WRN at telomeres: implications for aging and cancer

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Summary
 Werner Syndrome (WS) is a premature aging syndrome characterized by early onset of age-related pathologies and cancer. Since WS is due to a single gene defect, it has attracted much interest from researchers seeking to understand pathways that contribute to cancer and aging at cellular and molecular levels. The protein mutated in WS, WRN, appears to play a major role in genome stability, particularly during DNA replication and telomere metabolism. Much of the pathophysiology associated with WS, including the rapid onset of cellular senescence, early cancer onset and premature aging, can be attributed to a defect in telomere maintenance. Recent genetic evidence from the mTerc–/– Wrn–/– mouse demonstrates that mice with critically shortened telomeres display aging phenotypes reminiscent of human WS, further reinforcing the notion that telomere dysfunction is required for the manifestation of aging pathophysiologies in the setting of WRN deficiency.

Key words: Werner Syndrome, Telomere, DNA damage, Aging, Cancer

Introduction
 Werner Syndrome (WS) is a fascinating autosomal recessive disorder affecting ~10 per million individuals (Epstein et al., 1966). Patients appear normal until the second decade of life, when they develop pathologies that phenocopy many aspects of normal human aging, including alopecia, ischemic heart disease, osteoporosis, bilateral ocular cataracts, type II diabetes mellitus, and hypogonadism (Thannhauser, 1945). WS patients also experience an increased risk of rare non-epithelial cancers, especially mesenchymal neoplasms such as sarcomas (Goto et al., 1996). Death usually occurs in the fourth decade from cardiovascular compromise or cancer. Fibroblasts isolated from WS patients characteristically senesce prematurely in culture (Faragher et al., 1993) and display increased chromosomal aberrations (Salk et al., 1981; Melcher et al., 2000). Since WS is caused by mutation of a single gene, WRN, these observations suggest that the WRN gene product functions to maintain genome stability.

Cloning of WRN ten years ago revealed that it encodes a protein containing a highly conserved 3’ to 5’ DNA helicase domain of the RecQ family (reviewed by Martin and Oshima, 2000). RecQ helicase family members are involved in diverse biochemical processes, including DNA recombination, replication and repair, and WRN has been implicated in all of these (reviewed by Hickson, 2003). WRN also possesses several other conserved domains, including a 3’ to 5’ exonuclease domain, a nuclear localization sequence, and a multifunctional DNA/protein-binding domain (DPBD) that interacts with both DNA and proteins (reviewed by Orren, 2006). Through these domains WRN interacts with many factors that participate in diverse aspects of DNA metabolism beyond the scope of this commentary. Here, we focus on recent progress that indicates WRN might have a role at telomeres. Discussion of other aspects of WRN biology can be found elsewhere (Hickson, 2003; Comai and Li, 2004; Bohr, 2005; Orren, 2006).

Telomere structure and maintenance
 Telomeres are nucleoprotein structures that cap the ends of eukaryotic chromosomes and play crucial roles in maintaining genomic stability by providing both end-protection and a mechanism for generating chromosomal ends (LeBel and Wellinger, 2006). In mammals, telomeres consist of TTAGGG repetitive sequences that terminate in a 3’ single-stranded G-rich overhang. Telomeres can fold into a structure termed the t-loop, in which the 3’ single-stranded overhang invades a duplex region of the telomere to sequester the overhang, forming a single-stranded displacement (D) loop (Griffith et al., 1999; Murti and Prescott, 1999). This telomeric conformation probably protects natural DNA ends from being recognized as double-strand breaks (DSBs) that would otherwise activate DNA damage checkpoint responses or participate in aberrant recombination events (reviewed by d’Adda di Fagagna et al., 2004). However, an extended telomeric conformation must also exist to facilitate replication of telomeres during S phase. Telomeres are maintained by the enzyme telomerase, a specialized ribonucleoprotein complex that includes an RNA template (Terc) and a reverse transcriptase catalytic subunit (Tert) (Smogorzewska and de Lange, 2004). Telomerase is limiting in human somatic cells, resulting in progressive telomere shortening because conventional DNA polymerases cannot fully replicate the extreme terminus of the lagging DNA strand. Therefore, telomere attrition occurs with each round of DNA replication and eventually critically shortened, dysfunctional telomeres are generated that engage the DNA-damage response pathway, initiating the onset of p53-p21-dependent replicative senescence (Maser and DePinho, 2004).

