FEBS Letters 521 (2002) 170–174 FEBS 26187

Regulation of the Werner helicase through a direct interaction with a subunit of protein kinase A

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Received 2 May 2002; revised 13 May 2002; accepted 15 May 2002

First published online 29 May 2002

Edited by Gianni Cesareni

Abstract Werner syndrome is a hereditary disease characterized by cancer predisposition, genetic instability, and the premature appearance of features associated with normal aging. At the molecular level this syndrome has been related to mutations in the Werner helicase, a member of the RecQ family of DNA helicases which are required to maintain genomic stability in cells. Here we show by a yeast two-hybrid screen that the Werner helicase can directly interact with the regulatory subunit (RIβ) of cAMP protein kinase A (PKA). We confirm that this interaction occurs in vivo. Interestingly, serum withdrawal causes a redistribution of the Werner helicase within the nucleus of mammalian cells. Raising intracellular cAMP levels or increased expression of the regulatory but not the catalytic subunit of PKA inhibits this nuclear redistribution stimulated by serum deprivation. These results suggest that similar to lower organisms, gene products linked to genomic instability and aging may be directly regulated by growth factor-sensitive, PKA-dependent pathways. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Werner syndrome; Helicases; RecQ; Aging; Signal transduction

1. Introduction

Individuals with Werner's progeria manifest a premature onset of age-related phenotypes such as type II diabetes, osteoporosis and atherosclerosis [1]. Most afflicted patients succumb by their fourth decade usually from either a myocardial infarction or cancer. Genetic analysis has demonstrated that affected individuals have homozygous mutations in a gene product encoding a member of the RecQ family of DNA helicases. Disruptions in genes belonging to this family – BLM, WRN and RecQ4 – are associated with distinct clinical disorders: Bloom's syndrome, Werner's syndrome and Rothmund–Thomson syndrome, respectively. Patients with these disorders show a significant predisposition to the development of cancer, which highlights the important role of RecQ helicases in sustaining genomic stability and preventing tumor formation. Biochemical studies have implicated the Werner helicase in a host of diverse processes including DNA replication, repair and recombination [1–3]. This helicase seems to be responsible for resolving aberrant DNA structures that form after DNA damage or during normal DNA replication. Mutations in WRN prevent the repair of these structures and result in increased genetic instability. Cells derived from Werner syndrome patients show accelerated telomere shortening, chromosomal instability and shorter in vitro life span [4,5].

In this report we describe an association between the Werner helicase and a subunit of protein kinase A (PKA), and demonstrate that this association is involved in the dynamic nuclear re-distribution of the Werner gene product stimulated by nutrient withdrawal. Although the response to caloric restriction is undoubtedly complex, in yeast glucose is known to increase cAMP levels through a Ras-dependent signaling pathway. The rise in cAMP triggers the dissociation of the catalytic subunit of PKA (Tpk) from its regulatory subunit (Bcy1). The importance of this pathway is strengthened by a number of studies demonstrating that yeast life span is augmented either by caloric restriction or by mutations in certain upstream elements that regulate PKA-dependent signaling [6,7]. Recent evidence suggests that the life extension induced by caloric restriction requires Sir2, a member of the family of NAD-dependent histone deacetylases implicated in gene silencing and genomic stability [8]. Thus, with regard to longevity, Sir2 may act as a downstream effector of PKA signaling in yeast. Furthermore, PKA directly regulates UV-damaged DNA repair in cell-free yeast extracts [9]. Taken together, these results suggest that in yeast there is a pathway that couples nutrient supply, PKA activity and genomic stability. The interaction of the Werner protein with PKA described in this article offers a new perspective on the interrelation of these three processes in mammalian cells.

2. Materials and methods

2.1. Yeast and mammalian two-hybrid analysis

The C-terminus of WRN, WRN-C (amino acids 859–1432), was created by PCR amplification from full-length human WRN cDNA (kindly provided by J. Oshima, International Registry of Werner Syndrome, University of Washington, Seattle, WA, USA) and was subcloned into a pGBKT7 yeast expression vector (Clontech) in frame with the GAL4 DNA binding domain (BD). The BD-WRN-C bait was transformed into AH109 yeast strain, and then mated with Y187 yeast cells pretransformed with a human brain cDNA library fused to the GAL4 activation domain (Clontech HL4004AH). The two-hybrid screen was performed according to the manufacturer's recommendations with approximately 1.5×10^6 independent clones analyzed.

