diffusion. Furthermore, little is known on how the search dynamics are affected by the nuclear distribution of the proteins prior to the damage. We study the mobility of two GFP-tagged DNA helicases, WRN and BLM, in response to DNA double-strand breaks (DSB) at three locations: nucleoli, nucleoplasm and at the DSB. We find that both WRN and BLM employ a "DNA-scanning" mechanism. All three compartments have distinct dynamics: "effective diffusion" in nucleoplasm, "reaction-limited" in nucleoli and the presence of a WRN/BLM immobile fraction at DSB. Surprisingly, the accumulation of the WRN/BLM at DSB is not limited by search time, as the accumulation is much slower (∼1h) than either the nucleoplasmic or nucleolar dynamics.

Introduction

DNA repair proteins are crucial for maintaining genomic stability through a variety of pathways [1] . Because such repair processes invariably involve multiple steps several classes of proteins are involved, including helicases such as Werner syndrome helicase (WRN) and Bloom syndrome protein (BLM), as well as damage sensing proteins like xeroderma pigmentosum group C-complementing protein (XPC) and serine protein kinase ATM (ATM). Mutations in these proteins lead to devastating diseases, e.g. Werner syndrome and ataxia telangiectasia, with symptoms that include premature aging [3] and increased risk of cancer [2]. Most of DNA repair proteins (e.g. ATM, WRN, BLM) tend to accumulate at the site of DNA damage when cell is subjected to genotoxic stresses[3].

While extensive research has been done on the functions of DNA repair proteins, it is not well understood how they find the site of DNA damage: Do they do it by processively scanning genome, or in a distributive fashion by diffusion [4]. In the first scenario proteins scan genome by engaging in multiple weak and "unspecific" binding/unbinding events with the cellular chromatin. At the site of damage, proteins bind with a stronger binding affinity and thus accumulate at high concentration.

In an alternative scenario, DNA repair proteins do not scan, but instead diffuse freely until they at random encounter a site of damage, where they form active complexes and get bound to the chromatin. The search time and consequently the dynamics of damage response can further depend on how the proteins are distributed throughout the nucleus and their respective mobilities in nuclear subcompartments (e.g. nucleoli).

In this work we investigate the spatial dynamics of two DNA repair proteins (WRN and BLM) in three nuclear sub-compartments: the nucleoplasm, nucleoli and sites of DNA damage. To distinguish between the two scenarios discussed above we have performed Fluorescent Recovery After Photobleaching (FRAP) experiments. Comparing experimentally obtained recovery curves with mathematical reaction-diffusion (R-D) models we find that in the nucleoplasm the most probable scenario is that neither WRN nor BLM proteins diffuse freely; rather, they undergo rapid cycles of binding/unbinding, thus scanning for DNA damage through "effective diffusion". This "effective diffusion" is slower than free diffusion because the motion of WRN and BLM is impeded by binding/unbinding events that temporarily immobilize the proteins. The dynamics at nucleoli appear to be distinctly different, with the process effectively "reaction limited" by infrequent unbinding events rather than by diffusion. At sites of DNA damage the dynamics is of particular interest with both WRN and BLM existing as two subpopulations: a) a "mobile" fraction (~80%) with a binding-unbinding turnover of about 1 minute, and b) an "immobile" fraction (~20%) that remains bound to DNA for significantly longer. Furthermore, accumulation of the proteins at damage sites is surprisingly slow, saturating after roughly 2 hours, and can not be explained by diffusion from nucleoli.

Results

The spatio-temporal dynamics of proteins is often well described by the reaction-diffusion models. By fitting solutions of the R-D models to fluorescent recovery curves, one can identify whether there are distinct rate-limiting steps in the dynamics of interest. Furthermore one can distinguish whether the rate is limited by diffusion of the protein or rather the binding/unbinding reactions. To establish the nature of the rate limiting step, we followed the methodology by Sprague et al. [5] and compared the quality of fits for the three different mathematical models: a full R-D model, Diffusion model and Reaction model.

Diffusion and Reaction models are the simpler cases of the full R-D model where diffusion (reaction) are set to be rate-limiting. Thus if binding/unbinding reactions are much faster than diffusion, the diffusion is a rate limiting step, and thus the Diffusion model will fit the data better than the Reaction model, and nearly as good as full R-D model. If on the other hand, the binding/unbinding reactions are much slower than diffusion, the reaction is a rate limiting step, and fit of the Reaction model will better than the Diffusion model and comparable to the full R-D.

Each of the two simple models provides additional information about the protein dynamics:

Diffusion model: When data can be reliably fitted with a Diffusion model one can calculate the diffusion coefficient. slower, "effective" diffusion coefficient can be calculated. The impeded motion is a result of binding/unbinding events, and so we can calculate the ratio between the pseudo binding rate (k_{on}^{*}) and the unbinding rate (k_{off}). This pseudo binding rate (k_{on}^{*}) is the binding rate multiplied by the concentration of binding sites. From the ratio between the rates we can estimate the fraction of bound proteins.

Reaction model: When the data can be fitted by the Reaction model the limiting factor is the unbinding rate (k_{off}), which can be calculated directly from the fitting.

The parameters obtained from fitting can be used to differentiate between "DNA scanning" and free diffusion scenarios. The later is true if Diffusion model fits the data best and there is no binding, or the binding is negligibly weak ($\frac{k_{on}^{*}}{k_{off}} \ll 1$). Otherwise, any other parameter ranges are supporting the "DNA scanning" mechanism.

WRN and BLM undergo fast binding/unbinding cycles in nucleoplasm with about 99% of proteins bound to chromatin at any time.

We analyze WRN mobility as shown in Figure 1. We find that the *Reaction model* provides the worst fit, while the *Diffusion model* fits the data just as well as the *full R-D model*. Thus estimated WRN diffusion appears to be much slower (with diffusion coefficient $D_{WRN-eff} = 0.145 \frac{\mu m^2}{s}$) than diffusion of GFP ($D_{GFP} = 28 \frac{\mu m^2}{s}$) reported earlier in [6]. This difference can not be explained by the larger size of the WRN-GFP fusion alone. From the diffusion constant of GFP and protein sizes of WRN/BLM we estimate the diffusion of the fusion protein should be 100 times faster, with $D_{WRN-GFP} = 14.7 \frac{\mu m^2}{s}$ (see methods).

Sprague et al. [5] showed that such cases can occur when proteins engage in fast binding/unbinding reactions with chromatin and can be thought of as "effective diffusion" and described by the effective diffusion coefficient $D_{eff} = \frac{D}{1+\frac{k_{on}^*}{k_{off}}}$. (Here D is the diffusion constant of the WRN- or BLM-GFP fusion proteins.)

The results for BLM are the same and the effective diffusion coefficient $D_{BLM-eff} = 0.07 \frac{\mu m^2}{s}$ is similar to that of WRN within the order of magnitude (see supplementary).

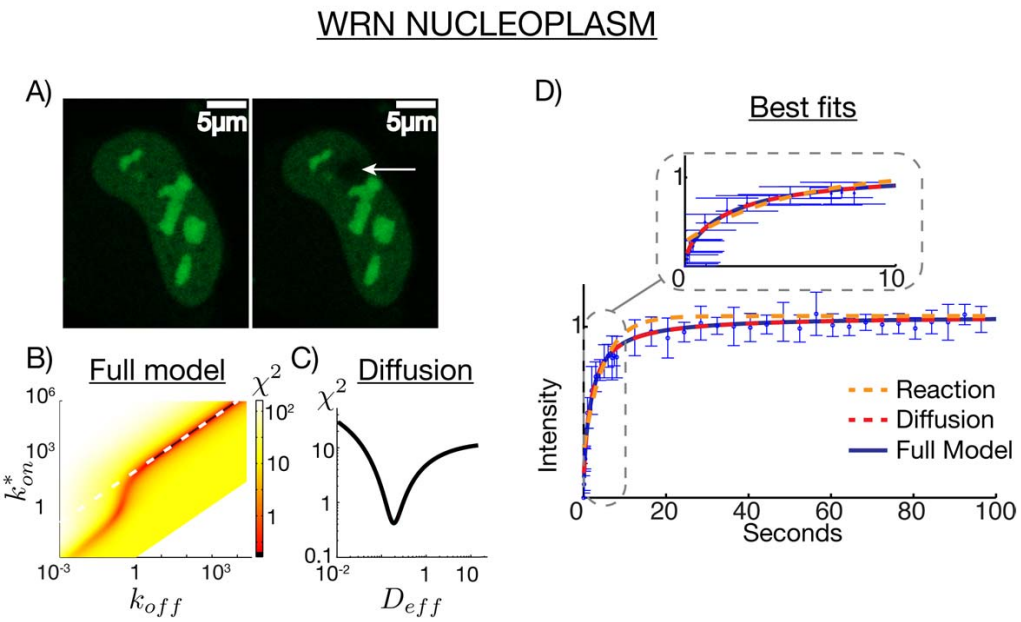

FIG. 1. Analyses of the WRN mobility in nucleoplasm.

A) *The nucleoplasm of a representative cell is shown before bleaching (left) and just after bleaching (right). The area of bleaching is indicated by an arrow.* ***B)*** *The deviation from data (χ^2) is plotted as a function of the pseudo on-rate (k_{on}^*) and off rate (k_{off}) for a full R-D model. The white dashed line indicates the best fit*

for the diffusion model, which coincides with the best fitting parameters for the R-D model (black). This indicates that the diffusion model fits the data as well as the R-D model. ***C****) Parameter scan for the diffusion model shows that the best fit occurs for the effective diffusion coefficient* $D_{WRN-eff} = 0.145 \frac{\mu m^2}{s}$. ***D****) Data is an average over 11 cells, with error bars representing standard deviation. The best fits for the 3 models (R-D as a solid blue line, diffusion as a dashed red line and reaction as a dashed orange line) are plotted alongside the experimental data (blue points with error bars).*

The fraction of unbound protein can be calculated from the ratio between the diffusion coefficient and the effective diffusion coefficient(see Methods). Remarkably, we thus find that only 1% of WRN (or 0.5% of BLM) is in the free unbound form. This means that although binding and unbinding occurs very rapidly, at any moment in time the vast majority of WRN and BLM (~99%) is in the bound state.

Expanding on this, we have calculated effective diffusion constants for a range of nuclear DNA repair proteins based on previous FRAP studies, as presented in table 1.

Nuclear Protein	**Estimated Diff. Coef.** $D_f \left[\frac{\mu m^2}{s}\right]$	**Effective Diff Coef.** $D_{eff} \left[\frac{\mu m^2}{s}\right]$	**Cell type**
Responders			
ERCC1-XPC	27	15	Hamster ovary cells[4]
PCNA	15	13	Hamster ovary cell[7]
RAD54	11	14	Hamster ovary cell [8]
RAD52	14	8	Hamster ovary cell [8]
RAD51	13	7	Hamster ovary cell [8]
NBS1	11	3	Human U2OS[9]
MDC1	8	2	Human U2OS[9]
Scanners			
Ku70	23	0.35	Hela cells and B cells[10]
Ku86	24	0.35	Hela cells and B cells[10]
BLM	14	0.17	B cells[11]*
WRN	**15**	**0.145**	**U2OS This study**
BLM	**14**	**0.07**	**U2OS This study**

Table 1: Effective diffusion coefficients can separate DNA repair proteins into scanners and responders. *The significant difference in diffusion coefficients allows us to separate proteins in two groups with "Scanners" characterized by ~100 fold difference and "responders" with less than 10 fold difference in estimated and measured diffusion coefficients. (*Diffusion coefficient not reported in reference, but calculated in this work from reported data.) The 2 studies of BLM shows that a 2 fold difference might be due to experimental setup, but the separation into responders and scanners is orders of magnitude and therefor still valid. For some DNA repair proteins a 100-fold difference between suggests constant binding/unbinding*

to chromatin or possibly exposed DNA stretches, similar to Ku proteins. For other DNA repair proteins, such as ERCC1-XPC and PCNA, the effective diffusion coefficient is close to the expected value, suggesting that they only bind to chromatin after a DNA damage response has been activated.

For many of these proteins the effective diffusion constant differs from the estimated diffusion constant by ~100 fold, corresponding to a bound fraction of $1-2\%$. Such slow effective diffusion cannot realistically be explained by complex formation or polymerization, as a 50-fold difference in diffusion coefficient corresponds to a more than 100,000-fold increase in mass. Our observation that the bound fraction far exceeds the free form thus appears to be a general feature for a class of DNA repair proteins, and has been proposed to provide a scanning mechanism when combined with fast unbinding rates [12]. In contrast to this scanning behavior, some repair proteins appear to diffuse freely in the absence of damage, and then become transiently immobilized[4]. All in all this indicates that DNA repair proteins can be classified into two categories, as shown in table 1: "scanners" with low effective diffusion coefficients due to constant scanning of DNA for damage, and "responders" which only bind to DNA after a DNA damage response has been activated.

WRN and BLM dynamics at nucleoli are reaction-limited

Under non-stressed conditions WRN and BLM are distributed unequally through the nucleus, tending to preferentially localize to the nucleoli [13], [14]. Nucleoli are subcompartments of the nucleus wherein ribosomal RNA is transcribed. They also may serve as storage depots for proteins [15]. There are no membranes separating nucleoli from nucleoplasm, yet a number of proteins (including WRN and BLM) show considerably higher concentrations in nucleoli than in the nucleoplasm. Given the scanning behavior identified in the previous section, such localization could result from two mechanisms (or a combination thereof): 1) a higher number of binding sites in nucleoli, or 2) tighter binding (manifesting as slower unbinding rates) in nucleoli compared to nucleoplasm. As shown in figure 2B, Reaction model fits FRAP measurements with the same quality of fit as the full R-D model. This holds both for WRN and BLM, with almost identical off-rates: $k_{off,WRN} = 0.12\ s^{-1}$ $k_{off,BLM} = 0.11\ s^{-1}$.

The fact that the Reaction model provides a good fit in nucleoli but not in the nucleoplasm implies that WRN and BLM have stronger affinities for the binding sites in the nucleoli compared to the binding sites distributed throughout the rest of the nucleoplasm. We can therefore rule out that localization to nucleoli is only due to a higher number of binding sites.

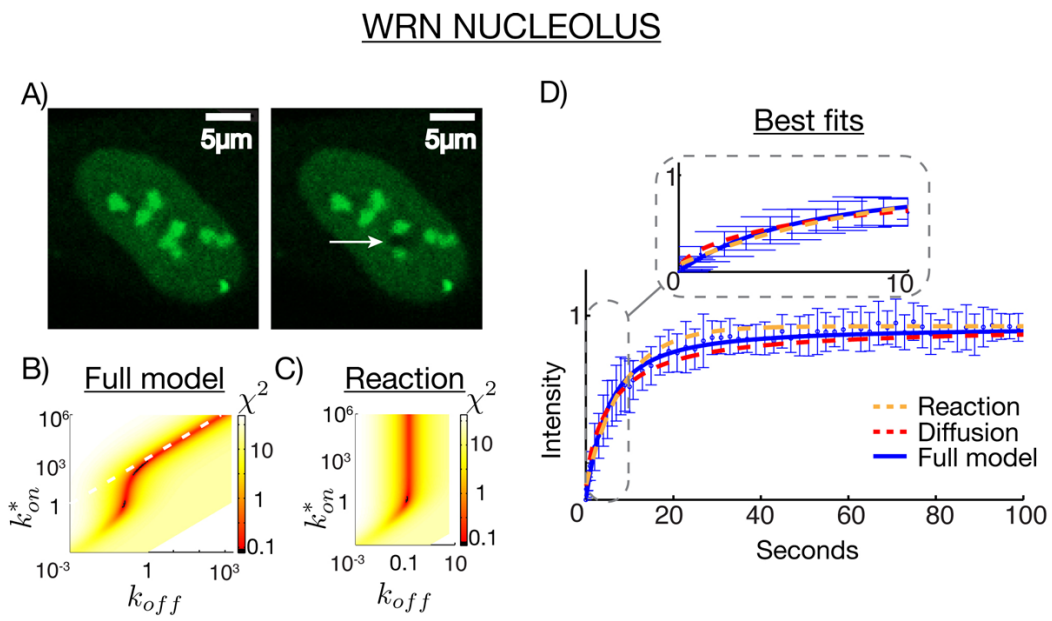

FIG. 2. Analyses of the WRN mobility in nucleoli.

