Identifying a role for POT1 as a regulator of the telomeric end in Aspergillus nidulans

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Abstract

Chromosome ends are capped by protective structures that maintain the genetic integrity and also play key roles in aging and cancer. These structures, termed telomeres, are protein-DNA complexes, their length being critical to their function. The filamentous fungus, Aspergillus nidulans, possesses very short and tightly regulated telomeres, but nothing is currently known about the mechanisms of telomeric length regulation. A telomere-binding protein, POT1, has been identified in A. nidulans, and it has been hypothesized to bind to the 3' overhang of the telomeres and function in length regulation and protection. However, no studies have been conducted to determine its importance to telomere maintenance in A. nidulans until now. By altering a previous method, I have developed a new approach to measuring the C-rich strand length of telomeres. Results show that the POT1 mutant displays heterogeneous telomere length at both the G-rich and C-rich strands, which is evident when compared to the tightly regulated telomeres of the wild-type. These findings demonstrate that POT1 is a telomeric protein in A. nidulans and a key player in telomeric length regulation.

Introduction

Curiosity is the force behind continuous discovery and an apparent constant in human nature. It is a quality that I have always possessed and something that has steered me to become a student of science and the scientific method. Scientific research is the result of natural causes, an outlet for this curiosity. There is nothing more intriguing than the unknown, a reality suggested in *Mein Weltbild* by Albert Einstein, one of the greatest minds to ever live:

> The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed. (Einstein, 1934)

In science, we constantly question mysterious phenomena. As we collect answers to these questions, we gain a better understanding of the world around us, helping improve our quality of life and our development as a people. With each theoretical answer comes endless novel questions, one of which I hope to answer in the following thesis.

What is DNA, and why is it important? This was a fundamental question of mine growing up. I watched many crime scene shows with my parents and often wonder what this three-letter word meant. How could a criminal be caught based on their DNA? What made his or her DNA different from everyone else's, and what was this mystical entity? I struggled with this concept for a long time, until I was presented with a simple analogy. DNA is an internal library for the body. Similar to books, the purpose of DNA is to store specific information (called genes), which are passed down over generations and are necessary to the body in order to perform every day maintenance and functions. The information that our DNA provides is stored as genes on double helices called chromosomes, which is comparable to the books in the library. Humans possess 46 chromosomes, and these same chromosomes are present in the nucleus of every cell in the body, no matter the type, excluding sex cells, which have half the chromosomes.

If every cell contains the same genetic information, how do they perform different tasks? In order to perform different tasks, specialized cells have the ability to either turn "on" or "off" certain genes in their genome. This mechanism can be compared to the fact that there are tons of books in a library, but we only read the ones that tell us what we wish to know.

In order to develop and maintain the body, these cells must divide. Cell division requires that the chromosomes be duplicated so that each daughter cell gets a complete set of the genome. During DNA replication, after the two strands of the DNA double helix separate, the enzyme primase synthesizes an RNA primer at the 3' end that is complimentary to the parent strand (Kornberg, 1984) (Figure 1). DNA polymerase recognizes this RNA primer and begins synthesizing the new strand. Once synthesis has begun, the RNA primer degrades, leaving a lack of replicated nucleotides (empty space) at both ends of the chromosome, in turn leaving chromosomes with one strand shorter than the other (a 3' overhang). In addition to having uneven ends, this process causes chromosomes to become shorter with each successive round of replication (Figure 1). This phenomenon is the end replication problem, defined by James Watson in 1972, and should be an issue, as the cell would theoretically lose important genetic information as it continues to replicate.

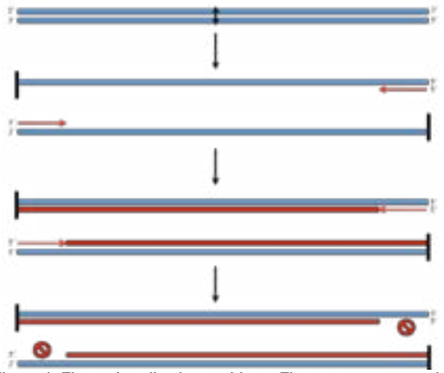

Figure 1. The end replication problem. The two parent strands of DNA (blue) are first separated. New strands of DNA are synthesized (red) that contain RNA primers (red arrows) used to initiate DNA replication. Degradation of this primer produces a 3' overhang on chromosomes, where the red strand is missing some DNA.