The telomeric t-loop is bound and stabilized by several telomere-specific binding proteins that form a complex, termed the telosome (Liu et al., 2004b) or shelterin (de Lange, 2005). This complex is composed of six core components: the duplex telomere-binding proteins TRF1 and TRF2, the single-
A large body of work in human cell culture systems and mouse models has documented the biological and genomic consequences of dysfunctional telomeres and how these consequences relate to the development of genomic instability (Wong et al., 2006). Primary human cells have a limited replicative potential owing to progressive telomere shortening due to the absence of telomerase, which eventually results in the onset of replicative senescence (Allsopp et al., 1992; Harley et al., 1994; Wright and Shay, 1992; Wright and Shay, 1995). Replicative senescence was originally defined by Hayflick (Hayflick, 1965) as the finite replicative lifespan of human fibroblasts in culture and has been shown to be caused by dysfunctional telomeres activating the p53-dependent DNA damage checkpoint and the p16-Rb tumor suppressor pathways (Itahana et al., 2001; Jacobs and de Lange, 2004; Campisi, 2005). This senescence response is not simply because of an arrest of cell proliferation. Rather, senescent cells adopt a complex phenotype as a result of changes in gene expression, including a flattened cell morphology, increased resistance to apoptosis, upregulation of p53, p21 and p16 proteins and senescence-associated β-galactosidase (SA-β-gal) activity (reviewed by Campisi, 2005).

Stochastic inactivation of p53 and pRB by antisense oligos (Hara et al., 1991) or by viral oncoproteins (Shay et al., 1991) can bypass replicative senescence to extend replicative potential, which drives additional telomere erosion and culminates in a period of massive cell death and rampant chromosomal instability termed ‘crisis’ (Counter et al., 1992). Dysfunctional telomeres are highly recombinogenic, leading to chromosomal breakage-fusion-bridge cycles that are potentially cancer promoting (Chang et al., 2003; O’Hagan et al., 2002). Depending on how fused chromosomes are resolved, it has been hypothesized that loss of heterozygosity or gene amplification could result in tumorigenesis (Chin et al., 2004). Indeed, it now appears that many human precancerous lesions progress through a phase of genomic instability likely to be generated in part by dysfunctional telomeres (Bartkova et al., 2005; Gorgoulis et al., 2005). Activation of a telomere-maintenance mechanism is thus crucial for tumor progression. This is achieved by the reactivation of telomerase in approximately 90% of human carcinomas, whereas the remainder maintains telomere length by a recombination-based mechanism termed Alternative Lengthening of Telomeres (ALT) (Kim et al., 1994; Bryan et al., 1997; Shay and Bacchetti, 1997). Telomere maintenance thus appears to be crucial for the formation of human tumors, removing the short telomeric barrier to tumor progression.

**Replicative senescence and human aging**

Cells derived from most multicellular organisms have finite replicative potential in cell culture, and considerable debate has surrounded whether this phenomenon is relevant to the biology of human aging. This potential link between replicative senescence and human aging has been strengthened by observations such as the accumulation of senescent cells with advancing age (Dimri et al., 1995), correlations between in vitro replicative potential and donor age (Martin et al., 1970), and the diminished in vitro replicative potential of cells derived from individuals with premature aging syndromes (Faragher et al., 1993). Although a definitive connection between telomere dynamics and normal aging in humans has yet to be established, accumulating evidence has strengthened the view that accelerated telomere attrition contributes directly to

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**Fig. 1.** (A) Telosome/shelterin complex and telomere structure. The telomere folds back onto itself to form a double-stranded t-loop and a single-stranded D-loop. This complex protects telomeres at the G2 phase of the cell cycle from inappropriate NHEJ- and HR-mediated processing of telomeric DNA. The six-component telosome/shelterin is shown schematically on the t-loop, with POT1 interacting with the D-loop. (B) During DNA replication, the presence of WRN at the replication fork is postulated to enable the replication complex to efficiently replicate telomeric DNA. (C) The presence of WRN at telomeres may facilitate unwinding of the D-loop, enabling telomerase to extend telomeres. The linear 3' overhang is probably protected by POT1.