A) *The nucleoplasm of a representative cell shown before bleaching (left) and just after bleaching (right). Arrow indicates area of bleaching.* ***B)*** *The deviation from data (χ^2) is plotted as a function of the pseudo on-rate (k^*_{on}) and off-rate (k_{off}) for a full R-D model. The white dashed line indicates the best fit for the diffusion model, which does not coalesce with best fitting parameters for the R-D model (black) and therefore does not provide a good fit for the data.* ***C)*** *A parameter scan for the reaction model shows that the reaction model reproduces the good parameters of the full R-D model for low values of k^*_{on} and k_{off}, with the best fit for $k_{off} = 0.12\ s^{-1}$.* ***D)*** *Data is an average over 12 cells, with error bars representing standard deviation. The best fits for the 3 models (R-D as a solid blue line, diffusion as a dashed red line and reaction as a dashed orange line) are plotted alongside the experimental data (blue points with error bars). The Reaction model provides a good fit of the data. For BLM the reaction model also fits the data as well as the R-D model, with a similar unbinding rate $k_{off,BLM} = 0.11\ s^{-1}$ (see supplementary).*

WRN and BLM dynamics at damage sites occur on two different timescales.

We performed FRAP measurements at sites of DNA damage by first using a high power laser beam ($1.8\mu W$) to induce damage, and two hours later using a low power laser beam (0.6μW) to photobleach the damage site and measuring recovery as for the other compartments (nucleoplasm and nucleoli). Control experiments were performed to show that the low laser power did not induce double stranded breaks (see supplementary). As illustrated in figure 3, the diffusion model accurately describes the behavior of WRN at damage sites just as it did for the undamaged nucleoplasm. The effective diffusion coefficient, $D_{WRN-eff} = 0.01\frac{\mu m^2}{s}$, is lower than in the nucleoplasm and thus implies that more than 99% of WRN at the damage site is in the bound state. For BLM we were not able to reduce the full model to any of the two simpler models, but the dynamics of both WRN and BLM at the damage site showed a distinctive feature in that only 80% of the signal is recovered after photobleaching (see figure 3D). This lack of recovery means that a mobile fraction of WRN and BLM (80%) exists alongside a smaller (20%) fraction that is

immobile at least on a timescale of minutes. This immobile fraction could represent proteins actively involved in DNA repair, and is incorporated in our analyses by rescaling the data as described in the methods section.

Further, the accumulation of WRN and BLM occurs on a timescale that takes several hours to peak (see figure 4). This period cannot be explained simply by the proteins diffusing and binding directly to DSBs because the breaks are created rapidly after laser irradiation and our FRAP measurements show that diffusion to the damaged site is complete after roughly one minute. The fact that accumulation at damage sites continues after this period means that a gradual creation of additional binding sites for WRN and BLM at the damage site must govern the accumulation. The additional binding sites are in such close proximity to the target of irradiation that they are indistinguishable at the resolution of our microscope (~250 nm). Since our microscopy measurements do not reveal the nature of these bindings site, we assume that upon induction of damage B^* potential binding sites are created. These potential binding sites are then at a constant rate converted into active binding sites for WRN/BLM followed by binding of WRN/BLM to the newly synthesized binding sites (which we denote *B)*. In our FRAP measurements we saw WRN and BLM find and bind to the damage site within 1 minute, which means that the find/bind step is negligible for accumulation occurring over several hours (see figure 4). We believe that the simplest model to account for the accumulation of WRN and BLM to the damage site is a conversion process of potential binding sites B^* into active B. The accumulation of WRN and BLM at the damage site is then limited by the rate of newly synthesized binding sites:

$$[B] = m(1 - \exp(-r \cdot t))$$

Here m is simply a factor to scale the saturation level. The enzymatic rates are $r_{WRN} = 0.6 \pm 0.03\ ms^{-1}$ and $r_{BLM} = 0.3 \pm 0.02\ ms^{-1}$ corresponding to half lives for the conversion of potential to actual binding sites of 20 minutes and 40 minutes respectively. Possible biological scenarios for the production of binding sites could be either complex formation of repair proteins assembling at the damage site, chromatin remodeling and/or histone modification. WRN has previously been shown to bind to γH2AX, a stretch of 2000 histones that are phosphorylated at DSBs [16]. While this histone phosphorylation occurs on a timescale of minutes [17], other slower histone modifications or resultant change in chromatin accessibility could be a possible mechanism for the creation of binding sites at DSBs [18].

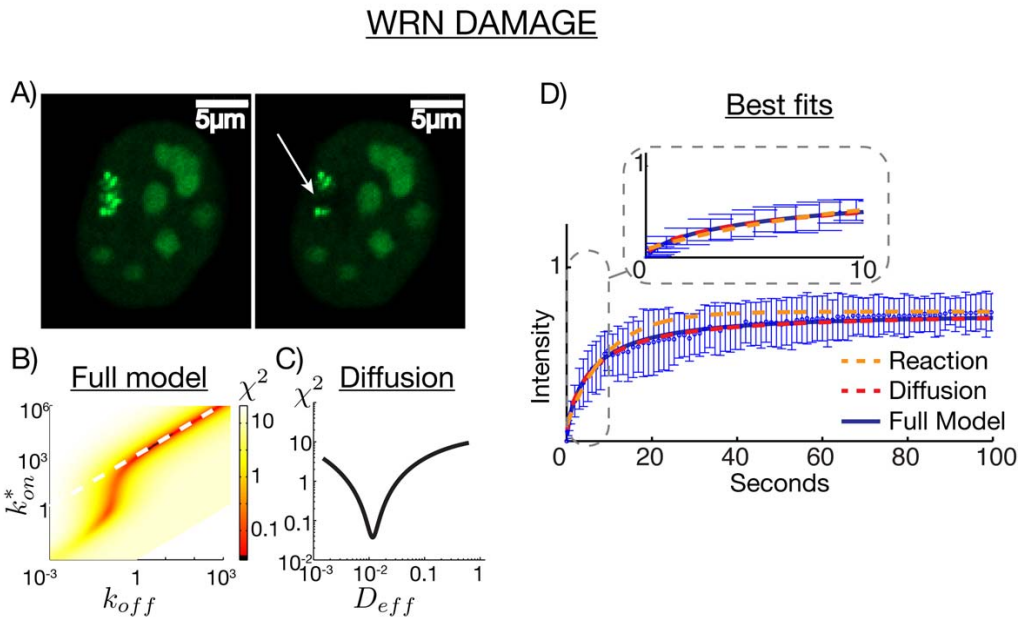

FIG. 3. Analyses of the WRN mobility damage site.

A) *The nucleoplasm of a representative cell shown before bleaching (left) and just after bleaching (right). Arrow indicates area of bleaching.* ***B)*** *The deviation from data (χ^2) is plotted as a function of the pseudo on-rate (k^*_{on}) and off-rate (k_{off}) for a full R-D model. The white dashed line shows the best fit for the diffusion model, which coincides with the best fitting parameters for the R-D model(black). This indicates that the diffusion model fits the data as well as the R-D model.* ***C)*** *Parameter scan for the diffusion model shows that the best fit occurs for the effective diffusion coefficient $D_{WRN-eff} = 0.01\frac{\mu m^2}{s}$.* ***D)*** *Data is an average over 15 cells, with error bars representing standard deviation. The best fits for the 3 models (R-D as a solid blue line, diffusion as a dashed red line and reaction as a dashed orange line) are plotted alongside the experimental data (blue points with error bars). The reaction model provides the worst fit, while the diffusion model fits the data almost as well as the full R-D model. For BLM we were not able to reduce the full R-D model to any of the simpler models (see supplementary).*

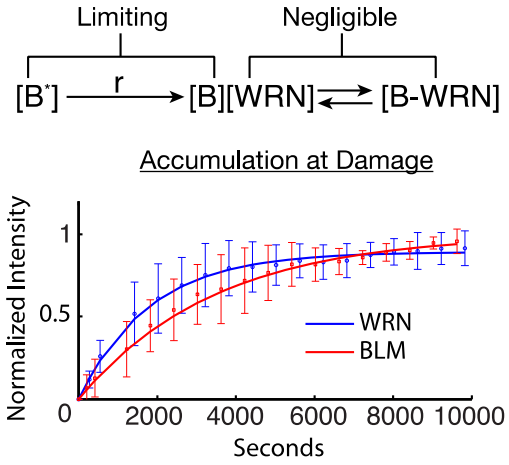

FIG. 4. The kinetics of the WRN and BLM accumulation at the site of damage

Data from 21 (WRN) and 9 (BLM) cells, with error bars showing standard deviation. Fitting to our simple model for generation of binding sites give half-lives of: $t_{\frac{1}{2}WRN} = 1152 \pm 58\ s$ *and* $t_{\frac{1}{2}BLM} = 2310 \pm 154\ s$*, corresponding to roughly 20 and 40 minutes.*

Discussion and conclusions

Our theoretical analyses and experimental data revealed several interesting points about the dynamics of WRN and BLM:

i) In the nucleoplasm WRN and BLM are characterized by the "effective" diffusion process. They undergo rapid cycles of binding/unbinding, with only a few percent of the proteins diffusing freely at any moment in time. This allows for a continuous "scanning" of the genome for possible damage sites.

ii) In nucleoli WRN and BLM are tightly bound to DNA with ten unbinding events occurring every second. This explains the higher concentration of BLM and WRN at the nucleoli under normal conditions. High affinity binding in nucleoli might occur at sites of rRNA transcription, given that WRN has been reported to interact with RNA polymerase I [19]

iii) WRN and BLM are present as two distinct species at damage sites, with an "immobile" fraction of ~20% that remains bound for at least several minutes and a "mobile" fraction that undergoes "effective" diffusion as in the nucleoplasm.

iv) The accumulation of WRN and BLM to damage was shown to be not limited by diffusion, but rather by the reactions at the damage. This, for example, could be a slow, occurring over several hours, creation of WRN/BLM binding sites at the chromatin surrounding DSBs .

In addition to these direct observations, our analysis of existing FRAP data (presented in table 1) illuminates a potential division of DNA repair proteins into "scanners" and "responders" based on their effective diffusion coefficients. We would hypothesize that scanners are generally recruited to DSBs earlier than responders, which has indeed been demonstrated for Ku versus RAD51[20]. This division might therefore be useful for characterizing repair proteins where the effective diffusion coefficients can be calculated.

Beyond this, our modeling approach allows us to make several important predictions regarding the dynamics of this system: most notably, the slow accumulation at damage sites belies an until now unidentified recruitment mechanism. If this does indeed include histone modifications (possibly as part of a chromatin remodeling response) identifying the relevant modification might give insight into a more general response that could help explain the recruitment of many DNA repair proteins. In line with this, because we propose that the accumulation is not directly mediated by the DNA lesions themselves, we expect triggering the DSB repair pathway without creating actual lesions would produce accumulation of WRN and BLM at the same timescale. An experiment along these lines has been done for mouse fibroblasts, but the focus was on the recruitment of other repair proteins [21]. More specific to the proteins studied here, because both the scanning behavior and the tight binding at nucleoli are presumably mediated by DNA binding domains, mutations that affect these binding affinities would be expected to change the dynamics in different regions of the cell. This would further depend on whether the same domain(s) mediate binding in the different regions: if a mutation exclusively affected the tight nucleolar binding, WRN and BLM might become well described by the diffusion model in this subcompartment, whereas mutations affecting DNA binding in general would change the effective diffusion constants throughout the cell.

We believe that these testable predictions can help direct further study on the dynamics of DNA repair proteins, and provide a framework to aid in the interpretation and classification of findings in this area.

Location	WRN	BLM
Nucleoplasm	**Diffusion** $(D_{WRN-eff} = 0.145\,\frac{\mu m^2}{s})$	**Diffusion** $(D_{BLM-eff} = 0.07\,\frac{\mu m^2}{s})$
Nucleoli	**Reaction** $(k_{off} = 0.12\ s^{-1})$	**Reaction** $(k_{off} = 0.11\ s^{-1})$
Damage	**Diffusion** $(D_{WRN-eff} = 0.01\,\frac{\mu m^2}{s})$	**Full Model** $(k^*_{on}; k_{off}) = (1105 s^{-1}\ ;\ 1.8\ s^{-1})$

Table 2. Quantitative summarization table: The models that result in the best fit with fewest parameters values shown for the 3 different compartments (Nucleoplasm, Nucleolus, Damage) and for both the proteins (WRN and BLM). In the nucleoplasm WRN and BLM are characterized by "effective diffusion", but in the nucleoli they are tighter bound and therefore best described by a reaction model. Last at the damage site WRN seems to be best described by "effective diffusion" whereas BLM can not be described by any of the two simpler models. Note that at the damage site ~20% of WRN and BLM was immobile, which means very tight binding.

Methods

Preparation of cells for microscopy

U2OS cells were maintained in DMEM (Gibco), supplemented with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 g/ml) (Gibco, Life technologies), and grown at 37°C in a humidified atmosphere containing 5% CO_2. One day prior to transfection approximately 10^5 cells were seeded in 15 mm dishes with thin glass bottoms (Mat-Tek). Cells were transfected with Lipofectamine LTX (Life Technologies Inc.) according to manufacturer's instructions using 1 µg of the relevant vectors.

Microscopy

For FRAP a spinning disk micropoint laser emitting a wavelength of 480nm is used. The objective is set to 40x magnification. For the FRAP experiments a laser power of 0.6 µW is used, and to create double stranded breaks the power is increased to 1.8 µW. The micropoint laser pulse is 5 ns. Volocity software was used to control the microscope. Cells were imaged at 37 °C and 5% O_2 using an environmental chamber.

FRAP analysis

The diffusion coefficients of the WRN and BLM helicases were obtained by fitting background corrected images, using matlabs *nlinfit* routine. Circular spots were bleached in the nucleoplasm, nucleolus and damage site. The spot diameter was 1µm in all cases except for WRN in the nucleoplasm, where it was 2 µm. The size of the spots is a trade-off between a good signal to noise ration and recording the first time points. Between 11 and 22 cells were used to create the recovery curves. The curves were fitted to both full model equations, effective diffusion and reaction limited as described in[5]. The Laplace transform of the FRAP recovery becomes:

$$\overline{frap(p)} = \frac{1}{p} - \frac{F_{eq}}{p}\left(1 - K_1(qw)I_1(qw)\right) \times \left(1 + \frac{k_{on}^*}{p + k_{off}}\right) - \frac{C_{eq}}{p + k_{off}}$$

Where w is the radius of the bleaching spot, F_{eq} is the equilibrium fraction of free protein and C_{eq} is the equilibrium fraction of bound protein. $I_1$and $K_1$are modified Bessel functions of respectively the first and second kind, and $q^2$is given by:

$$\frac{p}{D_f}\left(1 + \frac{k_{on}^*}{p + k_{off}}\right)$$

Where D_f is the diffusion coefficient, k_{on}^* is the pseudo on-rate and p is the complex argument for the Laplace transform. For a thorough and beautiful derivation of the 3 solutions (Reaction, Diffusion and R-D) used in this paper we refer to Sprague et. al[5] . To account for immobile fractions we modified the analysis by normalizing the data to the last few data points were the system has reached equilibrium.

$$frap(t)_{immobile\ correction} = frap(t)\sum_{N-n}^{N}\frac{frap(t)}{n}$$

Where n is the number of datapoints in equilibrium (in our case is roughly 10). If there is no immobile fraction our extra factor becomes 1 and therefore does not affect the analysis. The fraction of bound protein can be calculated from measuring the effective diffusion. Using that the effective diffusion is given as[5]:

$$D_{eff} = \frac{D_f}{1 + \frac{k_{on}^*}{k_{off}}}$$

And that the differential equation for binding/unbinding of a protein.

$$\frac{dB}{dt} = k_{on}^* F - k_{off} B$$

F is the free protein, *B* is the bound protein, demanding total amount of protein to be conserved *T=F+B*, and setting the reaction to equilibrium.

$$\frac{B}{F} = \frac{1}{\frac{T}{B} - 1} = \frac{k_{on}^*}{k_{off}}$$

We get fraction of bound protein *R*, is then given as:

$$R = \frac{B}{T} = \frac{1}{1 + \frac{k_{on}^*}{k_{off}}}$$

Which is the same as:

$R = \frac{D_{eff}}{Df}$

From the ratio of the diffusion coefficient of a freely diffusion protein and the effective diffusion coefficient the fraction of bound (and unbound) proteins can be calculated.