Telomeres

Telomeres are the reason why this mechanism of replication does not present an irrevocable problem for future generations of the cell. They are the protective caps found at both ends of eukaryotic chromosomes and are necessary for the stability of genetic information. Telomeres are short, specific, non-coding DNA-protein structures composed of specific

*This author wrote the paper as a part of a senior thesis under the direction of Dr. Kirk

repeating sequences of T, A, G, and C nucleotides—the same nucleotides that make up our DNA.

Telomeric DNA sequence and structure is similar across all eukaryotes, even in the most widely divergent species. An example of this would be the telomeric repeat of 5'-TTAGGG-3', which is found in all vertebrates (Meyne, Ratliff, & Moyzis, 1989), and numerous other species including slime molds (Forney, Henderson, & Blackburn, 1987), and some species of fungus, such as *Aspergillus nidulans* (Bhattacharyya & Blackburn, 1997; Kusumoto, Suzuki, & Kashiwagi, 2003) and *Neurospora crassa* (Schechtman, 1990). However, this sequence is not the same across all eukaryotes, as for example, *Tetrahymena thermophila* has the telomeric repeat of 5'-TTGGGG-3' (Blackburn & Gall, 1978). In addition to telomeric repeat sequence, the average length of telomeres differs across organisms as well. For example, human telomere length ranges from 1500-6000 bp, or 250-1000 telomeric repeats (Moyzis et al., 1988), whereas telomeres of *Aspergillus nidulans* are about 110 bp (Bhattacharyya & Blackburn, 1997; Vahedi Thesis, 2008), or only about 18 repeats.

As the solution to the end replication problem, telomeric sequences that do not code for genes flank chromosomes. Telomeres act as a buffering zone, allowing chromosomes to be replicated completely, without the loss of important terminal bases at the 5' end of each strand, leaving coding DNA intact (Olovnikov, 1973). This function, however, is only one of the many functions of the telomere, and an observation that came years after its discovery.

Going back in time, it was geneticist Hermann J. Müller who inadvertently discovered telomeres in 1938. While working with *Drosophila melanogaster*, he observed that, when exposed to x-rays, chromosome ends did not possess inversions or deletions unlike the rest of the genome (Müller, 1938). In other words, the interior of the chromosome possessed many mutations differing from the ends, which remained unchanged. From this discovery, he believed there must have been an additional component at chromosomal ends that provided stability towards genetic information. He accredited this finding to protective caps found at the ends of chromosomes that he called "telomeres" (Müller, 1938).

Just a few years later, Barbara McClintock saw the first glimpse of telomere function. She observed that chromosomes fused together when their natural ends, the telomeres, were removed (McClintock, 1941). From this experiment, she concluded that telomeres were necessary for the integrity of genetic information. Without telomeres, chromosome ends would be recognized as double stranded "breaks" and interchromosomal fusion could occur (McClintock, 1941). Chromosomes that do not have telomeres to cap their ends tend to bind to other "uncapped" chromosome ends. Such chromosomal fusions ultimately cause chromosome loss and cell death.

Although studies in the field appeared promising, the scientific community would not continue with telomere research until 30 years later when Watson (1972) identified the end replication problem. With respect to this problem, he suggested that human somatic cells might not be able to correct for the natural chromosomal shortening that occurred with each DNA replication event. The repeated telomere sequences at the ends of chromosomes could be acting as a buffer to prevent loss of important genetic information (Watson, 1972; Olovnikov, 1973). Around the same time that Watson made his findings, Olovnikov (1973) made a connection between Watson's study and a previous study by Hayflick and Moorhead (1961). Hayflick and Moorhead (1961) observed that human somatic cells no longer proliferated after approximately 50 cell divisions but instead entered a state of cellular senescence. Using these combined results, Olovnikov (1973) proposed that the end replication problem might cause the progressive shortening of telomere sequences with each cell division, which in turn could establish the potential number of rounds of DNA replication before the cell would reach cellular senescence.

Telomeres and Aging

A potential role for cellular senescence in aging was suggested when it was observed that there was a reduction in proliferative capacity in cells from donors with premature aging syndromes in comparison to control cells (Martin et al., 1970). If this finding was true, and the proposal made by Olovnikov (1973) was correct, then telomere length may therefore serve as a biological clock to determine the lifespan of a cell and, in turn, of an organism. At this time, the idea of telomeres being a predictor of aging was just speculation. In order to tie these two ideas together, it first had to be shown that telomere length was a specific predictor of how many times a cell could divide.

The connection between telomere shortening and cellular senescence was made in 1990. Harley, Futcher, and Greider (1990) performed a study in which telomere length was measured in aging human fibroblasts, making a significant correlation between telomere length and age of human fibroblasts. As these cells aged, telomeres were seen to shorten by approximately 50 bp with each round of DNA replication. Older cells showed significantly shorter telomeres than younger cells, thus showing for the first time that cellular aging could be linked to changes in genomic DNA (Harley et al., 1990).