stranded TTAGGG-repeat binding protein POT1 and the adapter proteins RAP1, TIN2 and TPP1 (Fig. 1). TRF1 is a negative regulator of telomere length (van Steensel and de Lange, 1997) and TRF2 is essential for telomere end protection (van Steensel et al., 1998; Celli and de Lange, 2005). Recent evidence suggests that the TRF1 and TRF2 complexes interact with each other to regulate telomere dynamics (Kim et al., 2004; Liu et al., 2004a; Liu et al., 2004b; Ye et al., 2004; Wu et al., 2006), and this may be mediated by POT1. POT1 is a negative regulator of telomere length, since reduction of endogenous POT1 levels or overexpression of a mutant POT1 that cannot bind telomeres results in telomere length elongation (Loayza and de Lange, 2003; Liu et al., 2004a; Ye et al., 2004).

**Telomere dysfunction and replicative senescence**

A large body of work in human cell culture systems and mouse models has documented the biological and genomic
acquired and inherited degenerative conditions and premature aging syndromes, such as WS (see below). In support of this notion, analysis of telomere lengths in cells derived from peripheral blood of humans over the age of 60 revealed that individuals possessing shorter telomeres than age-matched controls had significantly poorer survival rates, which was attributed in part to an elevated mortality rate from heart and infectious diseases (Cawthon et al., 2003).

It is postulated that senescent cells accumulate with normal aging and may contribute to age-related pathologies by inhibiting tissue regenerative capacities (Krotolica and Campisi, 2002; Itahana et al., 2001). Increasing evidence suggests that senescent cells do accumulate in aging human tissues. For example, SA-β-gal-positive senescent cells have been identified in aged liver, atherosclerotic plaques, and skin (Dimri et al., 1995; Vasile et al., 2001). Perhaps the best evidence that senescent cells exist in vivo comes from studies of aging baboon skin. Approximately 15% of aged baboon skin fibroblasts possess prominent foci of DNA damage markers, including γ-H2AX, 53-BP1 and phosphorylated Ataxia-telangietasia-mutated (ATM) kinase that colocalize with telomeres, indicating that telomeres are dysfunctional in aging skin (Herbig et al., 2006). These results suggest that in old primates, telomere dysfunction activates the ATM-dependent DNA-damage signaling pathway to initiate cellular senescence in tissues, probably negatively impacting upon tissue physiology and function.

**Cellular stress response and telomere dysfunction in WS fibroblasts**

Cells derived from WS patients exhibit a profound reduction of in vitro replicative lifespan and enter replicative senescence prematurely. Replicative senescence is a specific example of a more widespread cellular stress response termed cellular senescence, which can be initiated by many stimuli, including dysfunctional telomeres, expression of oncoproteins that induce replication-associated stress and oxidative damage (reviewed by Schmitt, 2003; Sharpless and DePinho, 2004; Bartkova et al., 2006; Di Micco et al., 2006). Given their compromised genome maintenance, it is likely that WS fibroblasts are particularly sensitive to agents that increase cellular stress. Indeed, WS fibroblasts are hypersensitive to various DNA-damaging reagents, including interstrand crosslinkers and inhibitors of DNA replication (reviewed by Orren, 2006; Pichieri et al., 2001; Zhang et al., 2005). Elevated stress response correlates with the activation of the stress-associated mitogen-activated protein kinase (MAPK) p38 (Iwasa et al., 2003). Activation of p38 stabilizes p21 and promotes entry into cellular senescence, and drugs that inhibit p38 activity extend the replicative lifespan of WS fibroblasts as well as reduce p21 levels to that observed in normal fibroblasts (Davis et al., 2005). Remarkably, the stress response prominent in young WS fibroblasts largely disappears after drug treatment. These results implicate the p38-mediated stress response as an important contributor to the decreased cellular lifespan of WS fibroblasts in vitro. Activation of p38 also results in the increased production of pro-inflammatory cytokines, which are abundantly expressed in senescent WS fibroblasts (Choi et al., 2001). Recent data also suggest that inflammatory factors secreted by senescent cells engender a local inflammatory response that can disrupt normal epithelial physiology and promote the development of preneoplastic lesions (Campisi, 2005). Accumulation of senescent cells could thus contribute to age-related changes in tissue function and pathology observed in WS.