To localize the putative binding domains, truncated WRN cDNAs were prepared by PCR amplification from full-length human WRN cDNA and subcloned into pGBKT7. These truncated baits were co-transformed with pACT2-PKA-RIβ into the AH109 yeast strain, and co-transformants were selected on SD/Leu-/Trp-/His-/Ade- with X-α-

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0014-5793/02/$22.00
PII: S0014-5793(02)02868-5

galactosidase to assess reporter gene activation. Full-length PKA-RIβ and variously truncated PKA-RIβ cDNAs were prepared by PCR amplification from a PKA-RIβ full-length cDNA obtained from Incyte Genomics (Clone ID 1290180), and subcloned into pGADT7 in frame with the GAL4 activation domain. All constructs were verified by direct sequencing.

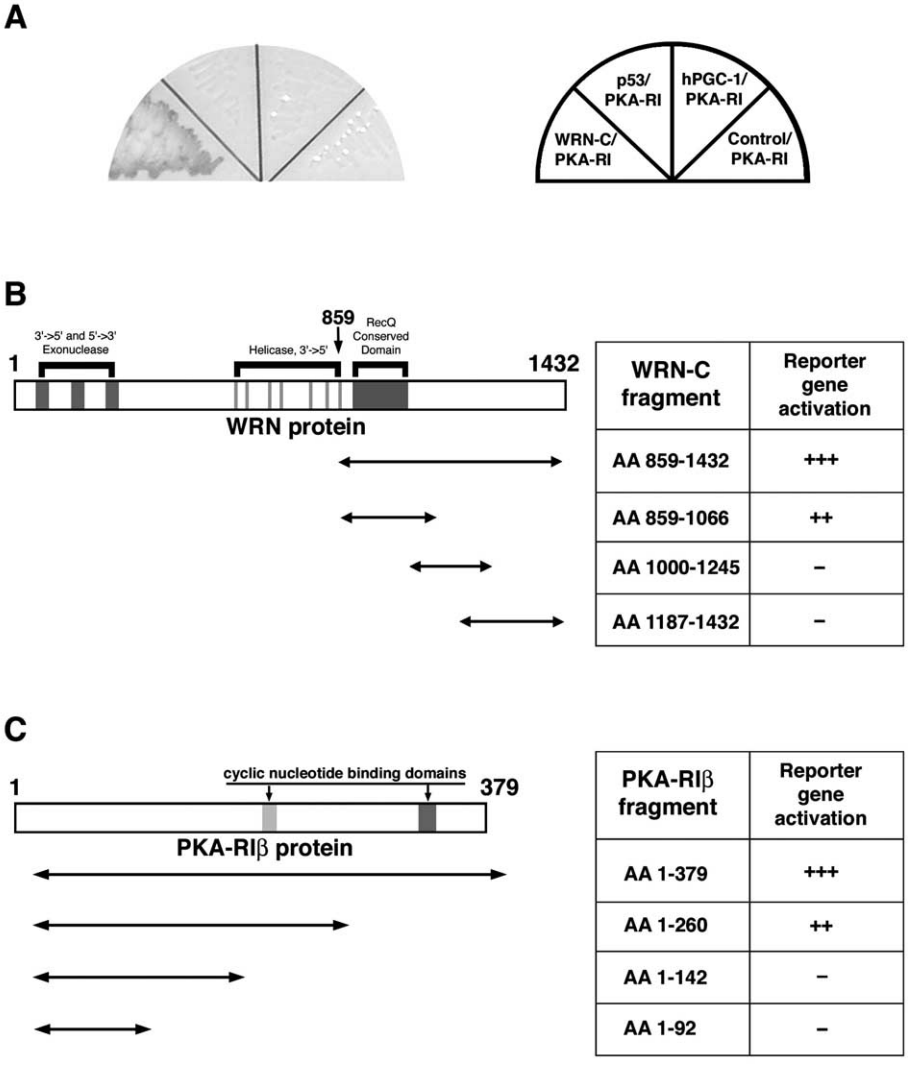
For the mammalian two-hybrid assay, WRN-C and full-length PKA-RIβ were subcloned into a pM-GAL4-DNA-BD and pVP-16 mammalian expression vectors, respectively (Clontech). pM-BD-WRN-C and pVP-16-PKA-RIβ were transiently co-transfected as described below into HeLa cells along with pG5CAT, a plasmid containing the CAT reporter gene under the control of five tandem GAL4 binding sites (Clontech). HeLa cells were harvested 72 h after transfection and the lysate prepared for a CAT ELISA assay following the manufacturer's recommendations (Boehringer Mannheim).