Even after these convincing results achieved by Harley et al. (1990), more positive evidence was needed in order to confirm that telomere length represented the cell's biological clock. This necessary evidence was found in a study done by Bodnar et al. (1998). In their research, they transfected human fibroblasts with TERT. This protein is a key component of the telomerase enzyme, which catalyzes the lengthening of telomeres and is normally absent or present at very low levels in human somatic cells. These TERT+ cells that had elevated telomerase levels showed elongated telomeres and were maintained in culture for an average of about 90 population doublings. In comparison, TERT- control cells exhibited short telomeres and senesced after about 55 population doublings (Bodnar et al., 1998). The fact that cell life span was extended through telomere lengthening gave stronger evidence that telomeres were directly tied to cellular aging.

Now that evidence had been presented linking telomere length to cellular life span, curiosity surfaced as to whether the same could determine aging at an organismal level. Herrera et al. (1999) studied mice that were genetically deficient for the telomerase enzyme. These mice were unable to elongate their telomeres, which allowed for the direct observation of mice with varying telomere lengths. Results showed that mice with shorter telomeres developed more diseases and had a shorter life span than mice with longer telomeres (Herrera et al., 1999). Through these results, the scientific community now had substantial evidence that telomere length in cells could be a determinant of life span at an organismal level.

Could the same be true in humans? Not only has recent research in the field shown a link between telomere length and lifespan in humans, but also to telomere length and early onset of age-related disorders. Similar to the many studies performed in mice, studies in humans have shown that telomere length negatively correlates with age (Jiang et al., 2008; Song et al., 2010). Interestingly, Cawthon et al. (2003) also showed

that individuals with shorter telomeres had a lower survival rate that was caused by an assortment of infectious diseases and heart disease. Additional studies have also been done linking accelerated telomere shortening to diabetes (Sampson et al., 2006), heart failure (Van der Harst et al., 2007), osteoporosis (Valdes et al., 2007), coronary heart disease (Fitzpatrick et al., 2007), and an increased cancer risk (Wu et al., 2003). From these observations it has been suggested that telomere length may serve as a biomarker determining general health, lifespan, and the pace of biological aging (Babizhayev et al., 2011).

Telomeres, Cancer, and Smoking

As explained previously, telomeres in human somatic cells shorten with each DNA replication event. This is because the enzyme that lengthens telomeres, telomerase, is normally absent or present at very low levels in these cells. Once a cell's telomeres reach a critical length, a DNA damage response is activated before the genetic material can be compromised, and the cell enters a senescent state (Harris & Levine, 2005). One of the speculations as to why organisms age has to do with the fact that cells enter this senescent state where they continue to live and function, but no longer go through cell division (Jeyapalan & Sedivy, 2008). Thus, aging would occur as a result of fewer new daughter cells being produced.

However, a problem arises when telomeres reach a critically short length, and this DNA damage response does not get activated. In these cases, senescence does not occur and the cell continues to divide but with severe telomere shortening, marked genetic instability, and massive cell death; this event is commonly called crisis. Most cells going through crisis die due to the occurrence of chromosomal abnormalities and the near complete loss of telomeres (Greider & Blackburn, 1996). However, as a result of possible mutations and chromosomal alterations that occur during crisis, some mutated cells survive that possess the ability to maintain their telomere length, either through the activation of telomerase or through an alternative pathway (ALT) that is thought to involve recombination (McEachern & Blackburn, 1996; Stewart & Weinberg, 2006).

The telomeric stabilization of genetically compromised cells in the crisis state contributes to the formation of cancers (Chin et al., 1999). These mutated cancerous cells divide rapidly, maintain very short telomeres, and are immortal, leading to the formation of malignant tumors. This claim is supported in a study done by Kim et al. (1994) where they found that almost all malignant tumor cells showed upregulated telomerase activity. Other research has also shown that cancer cell lines possess shorter telomeres than regular somatic cells (Shammas, 2011). Therefore, we should naturally want to maintain the length of our telomeres as long as possible, as we are at risk for many different life-threatening diseases once they get too short.