Statistics

We used reduced χ^2 to estimate the goodness of fit. Since our datasets vary in size, this method cannot be used to compare across datasets (e.g. if the diffusion model fits WRN in nucleoplasm data with χ^2=0.17 but fits BLM in nucleoplasm with χ^2=0.04, then BLM is not necessarily better fitted then WRN). However within one dataset reduced χ^2 can be used to distinguish between the models. Because the simpler reaction model and diffusion model are approximations of the full R-D model the latter will always fit the data better. If the difference between the fit of the R-D and the simpler model is below 30% we accept the simpler model. $\frac{2(\chi^2_{simple} - \chi^2_{R-D})}{\chi^2_{simple} + \chi^2_{R-D}} \leq 0.3$.

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Supplementary

	WRN	BLM
Nucleoplasm	Full Model: k^*_{on}=1470, k_{off}=15, χ^2 = 0.16 **Diffusion**: D_{eff}=0.145 **χ^2** = 0.17 Reaction k^*_{on}=0.6, k_{off} =0.2, χ^2=0.6	Full Model: k^*_{on} =2370, k_{off} =12.5, **χ^2**=0.03 **Diffusion: D_{eff}=0.07 χ^2 = 0.04** Reaction k^*_{on}=0.95, k_{off} =0.37, χ^2=0.17
Nucleoli	Full Model: k^*_{on} =291, k_{off} =0.45, **χ^2**=0.08 Diffusion: D_{eff}=0.01 **χ^2** = 0.18 **Reaction k^*_{on}=1.3, k_{off} =0.11, χ^2=0.08**	Full Model: k^*_{on} =1.2, k_{off} =0.11, **χ^2**=0.07 Diffusion: D_{eff}=0.01 **χ^2** = 0.30 **Reaction k^*_{on}=1.5, k_{off} =0.11, χ^2=0.08**
Damage	Full Model: k^*_{on} =3185, k_{off} =3.3, χ^2=0.03 **Diffusion: D_{eff}=0.01 χ^2 = 0.04** Reaction k^*_{on}=0.8, k_{off} =0.1, χ^2=0.12	**Full Model: k^*_{on} =1105, k_{off} =1,8, χ^2=0.04** Diffusion: D_{eff}=0.02 **χ^2** = 0.08 Reaction k^*_{on}=1.3, k_{off} =0.16, χ^2=0.13

Table S1: *Summary of the best fits for the 3 different models in the nucleoplasm, nucleoli and damage site. The χ^2 for the fits are given, and if the data can be fitted with a model with less parameters (reaction or diffusion) this model is in bold.*

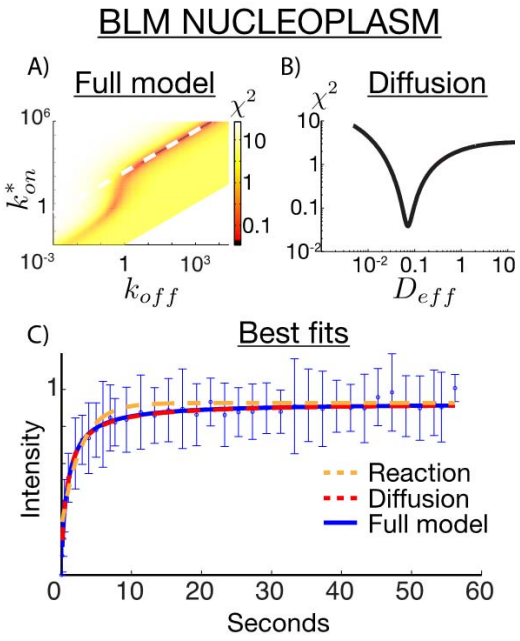

FIG. S1. Analyses of the BLM diffusion dynamics in nucleoplasm.

Data is an average over 12 cells, with error bars representing standard deviation. ***A)*** *The deviation from the data (χ^2) is plotted as a function of the pseudo on-rate (k_{on}^*) and off rate (k_{off}) for a full R-D model. The white dashed line indicates the best fit for the diffusion model, which coincides with the best fitting parameters for the R-D model (black). This indicates that the diffusion model fits the data as well as the R-D model.* ***B)*** *Parameter scan for the diffusion model shows that the best fit occurs for the effective diffusion coefficient* $D_{BLM-eff} = 0.07\frac{\mu m^2}{s}$. ***C)*** *The best fits for the 3 models (R-D as a solid blue line, diffusion as a dashed red line and reaction as a dashed orange line) are plotted alongside the experimental data (blue points with error bars). The reaction model provides the worst fit, while the diffusion model fits the data just as well as the full R-D model.*

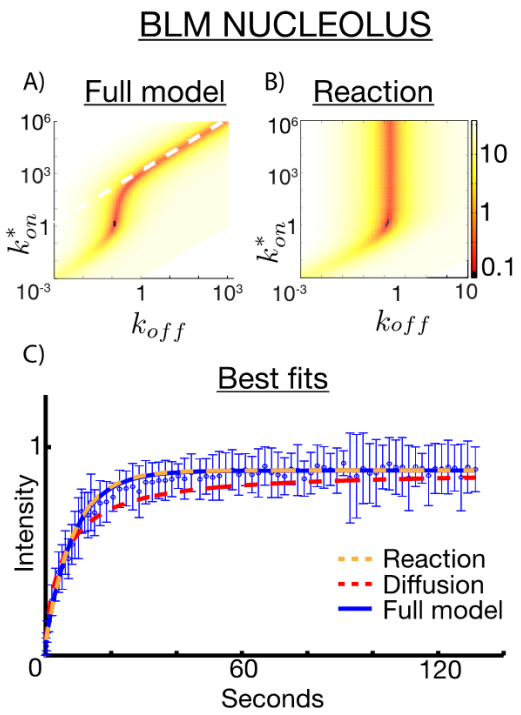

FIG. 2S. Analyses of the BLM diffusion dynamics in nucleoli.

Data is an average over 14 cells, with error bars representing standard deviation. ***A)*** *The deviation from data (χ^2) is plotted as a function of the pseudo on-rate (k^*_{on}) and off rate (k_{off}) for a full R-D model. The white dashed line indicates the best fit for the diffusion model, which does not coalesce with best fitting parameters for the R-D model (black) and thus does not provide a good fit for the data.* ***B)*** *A parameter scan for the reaction model shows that the reaction model reproduces the good parameters of the full R-D model for low values of k^*_{on} and k_{off}, with the best fit for $k_{off} = 0.11\ s^{-1}$* ***C)*** *The best fits for the 3 models (R-D as a solid blue line, diffusion as a dashed red line and reaction as a dashed orange line) are plotted alongside the experimental data (blue points with error bars). The reaction model provides a good fit of the data.*

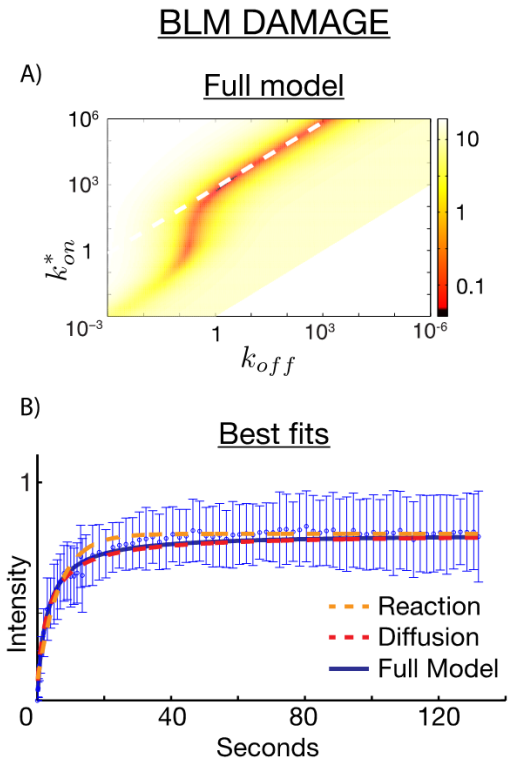

FIG. 3S. Analyses of the BLM dynamics at damage site.

Data is an average over 14 cells, with error bars representing standard deviation. ***A)*** *The deviation from data (χ^2) is plotted as a function of the pseudo on-rate (k^*_{on}) and off rate (k_{off}) for a full R-D model. The white dashed line indicates the best fit for the diffusion model. We are not able to reduce the R-D model to one of the two simpler models.* ***B)*** *The best fits for the 3 models (R-D as a solid blue line, diffusion as a dashed red line and reaction as a dashed orange line) are plotted alongside the experimental data (blue points with error bars). Visually it looks like the diffusion model is as good a fit as the R-D model but from the χ^2 of the fits we are not able to reliably reduce the model.*

Diffusion coefficient of fusion proteins.

Given that the relation between molecular weight and hydrodynamic radius, where R_h is in nm and M is in Dalton:

$$R_h = 0.718\sqrt[3]{M}$$

and the "Stokes-Einstein" relation:

$$D = \frac{k_B T}{6\pi\eta R_h}$$

We get that D scales with the mass M as:

$$D \propto \frac{1}{\sqrt[3]{M}}$$

Using the scaling relationship of the corresponding molecular weights:

$$\frac{D_{fusion\ protein}}{D_{GFP}} = \sqrt[3]{\frac{M_{GFP}}{M_{fusion\ protein}}}$$

With $M_{GFP} = 27kDa$ [22], $M_{WRN} = 165kDa$ [23] and $M_{BLM} = 170kDa$ [11] and $D_{GFP} = 28\ \frac{\mu m^2}{s}$ [6]. We can estimate the corresponding diffusion constants of the fusion protein:

$$D_{WRN-GFP} = 14.7\frac{\mu m^2}{s} \text{ and } D_{BLM-GFP} = 14.4\frac{\mu m^2}{s}.$$

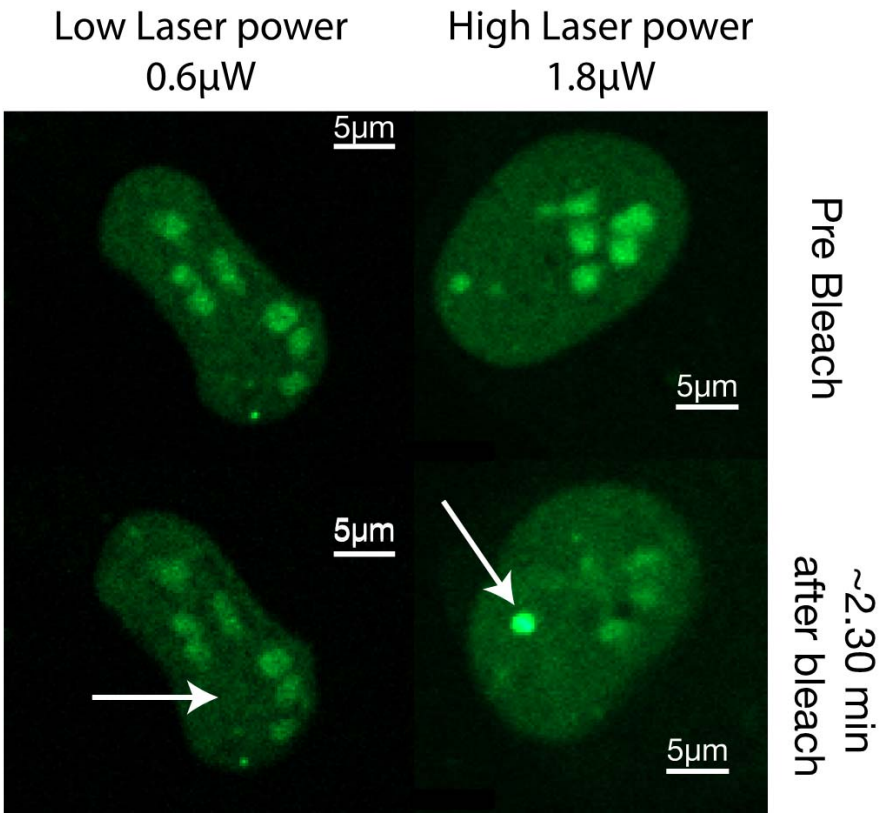

Fig 4S. No double stranded breaks were created with the low laser power(0.6µW). *As a control to see if the FRAP experiment induced double stranded breaks we bleached cells and monitored them for roughly 2 and a half minute, but no recruitment was seen.*

Chapter 3

Cockayne Syndrome

Another aspect of my work deals with Cockayne syndrome (CS), a rare disease that is commonly considered a clear-cut segmental progeria. It is an autosomal recessive genetic disorder for which two causative genes have been identified (*ERCC6* and *ERCC8*). The gene products have been named Cockayne Syndrome protein B and A respectively (abbreviated CSB and CSA), and while these proteins have been subject to a great deal of characterization, the underlying mechanism of CS remains elusive. Indeed, our current knowledge of CSA and B hardly serves to guide us towards a satisfactory explanation for the symptoms displayed by patients with CS: briefly, the two proteins are functionally very different and are only known to cooperate in TC-NER, whereby distorting DNA lesions in actively transcribed genes are efficiently repaired as prompted by stalled polymerases (see Section 1.4 for details). However, the symptoms of CS do not consummately match that of NER disorders, most notably because patients do not appear to be more prone to development of cancer [222]. Like the RecQ helicases, CS thus offers an opportunity to examine whether DNA damage is the direct cause of accelerated aging. In the following I will expound our mechanistic understanding of the involved proteins and concisely go through the analysis behind the hypothesis that (at least at the cellular level) CS may be a result of mitochondrial dysfunction.