2.2. Immunoprecipitation and immunoblotting

WRN-C and full-length PKA-RIβ were subcloned into pCMV-Tag-myc and pCMV-Tag-Flag mammalian expression vectors respectively (Stratagene). Equal amounts of WRN-C-myc and PKA-RIβ-Flag were transiently co-transfected into HeLa cells (2.5 μg total DNA/well of a six-well dish) using Lipofectamine 2000 according to the manufacturer's recommendations (Life Technologies). Extracts enriched in both nuclear and nucleolar proteins were prepared, with modifications, as previously described [10,11] Briefly, transfected HeLa cells were harvested on ice in lysis buffer (10 mM Tris–HCl, pH 7.4/10 mM KCl/2 mM $MgCl_2$/0.05% Triton X-100/1 mM EGTA) supplemented with protease inhibitor cocktail (Complete, Boehringer Mannheim). Extracts (approximately 2 mg total protein) were then sonicated on ice followed by centrifugation at $4000 \times g$ for 5 min. The first supernatant was retained and the remaining pellet was then additionally extracted in the above lysis buffer supplemented with 300 mM KCl. After a second centrifugation, the second supernatant was combined with the original. Nonidet P-40 was added to a final concentration of 1%. The nuclear-enriched extract was then pre-cleared with normal mouse IgG and Gammabind Plus Sepharose beads (Amersham Pharmacia) for 1 h at 4°C, centrifuged and normalized for protein concentration. The supernatant was then incubated overnight at 4°C with anti-Flag M2 affinity gel (Sigma). The affinity gel was precipitated by centrifugation and washed five times with 1×wash buffer (Sigma). Flag-tagged proteins were subsequently eluted and resuspended in sample buffer. Samples were analyzed on 12% SDS–PAGE gels and immunoblotted with either 1:1000 anti-Flag M2 mouse monoclonal antibody (Sigma), or 1:500 anti-myc mouse monoclonal antibody (Santa Cruz Biotechnology) and finally visualized by chemiluminescence.

2.3. Fluorescent microscopy

To obtain WRN–GFP, full-length WRN was subcloned in frame into the green fluorescent protein (GFP) expression vector pEGFP-C1



WRN-C fragment	Reporter gene activation
AA 859-1432	+++
AA 859-1066	++
AA 1000-1245	–
AA 1187-1432	–

PKA-RIβ fragment	Reporter gene activation
AA 1-379	+++
AA 1-260	++
AA 1-142	–
AA 1-92	–

Fig. 1. The carboxy-terminus of the Werner helicase and PKA-RIβ interact in yeast. A: Specific interaction between WRN-C and PKA-RIβ supports growth while no interaction is observed using p53, human PGC-1 or empty vector alone as bait. B: Growth and α-galactosidase reporter activity in yeast defines a region of WRN-C required for the interaction with PKA-RIβ, that encompasses a domain conserved among a number of RecQ helicases. C: The C-terminal domain of PKA-RIβ is not required for interaction with WRN-C.

(Clontech). HeLa cells were transfected as described above, and 18 h post-transfection cells were either maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% serum or placed in DMEM without serum. Transfected cells were then incubated for an additional 24 h prior to assessment with a Nikon TE300 fluorescent microscope. To determine the role of cyclic nucleotides, cells were incubated with either vehicle alone, 10 mM 8-bromo-cAMP, or 10 mM 8-bromo-cGMP, with each agent added at the time of serum withdrawal. In other experiments, cells were co-transfected with WRN–GFP (1.25 μg) and equal amounts of either pCMV-RIβ-Flag, pCMV-CIα V5 or empty vector. The initial CIα clone was a generous gift of Y.S. Cho-Chung (NIH), was amplified by PCR and subcloned into a V5 epitope-tagged expression vector and then confirmed by direct sequencing. Approximately 7–10 random fields (40×) were photographed for each condition and the number of punctates in 50–100 GFP-positive cells determined. All studies were repeated at least three times with similar results.