An important contributor to cellular stress in replicating primary cells is the presence of dysfunctional telomeres, which directly elicit a DNA-damage response to initiate cellular senescence (d’Adda di Fagagna et al., 2003; Takai et al., 2003). The observation that premature senescence characteristic of WS cells can be rescued by ectopic expression of telomerase suggests a crucial role for telomere integrity in the pathogenesis of WS (Wyllie et al., 2000; Choi et al., 2001; Crabbe et al., 2004). Recent evidence suggests that the elevated genomic instability characteristic of WS fibroblasts stems directly from increased telomere loss, which can be rescued by overexpression of telomerase (Crabbe et al., 2007). However, sensitive single telomere length analyses reveal that telomeres in WS cells erode at rates similar to those of normal controls (Baird et al., 2004), which suggests that accelerated global telomere attrition is not a mechanism that generates dysfunctional telomeres in WS cells. Rather, WS fibroblasts may be particularly sensitive to the presence of a few dysfunctional telomeres that arise stochastically owing to the loss of WRN function at telomeres, since even a single dysfunctional telomere is sufficient to limit cell survival in vivo (Hemann et al., 2001). As discussed below, WRN may be required for proper capping of telomeres by the telosome/shelterin complex as well as for replication of lagging telomeric DNA. Loss of WRN could therefore elicit the formation of dysfunctional telomeres that help drive entry into premature cellular senescence.

**The role of WRN at telomeres**

Increasing evidence indicates that WRN plays a direct role at telomeres. WRN functionally interacts with several members of the telosome/shelterin complex, including TRF1, TRF2 and POT1. In addition, WRN interacts with Ku70 and Ku80 (Ku70/80), a protein heterodimer involved in non-homologous-end-joining (NHEJ) that has a role in telomere length maintenance. The implications of these interactions are summarized below.