Mutations in CSB account for the majority of reported CS cases; these mutations are spread throughout the gene, and most frequently cause frameshifts or stop codons that result in truncation of the 168-kDa protein [223]. CSB is part of the SWI2/SNF2 subfamily by virtue of a central helicase-like domain, although no helicase activity has been demonstrated *in vitro* [224]. It has been shown to hydrolyze ATP in a DNA-dependent manner, and this activity is required both for CSB to act as a chromatin remodeller *in vitro* [225] and may be required for its repair activity *in vivo* [226]. On the other hand, ATP hydrolysis is not required for averting oxidative damage in mitochondria [227, 228]. CSB has been reported to localize to mitochondria [229, 230] where it may interact with mitochondrial transcription machinery [228], and the protein definitely plays a role in maintaining proper mitochondrial function [231]. Finally, several reports have suggested a role in transcription outside of TC-NER [232, 233], though the exact details remain elusive. CSA has meanwhile received rather less attention, in part due to being difficult to purify. A 44 kDa protein discovered in 1995 [234], it is part of the cullin-based E3 ubiquitin ligase complex CRL4 that in TC-NER is recruited to stalled polymerases in a CSB-dependent manner [235]. Although it accounts for a minority of CS cases, it is essential both for TC-NER and transcriptional restart after damage [236, 237], and the

patient phenotype of mutations in *ERCC8* is indistinguishable from those in *ERCC6* [223].
Given the various reported functions of CSA and CSB, it is perhaps not surprising that a number of theories exist to explain the pathology of CS. The shared role in TC-NER was identified first, and the idea of CS as a DNA repair disorder remains the most widespread [238, 239]. The strongest argument against this idea is as mentioned that the phenotype of CS differs somewhat from what is typically shown by DNA repair disorders. Most telling is a comparison of CS with xeroderma pigmentosum (XP): XP is caused by mutations in different proteins involved in global genome repair, a parallel pathway to TC-NER that identifies distorting lesions in the entire genome independently of transcription (again, see Section 1.4 for details), and in the subsequent repair machinery that is common to both pathways. There are similarities between the symptoms of CS and XP, notably cutaneous photosensitivity, but also salient differences: CS has a strong neurological phenotype, which manifests only in a subset of XP-causing mutations (and then usually less severely). Conversely, while CS patients do not appear to have higher risk of cancer, skin cancer incidence is increased 3000-fold in XP patients [239, 240]. For the XP mutations affecting global genome repair this difference might be ascribed to different sub-pathways, but the phenotypical differences also apply for XP cases where the mutated protein acts in the shared repair machinery (XPA, for example) and should therefore inhibit TC-NER as well. For this reason it has been proposed that the phenotype of CS results only partially from defective DNA repair (where it overlaps e.g. XP), while another role of CSA/B produces the more idiosyncratic symptoms. The most popular suspect for this role is transcription itself, championed especially by Jean-Marc Egly [241]. This would tie in well with the DNA damage theory of aging described in Section 1.3.2, since the distinguishing symptoms of CS are for the most part those that resemble accelerated aging. Alternatively, a recent bioinformatic tool has shown that CS clusters with various mitochondrial disorders based on symptoms reported in the literature [242], and as I've mentioned above and will elaborate in Section 3.1.1 there is increasing support for an important role in mitochondrial maintenance that could underlie the pathology of the disease.
Because the pathology of CS is still very much under debate, my work took an unbiased approach to look for commonalities induced by the absence of either CSA or CSB. Using microarray analysis to compare gene expression changes after knocking down either protein we were hoping to find common changes that might hint at the mechanism of pathogenesis in CS. As it turned out such common changes included both transcriptional and mitochondrial genes, as well as a milder response for DNA repair. Continuing the dispassionate investigation, we pursued several possible models encompassing all these previously reported observations in different constellations of cause and effect. I will spend a few words describing the reasoning that eventually led to the investigation presented in Paper III: For an upregulation of mitchondrial genes and (as we quickly discovered) ATP production to be the primary cause would require a direct and extensive role for CSA/B in (down)regulating mitochondrial function, which is not well supported by existing knowledge. It is also difficult to imagine how increased ATP production in itself would lead to pathology. We therefore sought to explain the mitochondrial phenotype via another dysfunctional process, most probably as a response to an increased energy consumption. Because ribosomal genes came up strongly upregulated in the microarray we briefly considered that an increase in translation (perhaps of incomplete

transcripts) could be the energy consuming process. However, preliminary experiments did not indicate a real increase in protein synthesis, and we consider it more likely that the translational upregulation is a secondary, compensatory effect of reduced transcription, which has been reported in the literature [243]. We then explored ways in which dysfunctional transcription could consume excessive amounts of ATP, and were encouraged by the induction of a similar mitochondrial phenotype in cells with drug-induced inhibition of transcription. An early idea was that an inability to remove blocking lesions and restart polymerases (previously reported for CSB-deficient cells [191, 244, 245]) could result in repeated cycles of unsuccessful transcription. However, nuclear run-ons did not suggest a greater failure rate for transcription in knockdown cells. While aberrant DNA repair could feasibly impose a large energetic burden on the cell, and assuredly reduce transcription, taking this to be the principal cause would intimate an energetic phenotype across various DNA repair disorders, which is not supported by the literature. In attempting to include both transcriptional and DNA repair defects as an interwoven source we invoked PARP-1, a known interaction partner of CSB [246]. PARP-1 is well known as a responder to strand breaks [247], but has also been shown to bind distorted helices and atypical DNA conformations [248, 249]. Through its substrate nicotinamide adenine dinucleotide (NAD^+) PARP-1 is tightly linked to both oxidative phosphorylation and cell death [250] and can thus be placed at the centre of our web of observations. By further examining PARP-1 activity in our knockdown and transcriptionally inhibited cells, we arrived at a model whereby CSA/B somehow turn off PARP signalling induced by stalling of RNA polymerases at blocking lesions/DNA conformations. Failure to turn off this signalling results not only in impaired transcription, but also depletion of NAD^+ and/or ATP which prompts the cell to upregulate its mitochondrial activity to avoid or postpone cell death.

My study of CS thus ended up bridging my work on DNA repair proteins with the secondary focus of my research, namely the role of mitochondria in the aging process, which I will describe in the following chapter.

3.1.1 Paper III

TRANSCRIPTIONAL DYSFUNCTION IN CSA AND CSB DEFICIENT CELLS RESULTS IN AN ENERGY-DEFICIENT PHENOTYPE THROUGH ACTIVATION OF PARP1.

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Keywords

ERCC6, ERCC8, CSA, CSB, Cockayne Syndrome, mitochondrial dysfunction, PARP-1, transcription

Running title

PARP overactivation in Cockayne syndrome

Abbreviations

CS, Cockayne Syndrome; CSA, Cockayne Syndrome protein A; CSB, Cockayne Syndrome protein B; DHE, Dihydroethidium; DPQ**,** 3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone; GO, Gene ontology; mtDNA, mitochondrial DNA; NAD^+, Nicotinamide adenine dinucleotide; OCR, Oxygen consumption rate; OXPHOS, oxidative phosphorylation; PAR, poly-adenine ribose; PARP-1, poly-adenine ribose polymerase 1; ROS, reactive oxygen species; TC-NER, Transcription-coupled nucleotide excision repair; TMRM, Tetramethylrhodamine; XP, Xeroderma Pigmentosum.

Summary

Cockayne Syndrome (CS) is a rare disorder with symptoms of accelerated aging, and is caused by mutations in the *ERCC6* and *ERCC8* genes, which encode the proteins CSB and CSA respectively. Though the proteins have been characterized to a degree, the pathogenesis of CS remains obscure. Here we report that cells depleted for CSA or CSB show a pattern of gene regulation that closely resembles that of cells with inhibited transcription: notably, ATP production and ribosomal proteins were strongly up-regulated, while transcription machinery was down-regulated. Both CSA/B knockdown and transcriptional inhibition increased mitochondrial content, membrane potential and ROS production, consistent with increased ATP

production, and knockdown cells show greater ATP flux *in vivo*. We further show overactivation of PARP-1 in both knockdown and transcriptionally inhibited cells, which could be a misregulated response to transcriptional stalling and account for the mitochondrial phenotype.

Introduction

Cockayne Syndrome (CS; MIM# 133540 & 216400) is) is an autosomal recessive genetic disorder caused by mutations in the genes *ERCC6* and *ERCC8*, which encode the proteins CSB and CSA respectively (Nance and Berry, 1992). CS only occurs in about one out of every two million births, but has garnered additional attention because it includes symptoms of premature aging (Navarro et al., 2006). Patients suffer from progressive growth failure, brain atrophy, photosensitivity, retinal deterioration and hearing loss, as well as a cachectic physical appearance and greatly shortened lifespan (Laugel et al., 2010). The onset and severity of the CS phenotype varies considerably between cases. Mutations in CSB account for the majority of cases, but no difference in phenotype has been linked with the genotypic origin of disease. Likewise, the cause of pathology remains poorly understood and no correlation is apparent between molecular defects and the phenotypic spectrum of the disease (Weidenheim et al., 2009).

The shared phenotype resulting from mutations in CSA and CSB is particularly puzzling given the dissimilar nature of the proteins. CSA is a 396 amino acid protein that is part of an E3 ubiquitin ligase complex (Henning et al., 1995; Groisman et al., 2003; Fischer et al., 2011). This association may be facilitated by several WD-40 repeats, which are thought to enable protein-protein interactions (Xu and Min, 2011). CSB is larger, 1493 amino acids, and belongs to the SWI/SNF2 ATPase family by virtue of a conserved helicase domain (Troelstra et al., 1992).

Though helicase activity has not been demonstrated, CSB does have ATPase activity which has been implicated in chromatin remodeling and translocation of DNA-bound proteins (Citterio et al., 2000; Berquist et al., 2012).

One process known to involve both CSA and CSB is transcription-coupled nucleotide excision repair (TC-NER), whereby bulky DNA lesions that cause stalling of RNA polymerases are identified, excised and replaced. Though their exact roles are not known, first CSB and then CSA are recruited to stalled RNA polymerase II following UV exposure and are required to repair lesions and restart transcription (Mayne and Lehman, 1982; Venema et al., 1990). This suggests that CS is the result of deficient TC-NER. While this theory is still prominent it has also been challenged, principally because the phenotype of CS is significantly different from that of xeroderma pigmentosum (XP) which is caused by defects in different steps of nucleotide excision repair (Waard et al., 2004). Alternative theories have sought to explain this discrepancy through additional roles of the CS proteins, including repair of oxidative damage (Dianov et al., 1999) and a more general role in transcription (Friedberg, 1996; Dubaele and Egly, 2002).

Some recent reports have also linked CSB to mitochondrial function: both CSA and CSB have been reported to localize to mitochondria where they interact with proteins involved in the base excision repair pathway and help maintain mitochondrial DNA (mtDNA) stability (Aamann et al., 2010; Kamenisch et al., 2010). CSB has also been shown to interact with mitochondrial transcriptional machinery (Berquist et al., 2012), and its absence may affect mtDNA stability (Kamenisch et al., 2010). This instability seems to be particularly pronounced in subcutaneous fat cells (Kamenisch et al., 2010) and $CSB^{m/m}$ mice are unable to accumulate fat due to dysfunctional mitochondria caused by a lack of mitochondrial autophagy (Scheibye-Knudsen et al., 2012).

In this study we have conducted an investigation of cellular dysfunctions shared between CSA and CSB deficient cells. Our findings link the proteins' role in TC-NER with transcription and mitochondrial function, and suggest a mechanism for the pathology of CS.

Results

CSA AND CSB DEFICIENT CELLS SHOW A CHARACTERISTIC PATTERN OF GENE EXPRESSION CHANGES THAT RESEMBLES INHIBITION OF TRANSCRIPTION

To find cellular changes common to CSA and CSB deficiency, we knocked down the two proteins independently in SH-SY5Y neuroblastoma cells (fig. 1**A**). We first confirmed the phenotype of our knockdown cells by measuring UV sensitivity, a hallmark of CS cells. As shown in fig. 1**B**, both CSA and CSB knockdown cells show increased sensitivity. We then performed microarray analysis on RNA isolated from these cells, and compared the resulting expression patterns of CSA and CSB knockdown to controls transfected with scrambled shRNA. As shown in fig. 1**C** & **D**, the knockdown of either protein lead to remarkably similar changes: more than half of the significantly altered genes were affected similarly for either knockdown and, as emphasized in table 1, the most strongly regulated gene ontology terms were nearly identical for both knockdowns. The microarray data was verified by RT-PCR, as shown in supp. fig. 1. Most noticeable among the downregulated gene ontology (GO) terms is transcription, while the upregulated terms almost exclusively relate to ribosomes/translation and mitochondrial oxidative phosphorylation (OXPHOS). Because transcription and mitochondrial function have previously been implicated in CS, we next asked whether the effect on these two systems might be connected. To gauge this we treated normal SH-SY5Y cells with the transcriptional inhibitors

α-amanitin and actinomycin D (mainly affecting RNA polymerases II and I respectively) at different concentrations, and performed analogous microarray analysis for comparison with the knockdown cells. The effect of such transcriptional inhibition was very similar to what was observed for the knockdowns, both in terms of overall gene expression profile (Fig. 2) and the most strongly regulated pathways.

GENE EXPRESSION CHANGES PRODUCE A PHENOTYPE OF INCREASED MITOCHONDRIAL ACTIVITY AND ROS PRODUCTION

To further test whether inhibition of transcription could cause the changes in mitochondrial gene expression, and to determine how these changes affect mitochondrial function in these cells, we used fluorescent dyes to measure different mitochondrial parameters by flow cytometry. Measuring uptake of Mitotracker Green and tetramethylrhodamine methyl ester (TMRM) by cultured CSA and CSB deficient cells revealed an increase in mitochondrial content and membrane potential respectively, consistent with increased production of ATP by OXPHOS (fig. 3**A**). These changes were accompanied by an increase in both mitochondrial and whole cell reactive oxygen species (ROS), as probed by MitoSOX and dihydroethidium (DHE) respectively. Although the trend was the same for both knockdowns, a greater effect was observed in CSB deficient cells. Expanding this analysis to cells treated with α-amanitin and actinomycin D (fig. 3**B** & **C**), we observe an analogous effect of increased mitochondrial content, increased membrane potential and higher ROS production. This effect was strongest for actinomycin D, was dose-dependent and at higher doses far surpassed that observed in the knockdown cells, suggesting that the absence of CSA or CSB leads to a (milder) transcriptional dysfunction that could produce the observed mitochondrial phenotype.

INCREASED MITOCHONDRIAL ACTIVITY CORRESPONDS TO HIGHER ATP FLUX

We speculated that this mitochondrial phenotype was a response to increased energy consumption in the cells, and to test this we measured oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in live cells using the Seahorse XF24 analyzer. OCR is indicative of mitochondrial respiration, while ECAR is used to measure glycolysis. As shown in fig. 4, both knockdown cell lines show an increase in both basal OCR and ECAR, consistent with a higher flux of ATP in these cells. CSB deficient patient cells have previously been shown to have higher OCR/ECAR as well as increased ATP consumption rates (Scheibye-Knudsen et al., 2012), and we assume that our findings here represent the same phenomenon.

ENERGETIC PHENOTYPE IS NOT CAUSED BY FUTILE CYCLES OF TRANSCRIPTION

To identify the origin of increased cellular ATP consumption we first tested whether the knockdown cells showed a greater incidence of failed transcription; the transcriptional inhibitors used here would naturally cause polymerase stalling, and given the previously reported role of at least CSB, it could be speculated that the absence of it or CSA would also limit successful transcription of genes. In such a scenario, futile cycles of abortive transcription could beget greater ATP consumption for essential levels of gene expression. To test this hypothesis, we performed nuclear run-on assays using two separate probes for each of three arbitrary genes, designed to capture transcripts at the beginning and end of the gene respectively. With this approach we could determine both the absolute transcriptional activity in these genes, which was reduced in both CSA and CSB deficient cells (fig. 5**A**), and whether initiation of transcription

occurred more frequently than successful elongation through to the end of the gene (fig. 5**B**). Although transcriptional failure is observed to some degree for *GAPDH*, there is no difference between knockdowns and control. The shorter genes *RPL35* and *EEF2* show neither unsuccessful transcription nor any difference between knockdowns and controls. In total, these results suggest that futile transcriptional cycles do not occur at an increased rate in the absence of CSA or CSB.

CSA/B KNOCKDOWN AND INHIBITION OF TRANSCRIPTION INCREASE PARP-1 ACTIVATION

Given the involvement of CSA and CSB in DNA repair, another possible source of increased energy consumption could be PARP activation. PARP-1 specifically is well known as a sensor of DNA strand breaks, but also recognizes abnormal DNA structures and interacts directly with CSB (Lonskaya et al., 2005; Thorslund et al., 2005). Ribosylation by PARP-1 uses the adenine derivative NAD^+ for substrate, whose biosynthesis is heavily dependent on mitochondria, and ultimately on adenine metabolism in general. Thus if PARP-1 is activated in response to stalled transcription by e.g. DNA lesions, and the cessation of this activity dependent on CSA/CSB, their knockdown could result in persistent PARP-1 activation which could deplete cellular ATP and/or NAD^+. To determine whether the absence of CSA or CSB results in overactivation of PARP-1, we measured cellular levels of its substrate poly-adenine ribose (PAR) by both Western blotting (fig. 6**A** & **B**), as well as the number of PARP-1 foci by immunocytochemistry (fig. 6**C** & **D**). Both methods suggest an increase in PARP-1 activation for both CSA and CSB knockdown. To confirm the link between the mitochondrial phenotype of CSA/B deficiency with transcriptional dysfunction, we similarly measured PAR levels and PARP-1 foci in cells treated with select concentrations of α-amanitin and actinomycin D. As shown in fig. 6, dysfunctional

transcription itself was sufficient to induce overactivation of PARP, representing a mechanism for this phenomenon in CSA and CSB deficient cells.

Discussion

The pathogenesis of Cockayne syndrome has been under debate for some time, based on the apparently diverse roles of CSB in particular. Since it has been proposed that this protein plays roles in transcription, DNA repair and mitochondrial function, it is difficult to trace the source of pathology back to a single cellular dysfunction. Accordingly, the findings presented here suggest a link between the reported functions of the CS proteins, where a failure to resolve transcriptional stalling interferes with the transcriptional machinery of the cell and affects mitochondrial function through runaway PARP signaling.