3. Results

To further extend the known functions of the Werner helicase we performed a yeast two-hybrid screen using the C-terminal domain (amino acids 859–1432) of the molecule as bait (WRN-C). This region of the molecule is devoid of any known exonuclease or helicase activity. A brain library was screened to potentially enrich for protein partners that were independent of DNA replication. From this library, we identified a number of potential interacting molecules including a subunit of the COP9 signalosome, Surf-4 as well as two expressed sequence tags of unknown function. In addition to these potential candidate interacting proteins we also identified the PKA regulatory subunit RIβ. As demonstrated in Fig. 1A, in yeast PKA-RIβ could specifically interact with the C-terminal domain of the Werner helicase. To determine which part of the helicase was required for this interaction we next made a series of C-terminal constructs. As demonstrated in Fig. 1B, amino acids 859–1066 of the helicase were sufficient for interaction while no activation was seen with various constructs comprising amino acids 1000–1432. Interestingly, the region comprising amino acids 859–1066 contains a domain of previously unknown function that is well conserved among several members of the RecQ helicases [12]. Similarly, analysis of PKA-RIβ demonstrated that the carboxy-terminus of this molecule was dispensable for interaction with the Werner helicase (Fig. 1C). As such, binding appeared independent of at least one of the two conserved cAMP binding motifs found in all regulatory subunits.

To confirm that the interaction observed in the yeast two-hybrid system occurs in vivo, we next prepared epitope-tagged forms of WRN-C (WRN-myc) and full-length PKA-RIβ (R1-Flag). Transfection of these constructs into HeLa cells demonstrated expression of both constructs using myc- or Flag epitope-specific antibodies (Fig. 2A). We next prepared nuclear extracts from cells transfected with both WRN-myc and RIβ-Flag. Immunoprecipitation of PKA-RIβ from nuclear extracts of transfected cells demonstrated an association with the WRN-myc gene product (Fig. 2B). Reciprocal immunoprecipitation of WRN-myc did not allow for unambiguous identification of co-precipitated PKA-RIβ since the regulatory subunit is similar in size to the immunoglobulin heavy chain (data not shown). In an effort to further confirm that the two proteins interacted in vivo we performed a mammalian two-hybrid assay [13]. As noted in Fig. 2C, the combina-

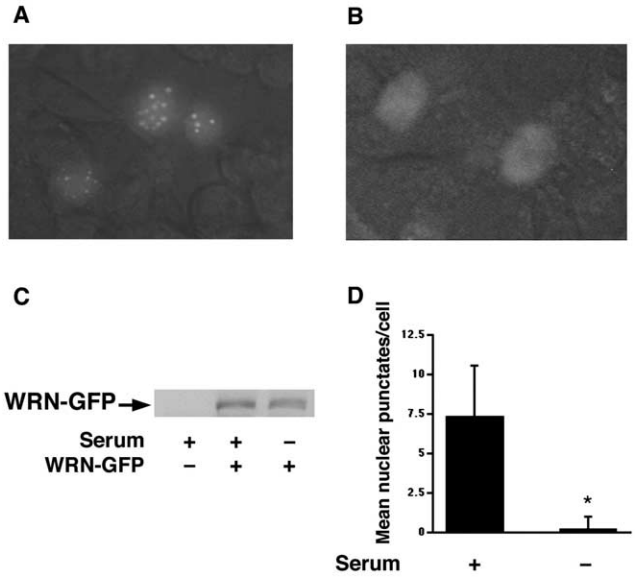


Fig. 3. Serum regulates the nuclear distribution of the Werner helicase. A: Under normal growth conditions, WRN–GFP in transfected HeLa cells shows a punctate nuclear distribution. B: Twenty-four hours after serum withdrawal the distribution of WRN–GFP is more diffuse within the nucleus. C: Total levels of WRN–GFP do not change appreciably following the withdrawal of serum. D: Quantification of the number of punctates within the nucleus confirms the redistribution stimulated by serum withdrawal.