**TRF1 and TRF2**

In telomerase-positive human cell lines, WRN localizes predominantly to the nucleolus. However, in human ALT cells a significant fraction of WRN appears to colocalize with TRF1 and TRF2 at telomeric DNA (Johnson et al., 2001; Opresko et al., 2002; Opresko et al., 2004). TRF2 directly interacts with WRN through the DPBD domain (Opresko et al., 2002; Machwe et al., 2004). Localization of WRN to telomeres appears to be confined to S phase of the cell cycle, as revealed by both live cell imaging (Opresko et al., 2004) and direct chromatin immunoprecipitation (CHIP) of synchronized cells (Opresko et al., 2004; Crabbe et al., 2004). In vitro studies revealed that the relatively weak 3'-5' helicase and 3'-5' exonuclease activities of WRN are stimulated through interactions with TRF2 (Opresko et al., 2002; Machwe et al., 2004), and these can resolve telomeric D-loops in vitro (Opresko et al., 2004). WRN may therefore play a role during normal telomere replication, perhaps by resolving aberrant secondary telomeric structures as the replication fork...
progresses through telomeres. This notion is supported by the observations that WRN is required for resolution of stalled replication forks: WS cells have a prolonged S-phase (Poot et al., 1992) and WRN colocalizes with replication protein A (RPA) in cells arrested in S phase by hydroxyurea (Constantinou et al., 2000). TRF1 and TRF2 are able to limit WRN exonuclease activity on the D-loop but do not block WRN helicase activity. These telomere-binding proteins may function to negatively regulate telomere length by competing with telomerase for access to the telomeric substrate (Loayza and de Lange, 2003; Kelleher et al., 2005; Lei et al., 2005). In addition, depletion of POT1 levels by RNAi (Veldman et al., 2004; Yang et al., 2005) or conditional deletion of the Pot1 gene in mice (Hockemeyer et al., 2006; Wu et al., 2006) results in increased chromosomal aberrations, which suggests that POT1 plays an important role in protecting telomeric ends. Recently, POT1 was found to physically interact with WRN and stimulate its helicase activity (Opresko et al., 2005). In the presence of POT1, WRN efficiently displaces a forked telomeric duplex and is able to release the invading tail of the lagging telomeric strand (Crabbe et al., 2004). Replication of the lagging-strand synthesis is compromised in the absence of POT1, which suggests that WRN and POT1 may cooperate to dissociate G-quadruplexes at the G-rich telomeric strand to facilitate replication fork progression. This notion is supported by data indicating that Pot1 deletion results in preferential loss of the G-rich telomeric strand (Wu et al., 2006). We postulate that the absence of WRN causes stalling of the replication fork as it encounters G-quadruplexes at telomeres, leading to telomere loss and the generation of dysfunctional telomeres that then engage canonical DNA-damage response pathways to trigger the onset of replicative senescence (Fig. 2) (Crabbe et al., 2004). Senescent human fibroblasts display molecular markers characteristic of cells bearing DNA DSBs, including phosphorylated γ-H2AX, 53BP1, NBS1 and CHK2, many of which colocalize with dysfunctional telomeres (d’Adda di Fagagna et al., 2003; Takai et al., 2003). Wrn-depleted fibroblasts also display these markers of damaged DNA (Chang et al., 2004; Szekely et al., 2005). These results suggest that inappropriate activation of the DNA-damage response throughout proliferating cellular compartments in WS patients may initiate p53-dependent checkpoint responses such as replicative senescence or apoptosis, which, in turn, results in organ degeneration and onset of premature aging phenotypes.

WRN is required for telomere metabolism
Direct evidence that WRN plays a role in telomere replication comes from observations that replication of telomeres by lagging-strand synthesis is compromised in the absence of WRN, which results in preferential loss of the G-rich lagging strand telomeric DNA (Crabbe et al., 2004). Replication of the lagging telomeric strand depends upon an intact WRN helicase domain; WRN may therefore resolve aberrant DNA structures that block replication fork progression through telomeric DNA. Indeed, WRN can efficiently resolve G-quadruplexes, thermodynamically stable intramolecular structures containing four hydrogen-bonded guanine residues, on G-rich telomeric sequences (Mohaghegh et al., 2001; Chang et al., 2004). The recent observation that POT1 can also resolve G-quadruplexes in telomeric repeats (Zaug et al., 2005) suggests that WRN and POT1 may cooperate to dissociate G-quadruplexes at the G-rich telomeric strand to facilitate replication fork progression. This notion is supported by data indicating that Pot1 deletion results in preferential loss of the G-rich telomeric strand (Wu et al., 2006). We postulate that the absence of WRN causes stalling of the replication fork as it encounters G-quadruplexes at telomeres, leading to telomere loss and the generation of dysfunctional telomeres that then engage canonical DNA-damage response pathways to trigger the onset of replicative senescence (Fig. 2) (Crabbe et al., 2004). Senescent human fibroblasts display molecular markers characteristic of cells bearing DNA DSBs, including phosphorylated γ-H2AX, 53BP1, NBS1 and CHK2, many of which colocalize with dysfunctional telomeres (d’Adda di Fagagna et al., 2003; Takai et al., 2003). Wrn-depleted fibroblasts also display these markers of damaged DNA (Chang et al., 2004; Szekely et al., 2005). These results suggest that inappropriate activation of the DNA-damage response throughout proliferating cellular compartments in WS patients may initiate p53-dependent checkpoint responses such as replicative senescence or apoptosis, which, in turn, results in organ degeneration and onset of premature aging phenotypes.