This connection opens the door to the complicated realm of PAR metabolism, which potentially influences disease progression at numerous levels. The substrate for PAR synthesis is the dinucleotide NAD^+, which influences cellular energy metabolism in two ways: first, its regeneration is an energetically costly process, consuming multiple molecules of ATP per NAD^+ (Hassa et al., 2006). Second, the cellular ratio of NAD^+ to NADH is an important regulator of OXPHOS and glycolysis (Wallace and Fan, 2010). In addition, poly(ADP-ribosyl)ation is important for a host of physiological functions, and substrate depletion caused by overactivation of a single one of these would expectedly have broad cellular effects. The PARP superfamily has at least 18 members, and from the results presented here we cannot say how each of these contributes to the observed changes in poly(ADP-ribosyl)ation. We would expect that PARP-1 is a central factor given that it is responsible for the greater part poly(ADP-ribosyl)ation in the cell

(Diefenbach and Bürkle, 2005), and from its known roles in DNA damage signaling, but further study is required to confirm this.

In addition to the gene expression changes we examined in more detail, we also observed an upregulation of ribosomal and translational proteins. We believe that this is a derivative effect with several possible causes: an increase in the cellular NAD^+/NADH ratio represses transcription of ribosomal DNA during caloric restriction, as mediated by SIRT1 and the eNoSC complex (Guarente and Picard, 2005; Murayama et al., 2008). CS decreases cellular NAD^+, which could produce the opposite effect of increased ribosomal biogenesis. Additionally it is possible for generally reduced transcription to selectively increase translation of specific mRNA. One example of this is mRNA containing 5'-terminal oligopyrimidine tracts, which includes many ribosomal proteins (Damgaard and Lykke-Andersen, 2011). Thus, the transcriptional deficiency in these cells could be producing a superfluous increase in ribosomal components.

An obvious continuation of this study would be to establish the exact mechanism by which the CS proteins act to prevent overactivation of PARP. CSB is required for both assembly of TC-NER proteins at transcription-blocking lesions (Fousteri et al., 2006) and, alongside CSA and dependent on its ubiquitin-binding domain, for repair to take place (Tu et al., 1998; Anindya et al., 2010). One possibility is therefore that PARP recognizes and is activated by the blocking lesion, and that its deactivation is dependent upon successful repair. In extension of this, PARP might (instead or additionally) be activated by abnormal DNA configurations that could evidently cause stalling of RNA polymerases and which have also been reported as direct recognition sites of PARP-1 (Lonskaya et al., 2005; Salvati et al., 2010). In this scenario, resolution of such structures could depend on chromatin remodeling by either CSB itself (Citterio et al., 2000) or by factors recruited via the CS proteins (Fousteri et al., 2006; Newman

et al., 2006). Alternatively, the CS proteins could play a more direct role in deactivating PARP signaling: CSB has previously been shown to physically interact with PARP-1 *in vivo* (Thorslund et al., 2005), is able to displace DNA-bound proteins (Berquist and Wilson, 2009; Berquist et al., 2012) and promotes the advance of stalled RNA polymerase II by a single nucleotide (Selby and Sancar, 1997). One could therefore imagine that the translocase activity of CSB physically removes PARP-1 from the site of stalling and thereby arrests poly(ADP-ribosyl)ation. Meanwhile, ubiquitinylation and subsequent degradation of PARP-1 can be caused by the E3 ubiquitin ligase CHFR (Kashima et al., 2012), and one could thus speculate on a similar method of deactivation mediated by CSA.

Another detail that begs analysis is how PARP activation would fit into the timing of events during transcriptional stalling. Poly(ADP-ribosyl)ation is often thought to mediate the recruitment of relevant proteins to damage sites, though in this case it is not immediately obvious what these might be. Because CSB itself is required for the recruitment of TC-NER proteins and is here shown to limit PARP activation in transcriptional stalling, it seems unlikely that PARP acts as a primary means of recruiting repair proteins. CSB interacts transiently with elongating RNA polymerase II, an interaction that is stabilized by induction of DNA damage (Van den Boom et al., 2004), and is itself a target of ribosylation by PARP-1 (Thorslund et al., 2005). It is possible that PARP present at the stalled polymerase stabilizes the interaction with CSB through ribosylation of the latter, thus allowing for activation of the TC-NER machinery. This machinery in turn acts as negative feedback for further ribosylation by deactivating PARP. In this manner, the specific activation of TC-NER by the general damage sensor PARP is rendered possible by the inherent transient interactions of CSB with RNA polymerases, and the failure to activate this

pathway in the absence of the CS proteins leads both to lack of repair and excessive PARP activation (which could possibly lead to activation of alternate pathways).

Conclusively, this study reports a connection between several known functions of the two dissimilar proteins implicated in Cockayne syndrome, and links these cellular deficiencies to aberrant PARP activity that could explain the phenotype of the disease. Several avenues of further research could be pursued to further test our hypothesis, and hopefully reveal targets of therapeutic intervention.

Experimental

Lentiviral production and knockdown: Lentivirus was prepared as described previously (Scheibye-Knudsen et al., 2012): vector cocktails against *ERCC6* and *ERCC8* (Santa Cruz Biotechnology), each containing three shRNA sequences in equal proportions, were co-transfected with viral vectors into HEK293T cells using X-tremeGENE 9 (Roche) according to manufacturer's instructions. Transfected cells were grown for 2 days, virus collected and filtered through a 0.45 μm pore filter and added to SH-SY5Y neuroblastoma cells. Cells were incubated at 37°C for 2 hours, rocked manually every 10 min, after which fresh media was added. These cells were split after 24 hours, and 10 mg/l puromycin added to the medium after another 24 hours. As no adequate antibody was available against CSA, knockdown efficiencies were tested by RT-PCR using the TaqMan system (Applied Biosystems) 2 days, 2 weeks and 5 weeks after treatment.

Cell culture: SH-SY5Y neuroblastoma cells are grown in a 1:1 mix of Dulbecco's Modified Eagle Medium and Ham's F12 medium supplemented with 10% FBS, 1% penicillin

streptomycin and grown in 20% O_2 / 5% CO_2 at 37°C. Cells harboring shRNA were grown in media further supplemented with 10 mg/l puromycin.

Microarray analysis: Total RNA was isolated from cells either 14 days after shRNA treatment or after 24 hours of treatment with actinomycin D or α-amanitin, using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Four separate samples analyzed for shRNA-treated cells, and three separate samples for each concentration of transcriptional inhibitors. A 0.5-μg aliquot of total RNA from the pooled normal or treated cell lines was labeled using the Illumina Total Prep RNA amplification kit (Ambion). A total of 0.85 μg of biotin-labeled cRNA was hybridized for 16 h to Illumina's 23,000-gene Sentrix HumanRef-8v2 Expression BeadChips (Illumina). The arrays were washed, blocked, and then hybridized. Biotinylated cRNA was detected with streptavidin–Cy3 and quantitated using Illumina's BeadStation 500GX Genetic Analysis Systems scanner. Image processing and data extraction were performed using BeadStudio version 15 (Illumina). Raw hybridization intensity data were log-transformed and normalized to yield z-scores. The z-ratio was calculated as the difference between the observed gene z-scores for the experimental and the control comparisons, and dividing by the standard deviation associated with the distribution of these differences. Z-ratio values ±1.5 were chosen as cut-off values, defining increased and decreased expression, respectively. A complete set of 522 cellular pathways was obtained from the Molecular Signatures Database (MSigDB, Broad Institute, Massachusetts Institute of Technology, Cambridge, MA). The complete set was tested for gene set enrichment using parametric analysis of gene set enrichment. For each pathway z-score, a p-value was computed using JMP 6.0 software to test for the significance of the z-score obtained. These tools were part of DIANE 1.0 (see http://www.grc.nia.nih.gov/branches/rrb/dna/diane_software.pdf for information).

Verification of microarrays by RT-PCR was performed using the RT^2PCR SYBR system (SABiosciences) according to manufacturer's instructions.

Cellular oxygen consumption: Oxygen consumption and extracellular acidification rate were measured using the Seahorse XF-24 instrument (Seahorse Biosciences) as described previously (Scheibye-Knudsen et al., 2012), with the following modifications: 4×10^4 cells were plated, and changed to unbuffered XF assay media at pH 7.4 (Seahorse Biosciences), supplemented with 5 mM glucose (Sigma-Aldrich), 2 mM sodium pyruvate, and 1 mM GlutaMAX (Life Technologies).

Nuclear run-ons: Nuclei from 5×10^8 cells were isolated by resuspending cells in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 20 mM NaCl, 5 mM MgCl2, and 0.25% vol/vol NP-40) and incubated on ice for 10 min. Nuclei were spun down and resuspended in 200 ml storage buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.1 mM EDTA-NaOH, pH 8.0, and 45% vol/vol glycerol) and frozen at -80°C. For analysis, Nuclei were mixed with 133 µl of 3x reaction buffer (450 mM KCl, 15 mM MgCl2, and 1.5 mM each of ATP, CTP, and GTP) plus 330 µCi a-$^{[32P]}$UTP (3,000 Ci/mmol, 10 mCi/ml; PerkinElmer) and incubated for 30 min at 30°C. Samples were then incubated with 100 U of RNase-free DNase I (Applied Biosystems) for 30 min at 37°C followed by 20 µg/ml of proteinase K and 0.2% SDS for 45 min at 37°C. Finally, the labeled nascent RNA was purified by phenol-chloroform extraction and isopropanol/ammonium acetate precipitation. RNA was hybridized to 1 mg DNA probes printed on Hybond N+ positively charged nylon membranes (GE Healthcare). The membranes were prehybridized overnight with 3 ml UltraHyb hybridization solution (Ambion) with 10 µg of Cot DNA (Life Technologies). The labeled RNA was then added in 1 ml of hybridization solution and membrane for 24 h in a rotisserie-style incubator at 45°C. The membranes were rinsed in 2 ×

SSC and 0.1% SDS, followed by washes in 0.1x SSC and 0.1% SDS twice at 58°C for 30 mins. Membranes were exposed for 2 days and scanned using a Phosphorimager (GE Healthcare). ImageQuant software (GE Healthcare) was used to convert the hybridization signals into raw intensity values.

Mitochondrial content, ROS and membrane potential: 2×10^6 cells were plated in 10 cm petri dishes and grown for 24 hours, then harvested by trypsin, and resuspended in DME without phenol indicator (Life Technologies). Cells were then stained with 50 nM TMRM (membrane potential, Life Technologies), 50 nM MitoTracker Green (mitochondrial content, Life Technologies), 3 μM dihydroethidium (cellular ROS, Life Technologies) or 3 μM MitoSOX (mitochondrial ROS, Life Technologies) for 15 min (TMRM) or 30 minutes (other dyes) at 37°C. Fluorescence was measured by a flow cytometer (Accuri C6).

Microscopy: 10^5 cells were seeded in 4-well chamber slides and grown overnight. The next day cells were fixed for 15 min in 3.7% paraformaldehyde in PBS, washed, and permeabilized in 0.125% Triton X-100 in PBS for 15 min. Subsequently, cells were washed in PBS and blocked overnight in PBS containing 5% FBS. The following primary antibodies were added, for 1 h at 37°C: rabbit anti-PAR at 1:1000 dilution (BD Pharmigen, #551813) and goat anti-PARP-1 (Cell signaling, #9542) at 1:250 dilution. After washing 6 × 10 min, fluorescent secondary antibodies were added at 1:1,000 and incubated for 1 h at 37°C. Cells were then washed 6 × 10 min in PBS before being mounted in ProLong Gold Antifade with DAPI (Life Technologies). Images were acquired at 60x on a confocal microscope (Eclipse TE-2000e), capturing brightfield, DAPI at 405 nm, anti-PARP-1 at 488 nm and anti-PAR at 647 nm. Exposure times varied but were constant within each experiment. 10 z-stacks were captured for each cell line in each experiment,

and quantified using Volocity software (Improvision). PAR was quantified as total signal per cell, and PARP-1 as number of foci per cell.

Western blotting: Whole cell extracts were prepared by lysing cells in ice-cold RIPA buffer (Cell signaling), followed by sonication and high-speed centrifugation for 30 min. The supernatant was collected, and protein concentration determined using the DC protein assay kit (Bio-Rad). 50 µg protein was size-fractionated using pre-cast NuPage gels (Life technologies), and transferred onto 0.2 µm pore size polyvinylidene difluoride membranes (Life technologies). Membranes were blocked with 5% milk for 1 h, followed by incubation with a specific primary antibody overnight at 4°C. Primary antibodies used were: anti-PAR (BD Pharmigen, #551813), anti-PARP-1 (Cell signaling, #9542), actin (Abcam, #ab6276), at 1:1000 dilution. After washing, membranes were incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (GE healthcare, 1:1000 dilution) for 1 h. Membranes were washed, incubated with enhanced chemiluminescent substrate (Thermo Scientific), and exposed in a ChemiDoc™ MP System (Bio-Rad). Quantification was done using the ImageJ software.

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Figures

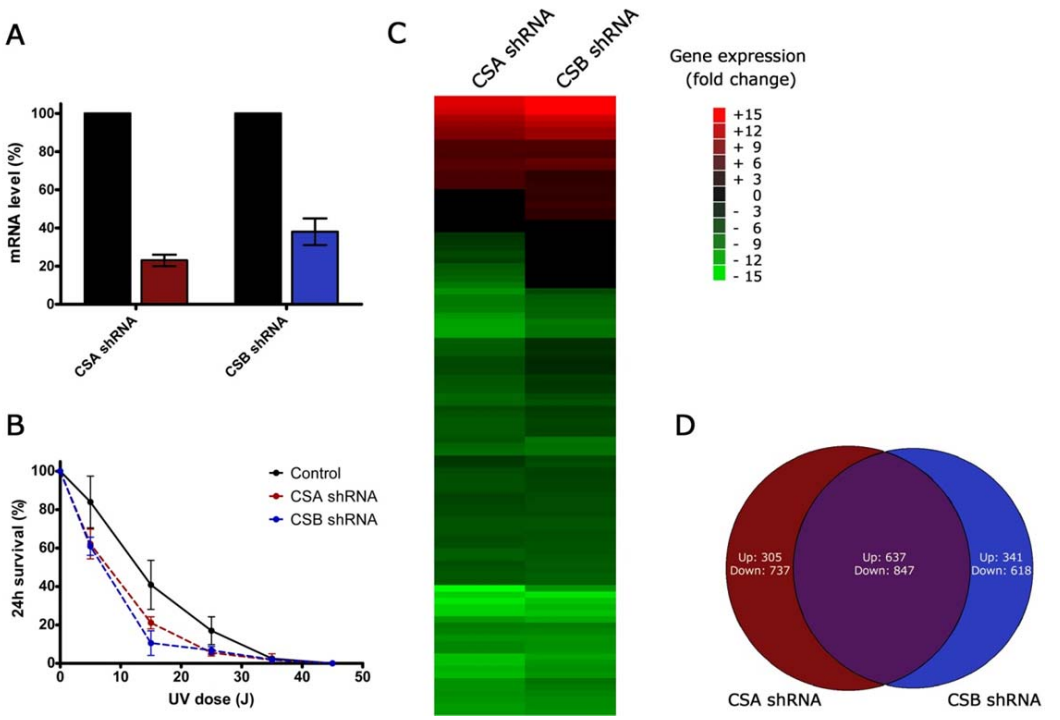


Figure 1 - Consistent gene regulation following knockdown of CSA/CSB. A) Expression of *ERCC8* (CSA) and *ERCC6* (CSB) two weeks after shRNA treatment. Levels are plotted relative to control treated cells, as determined by RT-PCR (n = 4). **B)** CS phenotype confirmed through UV sensitivity of shRNA-treated cells (n = 3). **C)** Similarity of gene regulation after knockdown of CSA or CSB. Bars represent gene ontology pathways (for details see table 1). **D)** Venn diagram showing overlap in up- and downregulated individual genes after knockdown.