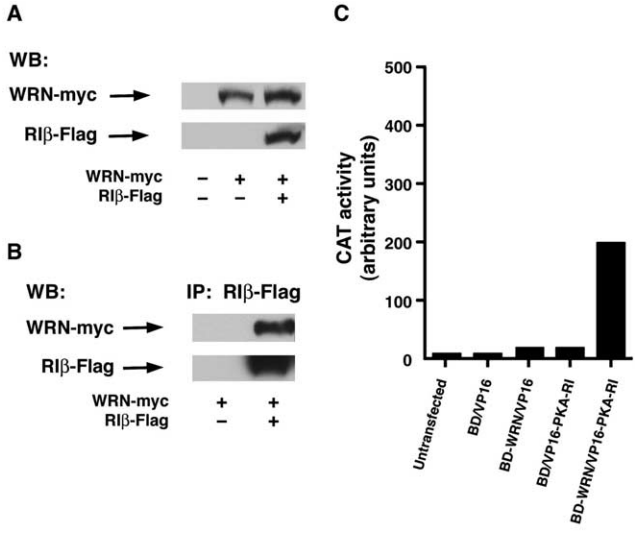


Fig. 2. The Werner helicase and PKA-RIβ interact in vivo. A: Expression as assessed by Western blot (WB) analysis of epitope-tagged Werner helicase (WRN-myc) and PKA-RIβ (RIβ-Flag) in transfected HeLa cells. Expression represents levels of transfected protein in 50 μg of nuclear-enriched protein lysate. B: Immunoprecipitation (IP) with Flag antibody of 80 μg enriched nuclear extract derived initially from 2 mg of total protein lysate. WRN-myc is only co-precipitated in cells expressing RIβ-Flag. C: Mammalian two-hybrid assay using plasmids expressing the GAL-4 DNA binding domain (BD) and the activation domain from VP-16. Fusion proteins encoding BD and Werner helicase (BD-WRN) as well as VP-16 and PKA-RIβ (VP16-R1) were tested independently and together for reporter activation. Only the combinations of WRN and PKA-RIβ fusion constructs were sufficient to activate transcription. Results are the mean values of duplicate determinations from one of two similar experiments.

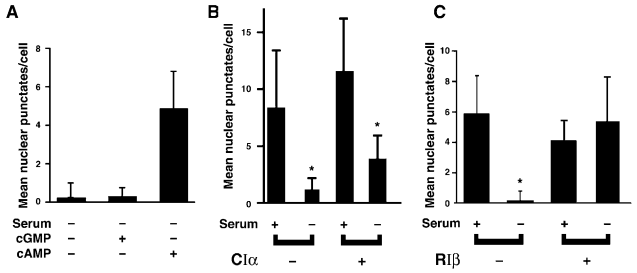


Fig. 4. The role of cAMP and PKA in Werner helicase localization. A: Incubation with a cAMP analogue inhibits the nuclear redistribution of WRN–GFP stimulated by serum withdrawal. B: The redistribution induced by serum withdrawal is not affected by over-expression of CIα. C: Expression of RIβ blocks Werner helicase redistribution stimulated by nutrient withdrawal.

tion of Werner helicase and PKA-RIβ strongly and specifically stimulated reporter activation.

We next constructed a fusion protein containing full-length Werner helicase and GFP (WRN–GFP) in order to obtain additional functional information regarding the role of RIβ in the regulation of the Werner helicase. Co-immunoprecipitation experiments demonstrated that this chimeric protein still interacted with PKA-RIβ (data not shown). As observed in Fig. 3A, expression of WRN–GFP demonstrated that, as has been previously described using immunohistochemical analysis of endogenous protein, the WRN–GFP molecule appears to localize within discrete sub-domains in the nucleus previously identified as the nucleolus [14,15]. In contrast, 24 h following serum withdrawal the distribution of WRN–GFP was more uniform within the nucleus (Fig. 3B). As previously observed, this serum withdrawal-induced redistribution occurred without a significant change in helicase levels [14]. This is also evident from Fig. 3C where it is demonstrated that in the presence or absence of serum, total levels of WRN–GFP are not significantly altered. In an effort to quantitate this effect, we counted the number of GFP-positive nuclear punctates per cell under normal growth conditions and in the setting of serum withdrawal. As demonstrated in Fig. 3D, consistent with the observed diffusion of the WRN–GFP, serum withdrawal resulted in a significant reduction in observed nuclear punctates per cell. In numerous experiments ($n = 6$), when compared to serum-containing conditions, the percentage decrease of nuclear punctates induced by serum withdrawal ranged from 60 to 90%.