The mTerc–/– Wrn–/– compound mutant mouse as a model of human WS
Extensive physiological analyses of telomerase-knockout mice revealed that late generation mTerc–/– animals that have short, dysfunctional telomeres exhibit decreased lifespan and a range of aging phenotypes including alopecia, hair greying, a reduced capacity to cope with acute and chronic stress and a modestly increased incidence of cancer (Rudolph et al., 1999). Age-matched animals at earlier generations that possess longer telomeres do not manifest these phenotypes, which suggests that telomere dysfunction is important for the emergence of premature phenotypes. However, only a subset of these human aging phenotypes are present in mice with dysfunctional telomeres, which suggests that other factors contribute to human aging.

Interestingly, despite the wide-ranging activities of the WRN helicase in human cells, Wrn-null mice do not exhibit any cellular or clinical phenotypes characteristic of human WS (Lombard et al., 2000). The observations that WRN is required for telomere maintenance, and that laboratory mice normally possess very long telomeres, suggest that manifestation of premature aging phenotypes in Wrn-null mice may require the presence of critically short telomeres. This hypothesis has
since received genetic support from studies of mice lacking both telomerase and Wrn (Chang et al., 2004; Du et al., 2004). Strikingly, these late generation mTerc\(^{-/-}\) Wrn\(^{-/-}\) mice display clinical symptoms resembling human WS, including early onset of age-related disorders such as defective wound repair, osteoporosis and skeletal fractures, hypogonadism, cataract formation, type II diabetes, an elevated incidence of osteosarcomas compared with the general population as well as premature death (Chang et al., 2004; Du et al., 2004). Quantitative telomere-fluorescence in situ hybridization (FISH) revealed that chromosomes from late generation mTerc\(^{-/-}\) Wrn\(^{-/-}\) mice display an elevated loss in overall telomere length that correlates with increased chromosomal fusions and nonreciprocal translocations (NRTs) (Chang et al., 2004; Du et al., 2004). Late generation mTerc\(^{-/-}\) Wrn\(^{-/-}\) mouse embryo fibroblasts (MEFs) exhibit prominent DNA-damage markers such as 53BP1 and H2AX and a profound reduction in replicative lifespan (Chang et al., 2004), which is similar to growth defects observed in human WS fibroblasts. Dysfunctional telomeres thus appear to cooperate with Wrn deficiency to activate the DNA-damage response and subsequent entry into replicative senescence. It is important to emphasize that the phenotype observed in the mTerc\(^{-/-}\) Wrn\(^{-/-}\) double mutant is not simply a worsening of aging phenotypes observed in the telomerase-null mouse, but a recapitulation of specific phenotypes encountered in WS patients that are not observed in late generation mTerc\(^{-/-}\) mice. Data from these mouse models therefore strongly support the hypothesis that manifestation of the diverse pathophysiological phenotypes observed in WS patients requires both the presence of dysfunctional telomeres as well as WRN deficiency.

**Elevated homologous recombination at telomeres and engagement of the ALT pathway in mTerc\(^{-/-}\) Wrn\(^{-/-}\) cells**

The mTerc\(^{-/-}\) Wrn\(^{-/-}\) compound knockout mouse is likely to inform us about the molecular mechanisms underlying clinical aspects of WS. For example, although WS patients exhibit an increased incidence of mesenchymal cancers such as osteosarcomas compared with the general population (Goto et al., 1996), little is known about the genetic pathways perturbed in these tumors, because few WS tumor cell lines exist. This increased incidence of mesenchymal cancers is intriguing in light of observations indicating that many human sarcomas maintain telomere lengths by the ALT pathway (Bryan et al., 1995; Bryan et al., 1997) (reviewed by Henson et al., 2002). The ALT pathway probably involves recombination-mediated telomere recombination. *Saccharomyces cerevisiae* lacking telomerase requires proteins involved in DNA recombination to survive, including RAD50 (Teng et al., 1999). These type II survivors also require the yeast ortholog of WRN (Sgs1p), and their chromosomal ends contain amplified telomeric DNA. Most human ALT cells possess telomeres resembling yeast type II survivors. However, deletion of both Sgs1 and telomerase could also promote cell survival by activation of a type I survival pathway to maintain telomeres (Johnson et al., 2004; Du et al., 2004). Late generation mTerc\(^{-/-}\) Wrn\(^{-/-}\) mice display an elevated loss in overall telomere length that correlates with increased chromosomal fusions and nonreciprocal translocations (NRTs) (Chang et al., 2004; Du et al., 2004). Wrn\(^{-/-}\) cells having dysfunctional telomeres as well as WRN deficiency. The resultant dysfunctional telomeres can initiate a p53-dependent DNA-damage response, leading to premature onset of replicative senescence.
2001; Huang et al., 2001; Cohen and Sinclair, 2001). Type I survivors have telomeres comprising tandem arrays of amplified units of subtelomeric elements and telomere repeats. Recently, an SV40-immortalized human cell line lacking both WRN and telomerase activities was found to maintain telomeres by a mechanism reminiscent of this type I survival pathway (Fasching et al., 2005; Marciniak et al., 2005); this suggests that a subset of human ALT cells could maintain telomeres by establishing tandem arrays. A similar mechanism also appears to operate in telomerase-null mouse ALT cell lines with dysfunctional telomeres (Niida et al., 2000), which suggests that tandem arrays of telomeric sequences can stabilize mammalian termini in the absence of telomerase.