In order to maintain telomere length, one must avoid factors that can contribute to their accelerated shortening. There are many different components that have been seen to speed up the shortening of our telomeres, including obesity (Furukawa et al., 2004), pollution (Hoxha et al., 2009), stress (Epel et al., 2004), and bad diet (Cassidy et al., 2010). It has been speculated through research that these factors cause telomere shortening due to a heightened exposure to agents that cause telomeric damage. With respect to pollution, the intake of genotoxic agents causes damage to telomeres, increasing their shortening process (Hoxha et al., 2009; Pavanello et al., 2010). Obesity, stress, and diets high in fats and proteins have been seen to cause high oxidative stress in the body (Furukawa et al., 2004; Epel et al., 2004; Cassidy et al., 2010). This increased amount of free radical species causes direct damage to telomeres, increasing the pace of their shortening. Furthermore, it has been seen that exercise has positive effects on telomeres. Research done by Werner et al. (2009) with the leukocytes of athletes showed they had elevated telomerase activity and longer telomeres in comparison to non-athlete

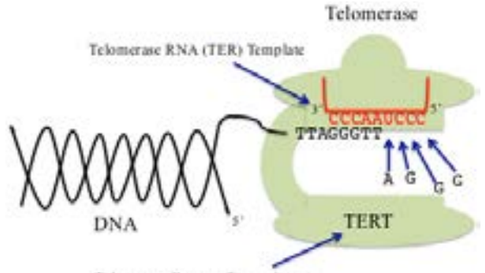

Figure 2. Schematic of the telomerase enzyme. Telomerase is a ribonucleoprotein that is composed of a Telomerase RNA (TER) and a Telomerase Reverse Transcriptase (TERT). The TER aligns at the end of the telomere, providing a template for nucleotide addition (red). TERT is the component of telomerase that facilitates nucleotide addition and telomere lengthening.

control subjects. In addition to these observations researchers believe that, by reducing body fat, exercise also reduces the amount of free radical species in the body and helps mobilize waste products (Shammas, 2011). This would lead to reduced oxidative stress, which would then lead to the preservation of telomeres. Another factor that has been seen to have positive effects on our telomeres is the consumption of antioxidants. Antioxidants could be protecting telomeres from free radical species and oxidative damage, thereby reducing the rate of telomere shortening (Farzaneh-Far et al., 2010).

One widely researched topic that has also been seen to severely accelerate telomere shortening is cigarette smoking. Cigarette smoking has been observed to increase oxidative stress in the body, which leads to accelerated telomere shortening (Babizhayev et al., 2011). In support of these results, a previous study conducted by Valdes et al. (2005) showed that the telomeres in white blood cells of non-cigarette smokers shortened at a rate of 25.7-27.7 bp per year, whereas cigarette smokers that smoked one pack per day lost an extra 5 bp from their telomeres per year. Therefore, if telomere length were to have a direct correlation to lifespan, by losing an extra 18-19 percent of your telomeres per year, smoking one pack of cigarettes per day for 40 years would theoretically shorten your life by about 7.2 to 7.6 years in that timeframe (Valdes et al., 2005). In addition, smoking causes an increased risk for developing cancer and other age-related diseases.

Telomerase: The Key to Immortality

The enzyme telomerase was identified by Elizabeth Blackburn and Carol Greider in 1987, a discovery that would later grant them the Nobel Prize in Physiology or Medicine in 2009 (Greider & Blackburn, 1987). Telomerase is a ribonucleoprotein enzyme that possesses the ability to elongate telomeres by adding nucleotides, in the form of telomeric repeats, to the end of the 3' G-rich strand of eukaryotic chromosomes (Greider & Blackburn, 1987). In somatic cells, telomerase is found at such low levels that it is almost undetectable. It has been observed, however, to be significantly more active in cancer, epithelial, and germline cells (Zhang, Kim, & Feigon, 2011).

Telomerase is considered a ribonucleoprotein because it is an enzyme that possesses both a protein component and an RNA component. The protein component is known as the Telomerase Reverse Transcriptase (TERT) and the RNA component is known as the Telomerase RNA (TER). In order for telomerase to elongate the telomere, it must first localize to the 3' end of the chromosome. After binding, telomeric repeats are added by TERT complementary to the template present in the TER component. For example, if the template region of TER contains the sequence 5'-CCCUAA-3', it will add a sequence repeat of 5'-TTAGGG-3' to the 3' end of the telomere. Once a telomeric repeat has been added to the 3' end of the G-rich strand, telomerase realigns its TER template to the end of this sequence in order to synthesize an additional telomeric repeat, continuing elongation of the telomere (Figure 2).