In an effort to assess whether the interaction of Werner helicase with a component of PKA played a role in the subcellular distribution of the helicase, we next examined the role of intracellular cAMP. As demonstrated in Fig. 4A, in the setting of serum withdrawal, levels of nuclear punctates were low in untreated cells. Administration of a cell-permeant form of cAMP but not cGMP throughout the period of serum deprivation resulted in maintenance of nuclear punctates. Since a rise in intracellular cAMP triggers an increase in the free levels of both the catalytic and regulatory subunits of PKA, we next sought to assess the role of each of these components separately. As demonstrated in Fig. 4B, increased expression of the catalytic subunit (CIα) had little effect on the redistribution of the helicase in the setting of serum withdrawal. In contrast, expression of the RIβ subunit prevented the nuclear redistribution induced by the withdrawal of serum (Fig. 4C).

4. Discussion

Our data demonstrate an interaction of the Werner helicase with a regulatory subunit of PKA and implicate this association with the nuclear distribution of the helicase. In particular, the redistribution of the helicase induced by serum withdrawal is inhibited by increased regulatory subunit expression and by cAMP but not by the catalytic subunit of PKA. This data suggest the regulatory subunit role in helicase localization is most likely independent of its role in the regulation of catalytic activity. Other examples of regulatory subunit activity that is independent of kinase activity have been previously described. For instance, in yeast, expression of $RAS2^{val19}$ leads to a number of nutritional alterations that can be suppressed by expression of MSI1, a subunit of chromatin assembly factor-1 [16]. Recent evidence suggests that MSI1 overexpression inhibits the $RAS2^{val\ 19}$ phenotype not by lowering cAMP levels or directly affecting catalytic (Tpk) subunit activity, but rather MSI1 appears to mediate its effects through the regulatory subunit Bcy1 [17]. Similarly, there is also evidence in mammalian cells suggesting that the regulatory subunit may have effects that are independent of its negative regulation of catalytic subunit activity. Indeed, some early studies demonstrated that the RII subunit interacts with DNA and possesses type I topoisomerase activity [18]. In addition, certain mutants of Rlα appear to confer increased resistance to the DNA-damaging agent cisplatin in a catalytic subunit-independent fashion [19]. Finally, recent evidence suggests that PKA regulatory subunits may potentially function as tumor suppressors [20].

Previous studies have demonstrated that the human Werner helicase gene product is localized to the nucleolus [13,14,21]. To date little is known about what regulates the import, export or retention of proteins within this sub-nuclear structure. Although the nucleolus is best known as the site for the processing and synthesis of an array of riboproteins, increasing evidence suggests that this structure is also important in sequestering a host of DNA repair and cell cycle regulatory gene products [22]. Although in dividing cells the human Werner helicase (hWRN) localizes to the nucleolus, the mouse gene product (mWRN), in contrast, is always found uniformly distributed throughout the nucleus [13,14]. Surprisingly, even when hWRN is expressed in mouse cells, the human helicase fails to localize within the nucleolus [23], suggesting a potential fundamental difference in nucleolar trafficking between species. Given that human and mouse WRN gene products have similar enzymatic activities, the failure of the mouse gene product to localize to the nucleolus has been proposed as an explanation for why mice with targeted deletions of mWRN fail to manifest symptoms of accelerated aging [23]. As such, it has been proposed that the nucleolar activity of hWRN may represent a critical aspect of the gene product's ability to regulate life span.

The Werner helicase has been shown to interact with a number of proteins including RPA, PCNA, δ-polymerase, KU, p53, DNA-dependent kinase and flap endonuclease 1 [12,24–30]. Each of these proteins is involved in DNA processing events such as repairing DNA damage or resolving aberrant DNA structures. These studies have strengthened the hypothesis that the Werner gene product plays an important role in the repair of DNA damage and in DNA replication. Nonetheless, other DexH-containing helicases which are

not associated with premature aging have very similar enzymatic function and can in some cases associate with similar proteins. As such, the results presented here provide additional insight into Werner function by providing a direct link between metabolic state, PKA activity and helicase sub-cellular localization. Furthermore, they strengthen the notion that at least some of the paradigms established in simple organisms such as yeast may have direct relevance to the study of human aging, genetic stability and carcinogenesis.

Acknowledgements: We wish to thank Y.S. Cho-Chung for the CIα clone and J. Oshima and the International Registry of Werner Syndrome for the full-length WRN clone. In addition, we thank S. Nemoto for helpful advice. D.T.N. was supported by the Howard Hughes Medical Institute Research Scholars Program.

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