Given the elevated number of chromosomal aberrations and robust DNA-damage response observed in organs and cells derived from late generation mTerc−/− Wrn−/− mice, it is tempting to speculate that a key factor underlying WS may be the inability of WS cells to suppress elevated genomic instability. When p53 is lost, the genomic instability induced by dysfunctional telomeres can provide a mutator mechanism enabling would-be cancer cells to achieve a threshold of cancer-promoting changes required for tumorigenesis (Artandi et al., 2000; Rudolph et al., 1999; O’Hagan et al., 2002). To understand the impact of Wrn deficiency on telomere maintenance, chromosomal stability and tumorigenesis, we allowed G5 mTerc−/− Wrn−/− mouse cell lines to undergo spontaneous cellular senescence. Surprisingly, loss of Wrn in this setting promoted escape from cellular senescence and the emergence of immortalized clones in which the p53 pathway is compromised (Laud et al., 2005). These clones continue to shorten their telomeres and exhibit marked chromosomal aberrations. However, telomere sister chromatid exchange (T-SCE), an indicator of homologous recombination (HR) between sister telomeres, specifically increases in these cells (Fig. 3). Restoration of wild-type, but not helicase dead, WRN to G5 mTerc−/− Wrn−/− cell lines decreases T-SCE levels, which suggests that the helicase domain of WRN is required to repress inappropriate telomeric recombination. Sgs1 represses recombination at homologous sequences (Myung et al., 2001; Sugawara et al., 2004), and this observation is consistent with a role for WRN in repressing HR at homologous telomeric sequences. The observation that human ALT cell lines also display elevated T-SCE (Bechter et al., 2004; Londono-Vallejo et al., 2004) suggests that HR-mediated telomere recombination mechanisms are important prerequisites for transition to the ALT phenotype.

When immortalized G5 mTerc−/− Wrn−/− clones are injected subcutaneously into severe combined immunodeficiency (SCID) mice, aberrant telomeric HR, coupled with the strong selective pressure to maintain telomere length in the absence of telomerase activity, results in the activation of ALT and rapid formation of tumors (Laud et al., 2005). T-SCE levels in ALT tumor cell lines are not repressed when telomerase is expressed in these cells, which is consistent with previous observations indicating that once ALT is activated, it is not normally repressible by telomerase expression (Cerone et al., 2001; Perrem et al., 2001; Londono-Vallejo et al., 2004). These results indicate that the increased incidence of chromosomal instability and cancer observed in WS patients may be caused by aberrant recombination at telomeres, which promotes the activation of ALT that maintains telomeres through a mechanism analogous to the type I survival pathway observed in sgs1−/−, telomerase-null yeasts. Although not all Wrn−/−, telomerase-null human cells maintain telomeres by this mechanism, the recent discovery of a Wrn−/−, telomerase-null SV40-immortalized ALT cell line that maintains telomeres in a manner reminiscent of the type I survival pathway supports this hypothesis (Fasching et al., 2005; Marciniak et al., 2005). The G5 mTerc−/− Wrn−/− compound knockout mouse thus offers a unique system to discover novel genes involved in ALT activation during tumor formation.