GO term	Cellular component	Δ_{shCSA}	Δ_{shCSB}
GO0005840	Ribosome	+13.32	+15.07
GO0003735	Structural constituent of ribosome	+13.16	+15.06
GO0030529	Ribonucleoprotein complex	+12.29	+15.07
GO0006412	Translation	+10.13	+12.22
GO0042254	Ribosome biogenesis and assembly	+8.94	+10.88
GO0005842	Cytosolic small ribosomal subunit (sensu Eukaryota)	+7.96	+9.13
GO0005830	Cytosolic ribosome (sensu Eukaryota)	+7.57	+9.35
GO0015935	Small ribosomal subunit	+5.11	+5.32
GO0019843	rRNA binding	+4.99	+5.95
GO0008137	**NADH dehydrogenase ubiquinone activity**	+4.66	+4.65
GO0005842	Cytosolic large ribosomal subunit (sensu Eukaryota)	+4.56	+4.87
GO0016469	**Proton transporting two-sector ATP complex**	+4.41	NS
GO0005743	**Mitochondrial inner membrane**	+4.29	+4.11
GO0015986	**ATP synthesis coupled proton transport**	+4.25	NS
GO0046933	**Hydrogen ion transporting ATP synthase activity, rotational mechanism**	+4.24	NS
GO0046961	**Proton-transporting ATPase activity, rotational mechanism**	+4.16	NS
GO0016021	Integral to membrane	-9.03	-10.07
GO0016740	Transferase activity	-9.11	-8.57
GO0006512	Ubiquitin cycle	-9.17	-8.26
GO0006350	**Transcription**	-9.55	-7.15
GO0015031	Protein transport	-9.64	-6.94
GO0003677	DNA binding	-9.68	-6.78
GO0005524	ATP binding	-10.41	-8.49
GO0006355	**Regulation of transcription, DNA dependent**	-10.88	-8.96
GO0005737	Cytoplasm	-11.20	-9.64
GO0000166	Nucleotide binding	-11.20	-8.86
GO0046872	Metal ion binding	-12.18	-11.50
GO0016020	Membrane	-12.18	-13.48
GO0008270	Zinc ion binding	-12.26	-10.69
GO0005515	Protein binding	-13.62	-11.67
GO0005634	Nucleus	-14.98	-8.87

Table 1 - Gene expression changes after knockdown of CSA or CSB: The most strongly regulated gene ontology terms are presented, with fold expression change shown for knockdown of CSA and CSB compared to control cells. Mitochondrial terms highlighted in blue, transcriptional terms in brown. NS = expression change not significant.

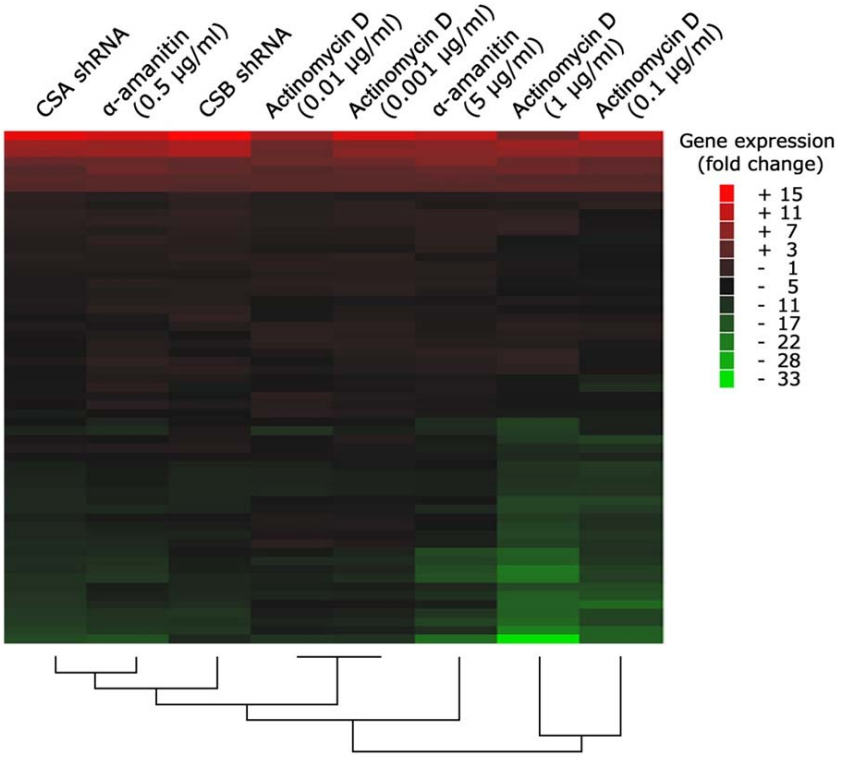


Figure 2 - Gene regulation after knockdown of CSA/CSB resembles transcriptional inhibition. Inhibition of RNA pol I (actinomycin D) and/or II (α-amanitin and high doses of actinomycin D) for 24 hours leads to gene expression changes very similar to those observed in CSA/B knockdown cells. Bars represent gene ontology pathways, while the dendrogram shows clustering by degree of similarity.

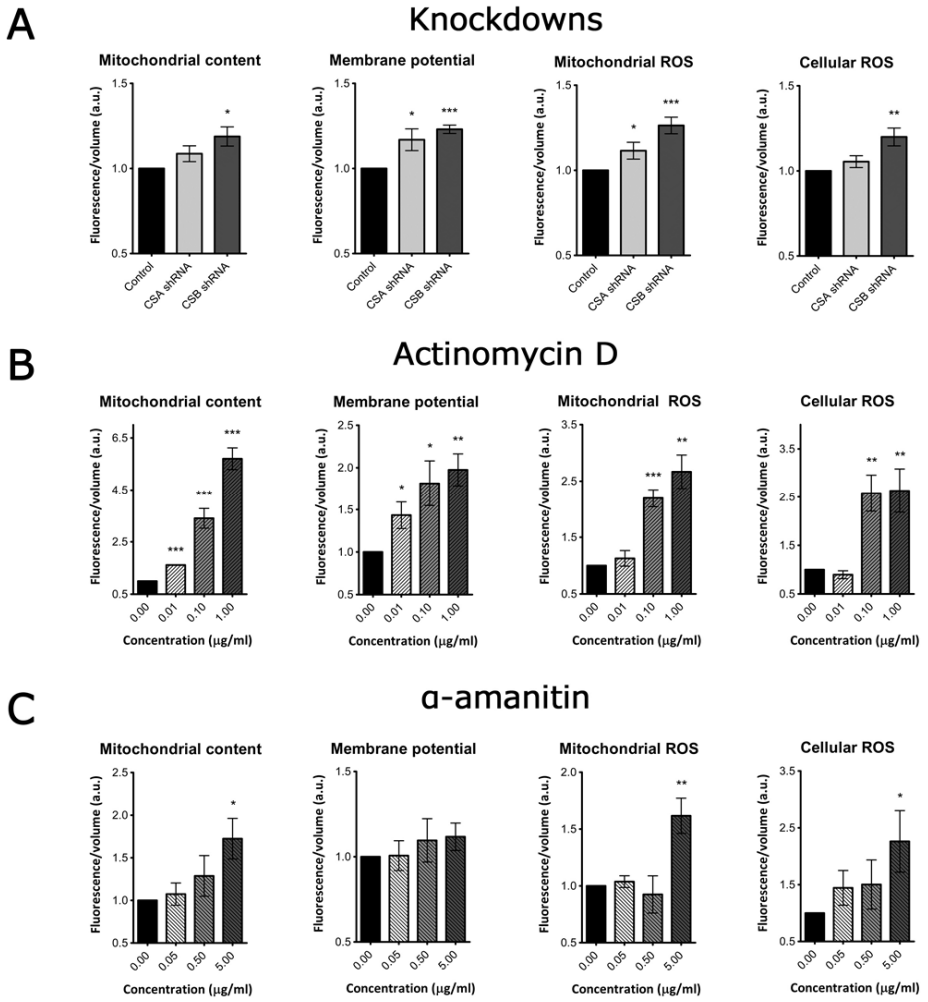


Figure 3 - Both CSA/CSB knockdown and transcriptional inhibition produces mitochondrial phenotype consistent with greater ATP production. Mitochondrial content and membrane potential, as well as mitochondrial and whole-cell ROS were measured using fluorescent dyes for CSA/B knockdown cells (**A,** n = 6) and for cells treated with actinomycin D (**B**, n = 3) or α-amanitin (**C**, n = 4) for 24 hours. All four parameters are increased in knockdown cells and dose-dependently for inhibition of transcription, consistent with increased ATP production in these cells (* to *** indicate $p = 5 \times 10^{-2}$ to 5×10^{-4} respectively).

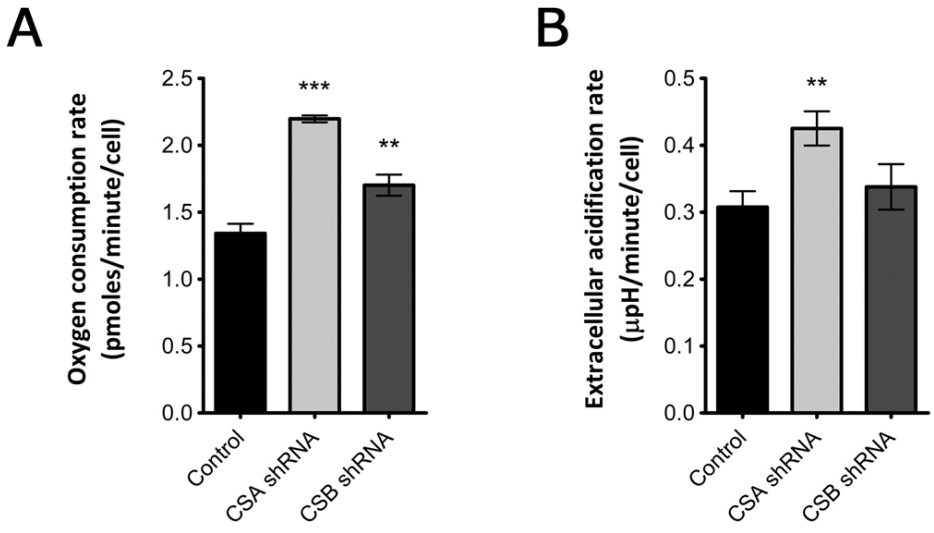


Figure 4 - Increased ATP flux in knockdown cells. Oxygen consumption rate (**A**, measuring OXPHOS) and extracellular acidification rate (**B**, measuring glycolysis) were measured in live cells using the Seahorse XF24 analyzer. Both parameters are increased for knockdown of CSA, and OXPHOS for CSB, consistent with greater ATP flux in these cells (** and *** indicate $p = 5 \times 10^{-3}$ and 5×10^{-4} respectively).

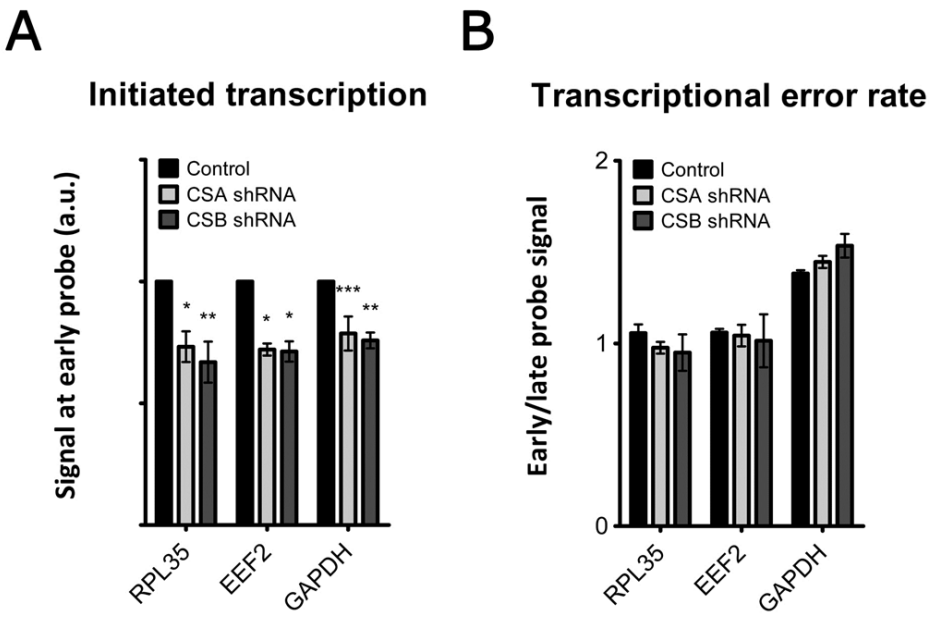


Figure 5 - Energetic phenotype is not caused by futile transcriptional cycles. Using nuclear run-on assays for three arbitrary genes, we tested levels of initiated transcription (**A**) as signal from a probe at the beginning of each gene and observed decreased transcription in CSA and CSB knockdown cells. Combining the early probe with a second probe at the end of each gene. we obtain the rate of unsuccessful transcription (**B**). The latter is unaltered for knockdown cells, suggesting that futile cycles of transcription to not occur (n = 3 for both measurements, * to *** indicate $p = 5 \times 10^{-2}$ to 5×10^{-4} respectively)

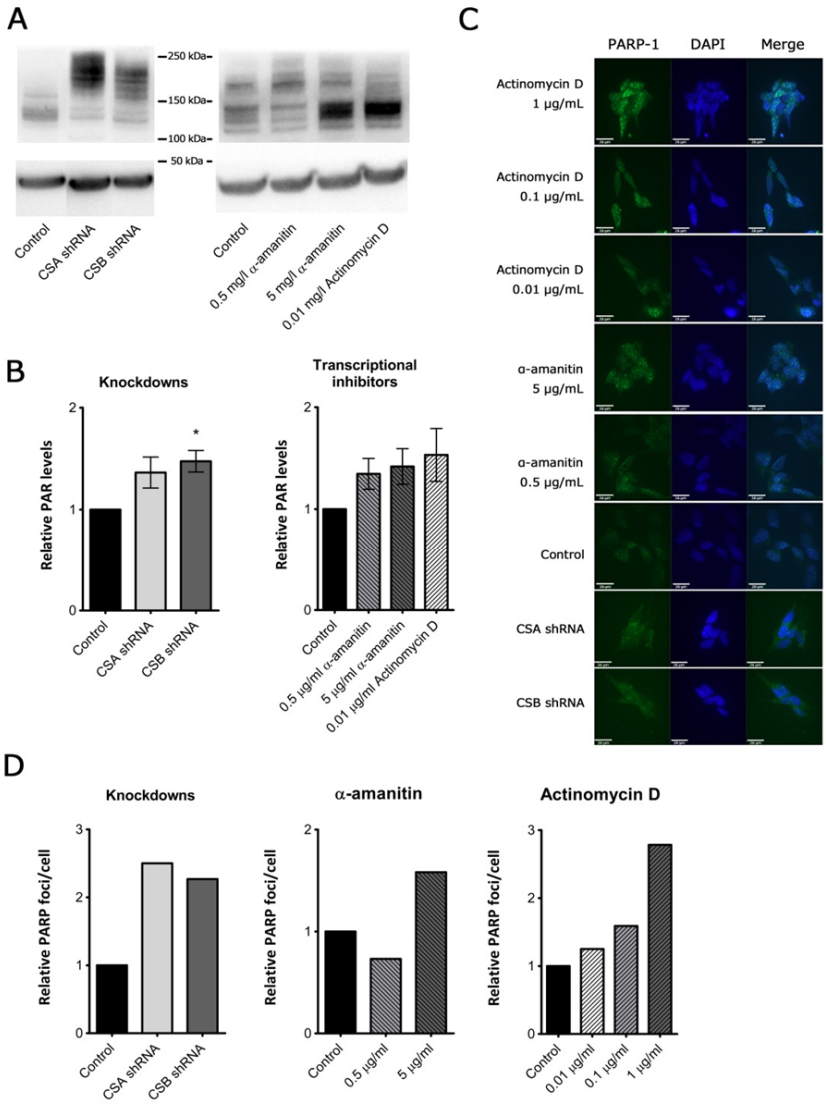
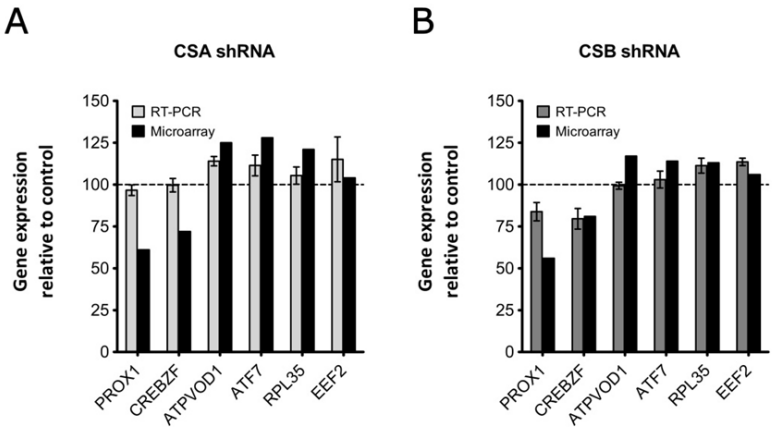


Figure 6 - Increased PARP activation in both knockdown and transcriptionally inhibited cells. Western blotting for the PARP product PAR in both knockdown and transcriptionally inhibited cells (representative blots in **A**, quantification in **B)** shows a trend of increased levels in all cases (n = 5, p values between 0.06 and 0.13). PARP-1 foci were also increased in knockdown and transcriptionally inhibited cells as gauged by immunocytochemistry (values in **D** averaged from 10 images, representative images shown in **C**, scale bar 26 µm).