Shelterin: The Master Regulator

Telomerase functions by extending telomeres, but this extension cannot go on infinitely. This activity is understood with the discovery of telomere end binding proteins, which have been observed to function in the telomeric length regulation of different organisms. At the vertebrate telomere, a six-protein complex termed shelterin is found that functions to protect the end of the telomere (de Lange, 2005). The three main shelterin protein subunits, which directly associate with repeats at the end of the telomere, are TRF1, TRF2, and POT1. These proteins are interconnected by three additional proteins, TIN2, TPP1, and Rap1 (de Lange, 2005). Research has shown that these components localize specifically to telomeres, are abundant at telomeres throughout the entirety of the cell cycle, and only function at the telomeres (Palm & de Lange, 2008). With each protein fulfilling a unique functional role towards the interactive system, this six-protein complex, as a whole, has been observed to control access of telomerase to the telomeres and allow cells to distinguish telomeres from DNA damage sites (Figure 3) (Palm & de Lange, 2008).

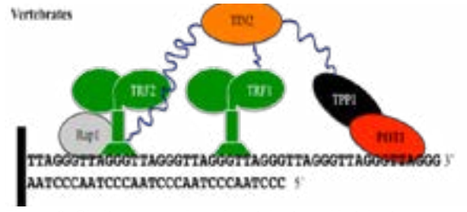

Figure 3. Shelterin complex of vertebrates. Vertebrates possess 6 proteins that bind the end of the telomere in a complex termed shelterin. POT1 is present in red. It has been shown to function in protecting the 3' G-rich overhang from a DNA damage response, and also controls telomerase access to the telomere. TPP1 is present in black. This protein connects POT1 to TIN2, and also possesses an OB-fold domain that has been seen to interact with telomerase. TRF1 and TRF2 are present in green, they are two proteins that bind the double-stranded regions of telomeres (both G-rich and C-rich strands), and act in recruiting TIN2, Rap1, TPP1, and POT1. Rap 1 is present in grey and its function is not fully known. TIN2 is present in orange, and associates with TPP1, TRF1, and TRF2. It functions in connecting TRF1 to TRF2, stabilizing TRF2 on the telomere. It also functions in recruiting TPP1/POT1 to the telomere, and connecting TPP1/POT1 to TRF1 and TRF2 (Palm & de Lange, 2008).

T-loop

In addition to the protective roles of the shelterin complex, telomeric DNA forms a protective t-loop structure (Griffith et al., 1999). In eukaryotes, the telomere is not blunt ended, but instead has a 3' single-stranded G-rich overhang which is required for telomeric extension by telomerase (Makarov et al., 1997; Froelich-Ammon et al., 1998). The current prediction on formation of the 3' overhang includes processing, or resection, of the telomeric C-rich strand by an unknown nuclease (Makarov et al., 1997). T-loops form when the single-stranded 3' overhang of the telomere invades the duplex region of the telomere, displacing the G-rich strand, forming a D loop (displacement loop) (Figure 4) (Griffith et al., 1999). These t-loops have not only been seen to form in vertebrates (Griffith et al., 1999), but also in ciliates (Murti & Prescott, 1999), trypanosomes (Muñoz-Jordán, Cross, de Lange, & Griffith, 2001), and *Caenorhabditis elegans* (Raices et al., 2008), possibly functioning in hiding the chromosomal end from DNA damage responses.

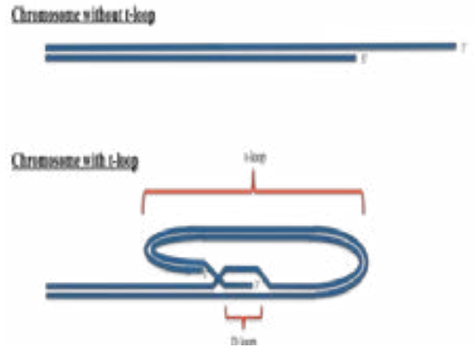

Figure 4. Schematic of t-loop structure at chromosomal ends. This diagram shows a chromosome that does not possess a t-loop (top) in comparison to a chromosome that possesses a t-loop (bottom). A t-loop forms as the 3' overhang at the chromosomal end invades dsDNA upstream of the telomere, forming a D loop (Griffith et al., 1999).

POT1: Protection of Telomeres

3' telomerie overhang was a very puzzling concept, but the past 15 years of research has revealed its significance. Research demonstrated that telomerase required this overhang in order to act on and extend the G-rich strand of telomeres (Froelich-Ammon et al., 1998). It was then hypothesized that the overhang, something of such high importance, had to have a protector in order to exist without being recognized as damaged DNA. It has since been observed that a protein ubiquitous in eukaryotes called POT1, for the protection of telomeres, binds the single-stranded 3' G-rich overhang present at the ends of telomeres, as well as inside the D loop (in eukaryotes that possess a t-loop). Generally, it has been shown to function in protecting the 3' G-rich overhang from a DNA damage response and also in controlling telomerase access to the telomere. Additionally, it is the most conserved component of the shelterin protein complex (de Lange, 2005).