### Conclusions and perspectives

Although we are still far from understanding the precise roles that WRN plays in maintaining genome stability, important clues regarding its function have emerged in the ten years since the WRN gene was cloned. For example, premature entry into replicative senescence, a hallmark of WS fibroblasts, is likely to be due in part to synergistic effects of dysfunctional telomeres activating both a p53-dependent DNA-damage response and a telomere-independent, p38-dependent cellular stress response, culminating in premature entry into cellular senescence. Whether cellular senescence directly contributes to organismal aging has been controversial. However, the demonstration that WS cells with dysfunctional telomeres accumulate markers of DNA damage (Chang et al., 2004; Szekely et al., 2005) and the intriguing observation that aged primate fibroblasts also bear identical markers of telomere dysfunction (Herbig et al., 2006) suggest a causal link between dysfunctional...
telomere-induced cellular senescence and organismal aging. This link is further supported by genetic evidence from the mTerc–/– Wrn–/– mouse model demonstrating that only mice with critically shortened telomeres that initiate a DNA-damage response manifest organismal aging phenotypes. Accumulation of prematurely senescent cells in vivo probably has profound adverse physiological consequences. We speculate that the transition from normalcy at birth to the premature aging phenotypes observed at the second decade of life in WS patients relates to the erosion of a population of telomeres to a critically short threshold that is capable of initiating a DNA-damage response. This view is in line with the emerging consensus that telomere maintenance, DNA repair, and DNA metabolism pathways are intertwined and function either to suppress or precipitate aging and cancer phenotypes, depending upon the status of the DNA damage pathway.

The increased incidence of mesenchymal cancers such as soft tissue sarcomas and osteosarcomas observed in WS patients may relate to the nature of the telomere maintenance program activated in these tumors. In contrast to carcinomas, mesenchymal tumors preferentially activate ALT to maintain telomeres. Activation of ALT occurs in sarcomas derived from mTerc–/– Wrn–/– cells that have dysfunctional telomeres and is probably because of increased HR at telomeres (T-SCE). We postulate that WRN is normally required to repress inappropriate telomeric recombination intermediates through its ability to resolve telomeric D-loops (Opresko et al., 2004). T-SCE, when unequal and coupled with non-random chromosomal segregation, may confer a proliferative advantage to cells that stochastically acquire longer telomeres, enabling escape from cellular senescence (Bailey et al., 2004; Muntoni and Reddel, 2005). The finding that human ALT cell lines also display elevated T-SCE (Bechter et al., 2004; Londono-Vallejo et al., 2004) suggests that acquisition of HR-mediated telomere recombination mechanisms is an important prerequisite for transition to the ALT phenotype. Although the mechanism underlying T-SCE in human ALT cells has not been identified, the data indicate that suppression of WRN function may be important for the initiation of aberrant recombination at telomeres. T-SCEs may be triggered when a replication fork encounters a block in one of the parental template strands, which leads to stalling of the DNA polymerase and pairing of the 3’-ended strand with its complement on the other, nascent sister chromatid DNA. This replication block may involve in vivo formation of G-quadruplexes. WRN can efficiently unwind G-quadruplexes in a helicase-dependent manner (Mohaghegh et al., 2001). We postulate that WRN normally resolves G-quadruplexes during DNA replication, which allows replication fork progression and complete synthesis of telomeric DNA. Lack of WRN helicase activity leads to replication fork stalling, resulting in preferential loss of the lagging-strand telomeric DNA (Crabbe et al., 2004) and elevated exchanges among sister telomeres. In addition, the recent observations that cells lacking POT1 (Wu et al., 2006) and Ku70 (Celli et al., 2006) also experience increased T-SCE suggest that a crucial function of the telosome/shelterin complex is to protect telomeres from aberrant HR. Since WRN interacts with both of these proteins, it is likely that WRN is also required for the telosome/shelterin complex to exert proper capping function and prevent the formation of dysfunctional telomeres.

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