Supplementary figure 1 - Verification of microarray by RT-PCR. Gene expression changes observed by microarray analysis (black bars) were qualitatively verified by RT-PCR (Two PCR experiments performed for each of four biological samples used for microarray analysis).

Chapter 4

Mitochondria

Numerous changes in mitochondrial function have been reported to occur in aging humans, though in all cases limited to observations of correlation. Levels of cytochrome C oxidase, complex IV of the ETC and a widely accepted marker for mitochondrial content, decrease with age in various rat tissues [251, 252]. This decrease also occurs in human post-mitotic tissues [253, 254, 255], with enough consistency that it has been suggested for age estimation in forensic medicine [256]. The amount of mtDNA, another marker for mitochondrial content, also drops with age in different tissues [135, 251, 252]. The decline of both these markers are generally reported to correlate with a decrease in tissue-level oxidative phosphorylation [135, 251, 252, 257, 258], though in some cases the activity of enzymes involved in oxidative phosphorylation did not change in age (for a thorough discussion, see [136]). Similarly, though overall protein levels and synthesis rates in mitochondria have been reported to decline with age [135, 259], as have drivers of mitochondrial biogenesis [258, 260], in some specific cases protein expression seems unchanged [251].

In accordance with the mitochondrial theory of aging, the mitochondrial genome suffers from instability over time, with both point mutations and deletions being reported in the brain and in at least some stem cell populations [261, 262]. These changes most likely contribute to heterogeneous populations of mtDNA within tissues, and even within single cells [263, 264]. While there is little doubt that loss of information in mtDNA adversely affects mitochondrial function, there is disagreement as to the relative importance of point mutations versus larger deletions and rearrangements [265]. As discussed more thoroughly in Section 1.3.2 there is an abundance of evidence showing an accumulation of oxidative damage with age, correlating with increased oxidative stress from mitochondria [266]. This picture is, however, somewhat obscured by the observation that in some cases antioxidant systems and mitochondrial DNA repair also seem to increase with age [134, 267, 268].

Despite all these indications, the mitochondrial theory of aging remains hypothetical. We cannot say whether the adverse changes that occur in mitochondria are in fact causative or merely a side-effect of aging (or even age-induced changes in e.g. physical activity). More specifically, the question of whether mitochondrial dysfunction leads to and/or results from DNA damage directly relates to the premise of the mitochondrial theory of aging. Further work is needed to establish this relationship convincingly, work that would ideally provide a test of causation between mitochondrial dysfunction and aging. The Polg-Mutator mouse described previously represents a step in this direction,

but as discussed it suggests only that mutations are causative of aging, without support for mitochondrial ROS producing these mutations. Verily, setting up such tests of causation is exceedingly difficult given the miscellany of interconnected factors affecting mitochondrial function, and the work presented in the following mainly provides new insight that helps to clarify the relationship between mitochondria and aging.

4.1 Mitochondrial function during aging

22855734 In line with the studies described above I have been involved in a project with the overall aim of expounding changes in mitochondrial function with age in humans. This was a collaboration with the laboratory of Flemming Dela at the Center for Healthy Aging, University of Copenhagen, and the project was organized by postdoctoral researcher Martin Hey-Mogensen. The primary goal of the study is to discover whether mitochondrial ROS production is higher in older humans, with a secondary aim of investigating whether level of physical fitness and activity influences age-related changes in ROS production. Ninety male subjects were recruited and divided into six groups based on age (young: age 20-30; old: age 60-75) and physical fitness/body mass index (BMI) (trained: BMI<25, with regular exercise; untrained: BMI<25, without regular exercise; obese: BMI>30). Aside from basic vital statistics and body fat percentage, subjects are characterized for physical fitness by peak oxygen uptake (VO_2 max) and insulin sensitivity (using the euglycaemic hyperinsulinaemic clamp test). Beyond this, muscle biopsies taken from quadriceps are used for further mitochondrial characterization: (**1**) Using a customized setup of an OROBOROS Oxygraph-2k linked to a fluorometer, mitochondrial respiration in permeabilized tissue is measured under constant substrate supply while membrane potential and ROS production are simultaneously probed using fluorescent probes. All three parameters are measured in states of ADP stimulated respiration, oligomycin-induced uncoupling and malonate-induced inhibition of the ETC, which allows determination of respiration and ROS production as a function of membrane potential. Furthermore, the ratio of respiration used for ATP production and thermogenesis can be determined at a given membrane potential. Such detailed analysis has not previously been reported using human tissue. (**2**) Intact mitochondria are isolated from tissue samples and mitochondrial DNA repair capacity estimated by measuring activity of BER enzymes in mitochondrial lysates from each subject. Activity is measured using *in vitro* biochemical assays where radiolabeled DNA substrates, incorporating sites of oxidative damage or abasic sites representing either damage or repair intermediates, are incubated with mitochondrial lysates and resolved for incision activity. Such an analysis has similarly never been reported for human samples. This part was my contribution to the study, and will be described in the following. (**3**) Levels of the antioxidant enzymes MnSOD and glutathione peroxidase will be determined in mitochondrial lysates by Western blotting. Finally, all these results are normalized to mitochondrial content, as determined by citrate synthase activity, mtDNA levels, and protein levels of complex I-IV and the voltage-dependent anion channel.

Since no existing literature was available for measuring incision activities in human samples, our protocols for both sample preparation and biochemical assays were adapted from previous studies using mice. The fundamental approach was unchanged, but numerous details were changed during optimization of the protocols. 50-100 mg tissue from each subject in the untrained young and old groups was received from Martin

Hey-Mogensen, as we found that lower quantities were unreliable in terms of mitochondrial yield. We isolated mitochondria from homogenized tissues using differential centrifugation and prepared lysates from both mitochondrial, nuclear and cytoplasmic fractions. We confirmed the purity of these fractions by Western blotting for proliferating cell nuclear antigen (nuclear), β-actin (cytoplasmic), voltage-dependent anion channel (mitochondrial, outer membrane) and cytochrome c oxidase (mitochondrial, inner membrane). As we were probing for differences in endogenous DNA repair activity between subjects, and could not rule out that such differences might legitimately arise from differential expression of relevant proteins, we normalized the lysates by total protein concentration. We then tested excision of both the most common type of oxidative lesion, 7,8-dihydro-8-oxoguanine (8-oxoG), and of abasic sites. In mitochondria the former is performed by 8-oxoguanine DNA glycosylase (OGG1), while the latter is performed by apurinic/apyrimidinic endonuclease 1 (APE1). For both OGG1 and APE1 activity assays we used 28 base pair duplex DNA substrate, with one strand radioactively labeled at the 5' end. In the former case the labeled strand had an 8-oxoG lesion at position 11, while in the latter had a tetrahydrofuran abasic site at the same position. 0.1 picomoles DNA substrate is incubated in the appropriate buffer with 1 μg/100 ng total protein for 24 hours/30 minutes to measure OGG1 and APE1 activity respectively, and the fraction of incised substrate resolved on a denaturing polyacrylamide gel. A representative gel (for APE1) is shown in Fig. 4.1**A**, with the activities of all examined subjects shown in 4.1**B**.

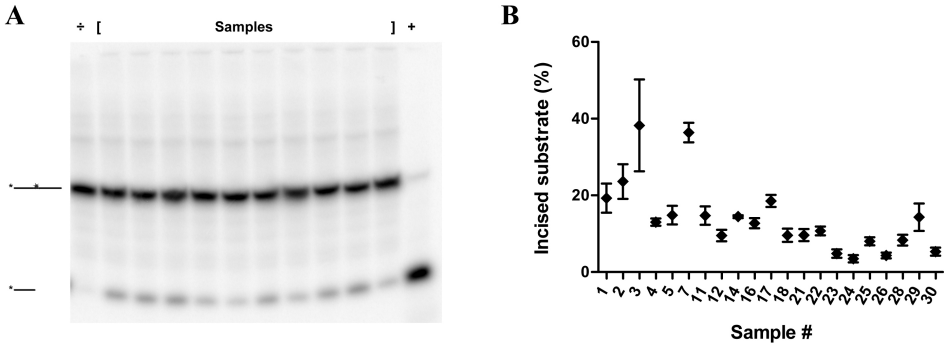


Figure 4.1: **APE1 incision data**. (**A**) Representative polyacrylamide gel showing separation of intact substrate DNA (top bands) and DNA cleaved at the damaged site (lower bands). Although the substrate was double stranded for the incision reactions, it runs as single strands on the denaturing gel. The 5' radiolabel is marked with an asterix. The first lane is a negative control without lysate, while the last lane is a positive control incubated with purified APE1. (**B**) Incision data from three independent experiments is pooled for each unidentified subject, and presented with error bars showing standard error of mean.

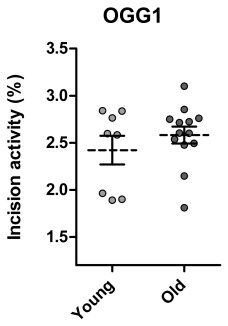
All sample preparation and activity measurements were performed blindly, and subject data received subsequently from Martin Hey-Mogensen. This data is shown in Fig. 4.2**A**, with enzymatic activities pooled by age group shown in 4.2**B** for OGG1 and 4.2**C** for APE1.
We clearly see no difference in incision activity for either protein as a function of age. While this is perhaps somewhat unexpected, there are several possible explanations:

First, there might be no difference in mitochondrial DNA repair with age. In this case the accumulation of damage described previously could result from an increase in damaging events with age, or simply from a small, age-independent fraction of damage escaping repair and thus accumulating over time. Another explanation could be that overall mitochondrial repair is impaired with age, but that the limiting factor is not the excision of damaged nucleotides. Finally, age-dependent changes in repair capacity might occur only in certain tissues, among which skeletal muscle in not included.
Regardless of interpretation, this data represents a novel observation on mitochondrial DNA repair as it relates to aging, and will be included in the manuscript currently in preparation by Martin Hey-Mogensen. Though far from conclusive, the absence of age-related changes in mitochondrial DNA repair could indicate that mitochondrial dysfunction in aging does not result from increased DNA damage to mitochondria themselves.

A

	Young	Old
Age (years)	23 ± 1	66 ± 1
Height (cm)	182 ± 2	177 ± 2
Weight (kg)	70.0 ± 1.2	77.4 ± 2.2
BMI	21.2 ± 0.3	24.8 ± 0.5
Body fat (%)	19.8 ± 1.5	27.1 ± 1.7
Fat free mass (kg)	56.7 ±1.5	57.5 ± 1.5
VO_2 max (ml/min/kg)	51.0 ± 3.1	31.9 ± 1.3
APE1 activity (%)	14.8 ± 3.8	13.4 ± 2.4
OGG1 activity (%)	2.4 ± 0.2	2.6 ± 0.1

B



C

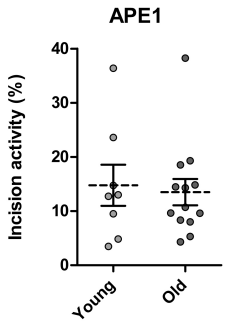


Figure 4.2: **Incision data by age group**. (**A**) Table showing some vital statistics for the two age groups, along with the average incision activities. $n = 8$ for the 'young' group and 13 for the 'old' group. Uncertainties are standard error of mean for the population. (**B**) OGG1 incision activities for the two groups represented as a scatter plot with average and standard error of mean. There is no significant difference between age groups. (**C**) APE1 incision activities for the two groups represented as a scatter plot with average and standard error of mean. Again, no significant different between groups.

4.2 Influence of telomeric dysfunction on mitophagy

The last part of my work for this thesis was to be a study elaborating on a recent report linking telomeric dysfunction [58]. Here, the lab of Ronald DePinho presented an extensive set of data showing p53-mediated downregulation of the mitochondrial regulators PGC-1α and PGC-1β in telomerase-deficient mice. In both $Tert^{-/-}$ and $Terc^{-/-}$ mice (which suffer telomere shortening over several generations) they observed generation-dependent, adverse effects in a number of tissues, including cardiomyopathy, reduced gluconeogenesis and reduced ability to reconstitute hematopoetic stem cells. These changes correlated with loss of mitochondrial content and function in these tissues, and with reduced transcription of PGC-1α & β. Meanwhile, removal of p53 increased expression PGC-1α & β and their downstream targets, partially restored mitochondrial content and attenuated heart and liver pathophysiologies. Clarifying how mitochondrial function is dependent on telomeres could provide a model where DNA damage affects

mitochondria by means of telomere shortening.
This study provides very strong support for a telomeric role in mitochondrial regulation, but despite its impressive scope there are of course still a number of details to resolve. One important topic that was left out of their investigation is that of mitophagy, the autophagic degradation of mitochondria. This process is constantly active and subject to multiple levels of regulation, and because it selectively targets damaged mitochondria it is important for both steady-state levels and quality control of mitochondria [184]. Simply put, the cell's population of mitochondria is a product of the ratio of biogenesis and mitophagy, and the condition of those mitochondria depends to a large extent on the overall flux resulting from the absolute levels of biogenesis and mitophagy. While Sahin *et al.* report lower mitochondrial biogenesis and content, that situation could come about under different conditions of mitophagy: one could imagine that inadequate biogenesis would repress mitophagy in an attempt to preserve essential mitochondrial content, which would attenuate the effect presented in [58]. Alternatively, the reduced mitochondrial content they observe could be the cumulative result of the reported pathway and a (possibly independent) increase in mitophagy. The former case, and the unexpected scenario of unaltered mitophagy, would imply poorly functioning mitochondria, likely with altered membrane potential, damaged mtDNA and more. Conversely, the latter case would predict the remaining mitochondria to be in good condition, perhaps as part of a defensive response against stressors causing telomere shortening (of which ROS is one).
The aim of this project is therefore to establish the mitophagic state in this mouse model of telomere dysfunction. Two subquestions of this are whether a putative effect varies between tissues with more or less active mitophagy, and whether any effect is specifically on mitophagy or part of a broader autophagic response. To test this we use the same $Tert^{-/-}$ mice as in [58], in order to maximize comparability between findings. The core of the project was based on Western blotting for mitophagic markers, and using fluorescence microscopy to assess colocalization between autophagic proteins and mitochondria. We used a cohort with nine mixed-gender mice in each of three groups: a control group of $Tert^{+/-}$ mice, which have normal elongation of telomeres [269]; a group of first generation $Tert^{-/-}$ mice, which are expected to show mild, if any, effects of telomere dysfunction; a group of fourth generation $Tert^{-/-}$ mice with drastically shortened telomeres. From these mice we use four tissues for Western blotting, with low (liver) or high mitochondrial activity (quadriceps and brain). In the brain we examine both cortex and substantia nigra, as the latter is implicated in Parkinson's disease where one of the causative genes encodes the mitophagic protein parkin [270]. In each tissue we quantitatively blot for the following proteins: LC3-II, a general marker for autophagy [271]; PINK1 and Parkin, both direct inducers of mitophagy [184, 272]; UCP2, an indicator of mitochondrial membrane potential; and FIS1, a mitochondrial fission protein that is essential for mitophagy [273, 274]. To evaluate mitophagy *in vivo* we use primary fibroblast cultures established from tail snips of three mice in each group, and measure colocalization of autophagosomes (using an antibody for LC3-II) with mitochondria (using MitoTracker Green) by standard immunocytochemistry [275]. If possible, we will also use a mitophagy assay currently under development, whereby mitophagy is measured directly using fluorescently labeled interstrand crosslinks. Because these lesions are not repaired in mitochondria [131], their removal (as evaluated by fluorescence microscopy over long periods) will occur exclusively by mitophagy and thus allow direct

quantification of mitophagic activity.
Regrettably I was not able to finish this project within the time frame of my Ph.D. After designing and organizing the project I performed all dissection/sample collection and started optimization of the quantitative Western blotting. I then handed the project over to Evandro Fei Fang at the Laboratory of Molecular Gerontology, National Institute on Aging, NIH, with whom I will be in correspondence to bring it to completion.