Its discovery dates back to work done with the ciliate *Oxytricha nova*. A complex was discovered in this organism, later named the telomere end binding protein (TEBP). It is composed of an α and β subunit and was seen to bind tightly to the G-rich strand overhangs of telomeres in this organism, maintaining telomere length by inhibiting telomerase from accessing the telomeric end (Price & Cech, 1987; Fang & Cech, 1993; Froelich-Ammon et al., 1998). It was later found that

binding occurred through the presence of OB-folds, three in the α subunit and one in the β subunit, which allowed the formation of a deep DNA binding cleft on the 3' overhang (Horvath, Schweiker, Bevilacqua, Ruggles, & Schultz, 1998).

Years down the road, a similar protein was discovered in *Saccharomyces cerevisiae* that had a role in protecting the end of the chromosome. This protein was called Cdc13, and like the TEBP of ciliates, was found to bind to the 3' overhang of telomeres (Nugent, Hughes, Lue, & Lundblad, 1996). In future studies, its role in telomeric end protection was discovered, as it was observed as a recruiter of telomerase to chromosome ends and a coordinator in synthesizing C-rich and G-rich strands (Pennock, Buckley, & Lundblad, 2001).

As interest on the topic of end protection grew, a similar protein was revealed in *Schizosaccharomyces pombe*, also known as fission yeast. It was discovered when researchers realized that an open reading frame in the genome had genetic similarities to the TEBP of *O. nova* discovered earlier (Baumann & Cech, 2001). In order to test its function, this genomic region was deleted, which resulted in the rapid loss of telomeric and subtelomeric DNA and was followed by segregation defects and chromosome end fusions (Baumann & Cech, 2001). In further support of its function, it was also observed that this protein, now referred to as Pot1, bound the G-rich overhang but not the C-rich strand or double-stranded region of the telomere *in vitro* (Trujillo et al., 2005). As further studies were performed, scientists realized that this protein had roles not only in telomere end protection, but also in telomere length regulation, something that was consistent with ciliate TEBP and budding yeast Cdc13 proteins (Miyoshi et al., 2008).

The next discovery was that of a human protein that was hypothesized to have similar functions to the fission yeast Pot1 due to a conserved sequence. However, when knockdown experiments were done with human POT1, mainly telomere elongation occurred (Ye et al., 2004). This result was puzzling to researchers who expected telomere degradation and chromosome end fusions (Ye et al., 2004). From this experiment, it was concluded that the function of POT1 in humans must have closer ties to telomere length regulation, rather than protection of the G-rich overhang. Further research with POT1 recovered another interesting quality. In humans, it was found that the telomeric C-rich strand almost always ended with the sequence CAATC-5', and other permutations were only seen about 20 percent of the time (Sfeir, Chai, Shay, & Wright, 2005). When a knockdown of POT1 was performed, permutation at the end of the C-rich strand became randomized, suggesting that POT1 had another role in processing the C-rich strand (Hockemeyer et al., 2005).

The experiments done in humans were only knockdowns of POT1, meaning a depletion of POT1 rather than complete removal. Thus, the true purpose of vertebrate POT1 was not discovered until complete POT1 knockouts were performed in chickens. In these knockout experiments, the same results were observed: telomeres elongated and 3' G-rich overhangs increased in length (Churikov, Wei, & Price, 2006). However, this time, a DNA damage response was seen at the telomeres, along with arrest of the cell cycle in the G2 phase (Churikov et al., 2006).

These results seemed to be more consistent with the thought that POT1 protected the end of the chromosome in addition to its properties in controlling access of telomerase to the telomere via length regulation. Even though a DNA damage response occurred, there were rarely chromosome end fusions as seen before in fission yeast (Baumann & Cech, 2001). Scientists believed that this difference existed because even though the telomeres elongated during POT1 removal, the G-rich 3' overhang remained intact, therefore preventing chromosomal repair by non-homologous end joining (NHEJ) (Zhu et al., 2003).