Chapter 5

Conclusions

As I wrote in the opening paragraph, the goal of this thesis has been a contribution to our understanding of how genomic and mitochondrial maintenance function in the context of aging. Accomplishing this goal in turn comprises a tiny contribution to grander aim of explaining both why and how the phenomenon of aging occurs to most organisms, a relation that I will expand upon somewhat in this chapter. To pursue this goal I have taken a broad approach, touching on several potential factors in aging at several levels of detail. The main theoretical basis for my work has been the DNA damage and mitochondrial theories of aging, both operating around the idea of damaging molecules (largely ROS) create lesions in DNA and prevent its use in the vital operations of the cell. Within this framework I have focused on segmental progerias as a model system, because their accelerated phenotypes facilitate the search for cellular processes involved in normal (slowly manifesting) aging.

The first publication presented in this thesis looks closely at how disease-causing point mutations in *RECQL4* affect the functional activities of the resulting protein. By purifying both wildtype and mutant proteins and subjecting these to a range of biochemical, structural and *in vivo* assays, we were able to uncover a consistent pattern of deficiency in three separate patient mutations. This deficiency manifested in the ATP-dependent helicase activity of the protein that may well be required for a secondary cellular function, as the activity is not mediated by the N-terminal domain required for initiation of replication and the helicase domain does not seem essential for this known function of the protein. One could thus speculate that while its role in replication is essential, RECQL4 has a second, non-essential function whose breakdown results in the observed disease phenotypes.

In the second publication we tracked the movement of two other disease-implicated helicases (WRN and BLM) within the nucleus, in order to explain both their distribution under non-stressed conditions and how they accumulate at sites of DNA damage. By combining FRAP microscopy with mathematical modelling of reaction-diffusion processes we were able to identify limiting factors for the localization of these proteins to different subcompartments, and as it turns out the dynamics governing this localization vary greatly between subcompartments. By this approach we could further establish that while the binding of these proteins at sites of damage is a diffusive event, their gradual accumulation is determined by the slow creation of binding sites at or in close proximity to the damaged site. While we cannot determine the nature of such binding sites with this setup, they likely represent prerequisite recruitment of other repair

proteins and/or histone modifications surrounding the lesions. Finally, by applying our model to previously reported data, we can classify DNA repair proteins as either 'scanners' that constantly interact with DNA, or 'responders' that have no such interaction prior to damaging events.
Our study of Cockayne syndrome was based on an unbiased search for pathways affected both by the knockdown of CSA and CSB, thereby hoping to identify cellular functions involving both proteins and which could therefore be important for the development of CS. Using microarray analysis we identified a strong upregulation of mitochondrial ATP production for both knockdowns, alongside downregulation of transcription. In pursuance of a connection between these observations, we found that treatment with different transcriptional inhibitors produced a phenotype of increased mitochondrial gene expression and activity similar to what we see in the knockdown cells. Further investigation uncovered a link between these characteristics in increased activation of PARP-1, quite possibly in response to stalling of polymerases during transcription. Thus CSA and CSB may cooperate in terminating PARP signalling in these events, such that their absence results in excessive poly-adenine ribosylation. PAR metabolism is tightly linked to mitochondrial function, and its imbalance could lead to a compensatory response in mitochondrial regulation. In CS cells such an imbalance would be permanent, and in the course of time mitochondrial failure would lead to the death of the cell. Thus, at least in this specific disease, mitochondrial dysfunction appears to be downstream of DNA damage.
Due to the ongoing nature of the manifestly mitochondrial projects fewer conclusions can be drawn from this part of my work. Nonetheless, my contribution to the project of Martin Hey-Mogensen provides strong support for the absence of decline in mitochondrial DNA repair capacity with age. While my measurements of incision activity naturally only represent a subset of the reactions that constitute mitochondrial DNA repair, the lack of change for APE1 in particular is convincing because this endonuclease is a point of convergence for several mitochondrial repair mechanisms [276]. It will be interesting to see how other aspects of mitochondrial function do change with age, once this study is published.

To put these findings into a broader perspective, it is relevant to give some thought to the segmental progerias used as models systems for the majority. One observation arising from our examination of CS is that the known segmental progerias may represent the failure of different components within the body, components that decline more uniformly in normal aging. As an example, let us look at the manifestation of CS as compared with Werner syndrome. From the results presented in Chapter 3 I would argue that at least a large part of the CS phenotype stems from cell death arising from progressive energy deficiency. This deficiency arises from excessive PARP activity in response to transcriptional stalling and would be expected to manifest most strong in the brain, both due to higher transcription in the brain [114] (which could lead to more frequent activation of the signalling) and because of the vast amount of energy required by the brain (thus rendering it more sensitive to deficiencies). This could explain why CS includes such a strong neurological phenotype, which occurs only rarely (and as a rule less severely) in XP, and is compatible with the observation that $CSB^{m/m}$ mice only show mild neurological impairment [277]: because the fraction of basal metabolism required by the brain is approximately tenfold higher in humans than in mice [278], one

would expect an inadequate energy supply to have a more serious effect on the brain in humans. On the other hand, the systemic effect of CSB deficiency in these mice is consistent with the non-neurological symptoms of CS patients, and with an imbalanced metabolic state: despite consuming more food than their wildtype counterparts (and having similar levels of physical activity), their body weight is significantly lower and they show markedly less accumulation of fat with age [231, 277]. This closely resembles the cachexia of CS patients, who also lose subcutaneous fat and are unable to gain weight. Aside from cachexia and neurodegeneration, several symptoms of CS are amongst the most common in mitochondrial diseases, including muscle failure, deafness and retinal problems [242]; conspicuously, these symptoms are also common in late aging.
Turning to Werner syndrome, we see only a few symptoms (cataracts, growth failure) shared with CS. On the other hand, perhaps the majority of symptoms in WS are characteristic of normal aging: thinning and greying hair, wrinkled skin, osteoporosis, type II diabetes, tissue calcification, atherosclerosis, hyperpigmentation and more. In further contrast with CS, many of these symptoms parallel features of aging that emerge progressively from midlife and are not typically considered disease conditions. While the origin of pathology in WS is not known, current evidence points towards increased genomic instability, telomere shortening and cellular senescence. Thus we have a difference both in the cellular dysfunction and in the resulting patient phenotype of WS contra CS, and it is attractive to surmise that each of these progerias arise from one of several specific dysfunctions occurring in parallel during aging. Hence, age-related neurodegeneration and deafness might be the result of mitochondrial dysfunction that normally occurs in conjunction with (and is likely interdependent with) senescence-dependent tissue wasting. In relation to my first research question, this would suggest that while some aspects of aging result directly from DNA damage, others are secondary effects. Understanding such distinctions in age-related decline could be important for identifying the underlying mechanisms, and at the same time guide us to the appropriate choice of segmental progeria (or alternate models) for studying specific mechanisms of aging.

In contrast, my findings on the workings of RecQ helicases are rather less conducive towards broad conclusions. Understanding activities and interactions at the protein level is essential for deciphering the bigger picture of how pathology results from cellular dysfunction, but charting these details is akin to filling out the pieces of a puzzle; most of these pieces will be added along the established edges of the puzzle, and only incrementally build towards important changes in perspective. Nevertheless, a look at what types of proteins are studied in this context does bring up some general questions whose answers may help guide future research. One of these is why we so often find helicases playing central roles in our models of aging? This can be seen as a subquestion of why DNA repair proteins are so often involved, which should be justified from the theoretical base presented earlier. But why do we not see segmental progerias caused by mutations in glycosylases or DNA ligases? A possible explanation is that the mutations we are able to observe in patients represent milder deficiencies in DNA maintenance, while mutations in more crucial genes simply lead to loss of viability. This would suggest some degree of redundancy in the function of helicases involved in DNA maintenance and repair, which may not be the case for e.g. ligases and structural proteins. On the other hand, mouse models lacking single glycosylases generally do not show a strong phenotype, which could suggest that the lack of glycosylase-linked proge-

rias results from even greater redundancy at the incision step [279]. The idea that serious disease results from compromise to a system within a certain range of redundancy fits with the findings presented in Paper I: the majority of patient mutations in *RECQL4*, including those studied here, fall in and around the helicase domain rather than the N-terminal Sld2-homology domain that seems to be essential for replication. This could signify that mutations in the Sld2-homology domain are not viable, while the helicase domain mediates a less critical function of the protein. Such a scenario could pertain to my first research question, depending on whether damage processing or secondary roles are most frequently implicated in disease. This situation is particularly plausible for the RecQ helicase family, where the five human members are probably all derived from the single RecQ helicase in *E. coli* and share a large degree of homology. Interactions between members have been reported in several cases, as well as amplified phenotypes in double-knockout cells [280, 281, 282]. Thus, while the human RecQ helicases have evolved into specialized roles, they may still have some residual redundancy from their common ancestry; resolving this hypothesis requires a lot more pieces in the puzzle.

Another issue concerns the apparent involvement of RecQ helicases in different DNA repair pathways. As evidenced in Paper II, and numerous previous studies, several RecQ helicases are recruited to DNA double-strand breaks [283] and interact with participants of other DNA repair pathways [188]. This association spans quite widely, even where a specific function has already been identified (e.g. resolution of double Holliday junctions by BLM). Because it is unusual for proteins to have an array of independent functions, it is appealing to imagine that the involvement of RecQ helicases in different DNA repair pathways is somehow mediated by a common mechanism. Though such commonality would be expected to originate in the homologous sequence of the proteins, there is little to hint at which (if any) domain(s) could be responsible for a general recruitment to DNA damage sites. This idea is of course purely hypothetical, but the fact that at least WRN and BLM fall into the category of proteins that continuously scan DNA would support this interpretation. One possible scenario is that these proteins are readily recruited to compromised DNA, and once there coordinate with further sensing by other proteins to initiate the appropriate repair response. An argument to support this idea is that the DSBs observed to prompt recruitment are typically generated using micropoint laser irradiation at relatively high intensities [213]; such irradiation does not selectively create DSBs [284], and the aggregate damage site thus produced could attract DNA repair proteins in several different capacities. This could further explain the interactions with proteins from other repair pathways, which could be stimulated once their necessity for the damage response is established. Again, this interpretation is highly speculative, but presents a possible scheme that may be relevant in light of future data.

Further elaboration on the pathogenesis of CS could also be pursued by several avenues. Immediately at hand is a more detailed description of the imbalanced PAR metabolism in cells lacking CSA or CSB. We focus on PARP-1 based on its known role in DNA damage recognition, and the fact that it is responsible for the majority of poly-adenine ribosylation in the cell [285]; that we observe an effect on the cellular phenotype after relatively specific inhibition of PARP-1 also support a dominant role for this polymerase. Even so, we cannot rule out the involvement of other PARP family members, and clarifying this situation should help understand the details of pathogenesis. Further,

while we show increased PAR levels in these cells it is not clear how the relative ribosylation and PAR degradation activities are affected. Closer inspection of levels and activity of the PAR depolymerizing enzyme poly-adenine ribose glycohydrolase thus offers an obvious expansion of our study.
A closely connected question is whether cell death in CSA/B deficient cells happens because an inadequate ATP supply eventually leads to a failure of vital cellular processes, or whether depletion of NAD^+ triggers apoptosis by a more direct pathway. It is not difficult to imagine that the cell would enter apoptosis when starved of ATP for any significant period of time, and stimulation of apoptosis following glucose-deprivation has been reported previously [286]. Such a state would presumably affect a wide spread of processes, any or all of which could contribute to induction of apoptosis and/or necrosis [287]; given the important role of mitochondria in apoptotic signalling, the cells might already be sensitized towards apoptosis by their compromised metabolic state. Alternatively, overactivation of PARP-1 rapidly leads to depletion of cellular NAD^+ [288], which in itself has been proposed to induce apoptosis [289]. At least two mechanisms have been reported that could mediate this effect: the first of these is the release of apoptosis-inducing factor (AIF) from mitochondria following PARP-1 activation by alkylating agents, oxidative stress and stimulation of glutamate-receptors [288]. This effect was caspase-independent, but was blocked both by neutralizing AIF using antibodies and by preventing its release from mitochondria. This release was not stimulated by isolating mitochondria in NAD^+-free medium, suggesting that signalling is tied to consumption of NAD^+. The second mechanism is based on substrate competition with the sirtuin deacetylases. Sirtuin 1 prevents cell death by deacetylating pro-apoptotic factors like p53 using NAD^+ as a substrate [290], and its stimulation by increased NAD^+ availability has been shown to prevent axonal degeneration [291]. Conversely, the depletion of NAD^+ by PARP-1 overactivation was shown to mediate caspase-independent cell death through inhibition of the mouse orthologue of sirtuin 1 and consequent hyperacetylation of p53 [292]. Although this effect was proposed to be independent of AIF-release, the situation is somewhat muddled by the observation that knockout of sirtuin 1 leads to overactivation of PARP and release of AIF [293]. Whether these two mechanisms are independent or connected, closer inspection of caspase pathways in the CSA/B knockout cells is a promising possibility for determining the method of cell death.
Beyond these specific investigations, it is clear from both these findings on CS pathology and my mitochondrial work that mitochondrial function is linked to a range of factors implicated in aging, including inflammation, DNA repair and apoptosis. Though it feels almost tautological to point out, sorting out the intricate role of mitochondria as instigators, mediators and regulators in various processes implicated in aging is likely to be critical for our understanding of its deteriorative effects.

As I have repeatedly emphasized, aging is a multifaceted disorder that is probably caused by the concurrent malfunction of numerous physiological systems. In something as synergistic as a living organism these systems are likely to be interdependent. Thus the relatively uniform progression of aging in different organisms could represent a wave of malfunction that may have been spearheaded by a specific failure, but which carries in its wake other failures that produce a recognizable set of symptoms. Returning to an evolutionary perspective, natural selection would be strong against whatever constituted the dominant cause of death and weak against other potential causes. In terms of aging

this implies that any 'weakest link' in the declining organism would in turn be shored up through evolution, offering further support for a comparable decline of multiple systems. While this interpretation condemns biogerontology to the challenging study of discrete-yet-interconnected systems, it also brings up interesting questions. Major causes of death for humans today may be evident because we are essentially not adapted to modern society: cancer is common because of our 'unnaturally' long lifespans, and the brain's paramount importance in (evolutionarily) recent history accentuates the impact of neurodegenerative disorders. On the other hand, such aspects of aging would have evolved in environments where extrinsic mortality was the norm, and may in fact not be evolutionarily stable in modern society. Given that modern society changes much faster than biological evolution would be able to adapt to it is difficult to say what implications this hypothesis would have for the process of aging, but it may be stimulating to some researchers to consider aging an evolutionary remnant on par with goosebumps. We may not get the answer to this any time soon, but the field of aging offers a host of interesting biological questions for the meantime.

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