As the G-rich overhangs of telomeres were being kept intact, it was believed that there must be an additional protein binding as a form of protection from nucleases in the absence of POT1. When the DNA damage response that occurred by POT1 removal was looked at in further detail, it was observed that ataxia telangiectasia and Rad3 related protein (ATR) was activated rather than ataxia telangiectasia mutated protein (ATM) (Churikov & Price, 2007). ATR is the DNA damage response that occurs during issues with replication forks, including single-stranded DNA damage, as opposed to ATM, which occurs during double-stranded DNA breaks. This indicated that the protein which bound to, and protected the G-rich 3' overhang during POT1 removal was Replication Protein A (RPA). This was hypothesized because RPA was the protein that would bind to the damaged site, necessary for recruiting ATR. This theory was proven correct in future ChIP studies, which proved that RPA was loaded onto telomere ends after POT1 removal (Churikov & Price, 2007).

Through the research that has been performed over the last 25 years, we understand that although it seems to have slightly different roles in each organism, POT1 exists in almost all eukaryotes. In general, it is considered the gatekeeper of the 3' telomere end. POT1 has been observed to protect the end of the chromosome by binding to the 3' G-rich overhang, preventing telomeric degradation, activation of DNA damage responses, and cell cycle arrest. It also has been seen to prevent access of telomerase to the end of the telomere, in turn regulating its length. As research continues, we hope to advance our knowledge on this protein's important functions.

A. nidulans as a Model Organism

At some point in his or her life, every person has observed a green colored mold growing on bread that has been left sitting out on his or her countertop. This mold that everyone despises belongs to the genus *Aspergillus*. The *Aspergilli* are a group of filamentous fungi that cover an evolutionary span of more than 200 million years and consist of over 185 different species (Galagan et al., 2005). Along with serving as the simple mold on our bread, the *Aspergilli* impact our lives in many other ways. There are 20 species of *Aspergillus* that act as human pathogens, and, in contrast, many other species are used in the production of food and industrial enzymes (Galagan et al., 2005). As an example, *A. oryzae* is a non-pathogenic species that is used in the production of sake.

The most widely used model organism in this genus is *A. nidulans*. *A. nidulans* is a non-pathogenic species of Aspergillus that has been used in many different aspects of research. It has improved our understanding of metabolic regulation, development, chromatin structure, cell cycle control, DNA repair, pH control, cytoskeletal function, morphogenesis, mitochondrial DNA structure, and human genetic diseases (Galagan et al., 2005). This organism is easy to grow, inexpensive, and simple to maintain, making it a perfect model organism for research. In addition to these qualities, it possesses well-characterized sexual and asexual life cycles and a fully sequenced genome. In recent years, *A. nidulans* has been the organism that our lab uses to study telomeres, and we recently published a paper identifying the TER gene in many different *Aspergilli* (Kuprys, Davis, Hauer, Meltser, Tzfati, & Kirk, 2013). Why should we be interested in the telomeres of this organism?

Telomere Regulation is Extremely Tight in *A. nidulans*

The first research studies on telomeres in *A. nidulans* showed some interesting results. When the telomeric repeat was cloned and sequenced for the first time, it was seen to be 5'-TTAGGG-3', the same repeat found in all vertebrates (Bhattacharyya & Blackburn, 1997). However, when the length of these cloned telomeres was measured, there was a noticeable difference between those of *A. nidulans* and vertebrates. Vertebrate telomeres vary in length from approximately 5.0 to 150 kb (Bhattacharyya & Blackburn, 1997), with the typical human telomere having a length of 1.5 to 6.0 kb (Moyzis et al., 1988). These telomeres are long, with a highly variable length. In comparison, it was found that the telomere length of *A. nidulans* was approximately 0.1 kb (or 18 to 19 repeats per chromosomal end) (Vahedi Thesis, 2008).

Compared to the size of its chromosome, *A. nidulans* has the shortest and most tightly regulated telomeres of all organisms in existence today, and that is why we are interested in its telomeres. In past studies, scientists attempted to disrupt this length regulation. Bhattacharyya & Blackburn (1997) grew the organism at an assortment of different temperatures and observed that the telomere length was unchanged. This very strict, stable, and tight regulation may be due to the fact that this organism defines the minimum length that is required to maintain stable telomeres (Bhattacharyya & Blackburn, 1997).

In furthering research on the telomeres of *A. nidulans*, I would like to understand its method of length regulation. The first step towards my goal would be attempting to disrupt the tight regulation of these telomeres. If certain components important to the telomeres were altered or removed, and a change in length regulation occurred, with the proper controls I could pinpoint the components involved.

Narrowing the Gap in Knowledge

When pondering a way to disrupt telomere length regulation, one should turn to a protein that has been seen to play a role in telomere length maintenance in the shelterin complex. One such protein is POT1, which is considered the gatekeeper of the 3' telomeric end. It has been seen in other organisms as a protector of the end of the chromosome by binding to the 3' G-rich overhang, preventing activation of DNA damage responses and cell cycle arrest. In addition, it has been seen as a telomere length regulator by preventing access of telomerase to the end of the telomere. This was the protein that I decided to target.

The genetic location of the POT1 protein in *A. nidulans* was identified in 2004 when a group of scientists were looking at changes in cell cycle progression in a group of various temperature-sensitive mutants (Pitt, Moreau, Lunness, & Doonan, 2004). Temperature-sensitive mutants are called so because their cells function normally at permissive temperatures but display mutant phenotypes when exposed to restrictive temperatures. These mutants were created through random mutation by Morris (1976) and named based on the problems that they caused during cell cycle progression. Pitt et al. (2004) observed that the temperature-sensitive *nimU24* (never in mitosis) mutant led to a variety of mitotic defects, but never lead to cell cycle arrest. The cell cycle progressed in this mutant strain when grown at the restrictive temperature, but rather than going through mitosis, possessed enlarged nuclei due to continuous DNA replication without cell division (Pitt et al., 2004). These studies concluded that inactivation of the *nimU* gene lead to increased chromosomal instability, segregation errors, and loss of viability (Pitt et al., 2004).

When the *nimU* gene was cloned and sequenced, it was further observed that it encoded an essential protein in *A. nidulans*. When searches of homology were done with this gene among the genomes of other organisms, tight homology was seen with fission yeast and human POT1 proteins (Pitt et al., 2004). This similarity was also observed in their secondary structures. However, the *nimU* gene also showed homology, similar to fission yeast and human POT1, with the TEBPα and TEBPβ subunits of *O. nova*, overlapping with oligonucleotide/oligosaccharide-binding (OB) folds of the α and β proteins that interact with telomeric ssDNA (Pitt et al., 2004). In addition, Pitt et al. (2004) searched the *A. nidulans* genome with the fission yeast Pot1 gene, *O. nova* TEBPα and TEBPβ genes, and the *nimU* gene itself but could not identify any other gene besides *nimU* that had a similar coding sequence. This *nimU* gene represented the sole POT1 homologue in *A. nidulans*, and the NIMU/Pot1 protein (which will be referred to throughout the rest of this thesis as POT1) most represented a fusion of the protozoan TEBPα and TEBPβ subunits (Pitt et al., 2004).

Based on these observations, I acquired the *A. nidulans* SJ203 temperature-sensitive (ts) mutant from Dr. Peter Mirabito at the University of Kentucky. This mutant is the original ts mutant created by Ron Morris in 1976 possessing an identical mutation to the nimU24 strain used by Pitt et al. (2004) (Peter Mirabito, personal communication, 2012). Its permissive growth temperature is 25°C and its restrictive growth temperature is 42°C. The POT1 protein is made up of 614 amino acids, and is flanked by a zinc-finger protein at its 5' end and a karyopherin α protein at its 3' end. The SJ203 strain possesses a single T to A missense mutation in the *nimU* gene, which alters the leucine at amino acid 536 to a glutamine (L536Q) (Figure 5) (Pitt et al., 2004).

Figure 5. Shows the location of the nimU24 mutation in the SJ203 A. nidulans strain. The SJ203 mutant is temperature sensitive for growth. It is a single T to A missense mutation that alters the leucine (L) at amino acid 536 of the POT1 protein to a glutamine (Q). POT1 is predicted to be a 614 amino acid protein. Homology shows POT1 to be most closely related to human and fission yeast POT1, and also the TEBP α and β of ciliated protozoa (Pitt et al., 2004).

Although cell cycle studies had been done, nothing was known about the telomeres or their length in this POT1 mutant. Measuring telomere length in the absence of functional POT1, therefore, was my next project. The results of this study would give insight into the mechanisms of telomere length maintenance of both the G-rich and C-rich strands of the short and tightly regulated telomeres of *A. nidulans* (see Figure 3 for G/C-rich strand clarification).

HYPOTHESIS

Due to the homologous similarity of POT1 in *A. nidulans* to POT1 in humans, I propose that similar results upon POT1 dysfunction will be observed. When a POT1 knockdown was performed using RNAi in human cells, telomere elongation occurred at the G-rich strand, followed by similar elongation at the C-rich strand (Ye et al., 2004). Based on these

prior observations, I hypothesize that a significant increase in telomere length will be seen in the *A. nidulans* POT1 mutant when compared to the short and tightly regulated telomeres of the wild-type strain. I predict that this increase in telomeric length will be seen at both the G-rich and C-rich strands.

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