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The molecular role of the Rothmund-Thomson-, RAPADILINO- and Baller-Gerold-gene product, RECQL4: recent progress

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Abstract. The RecQ family of DNA helicases is highly conserved throughout evolution and plays an important role in the maintenance of genomic stability in all organisms. Mutations in three of the five known family members in humans, *BLM, WRN* and *RECQL4*, give rise to disorders that are characterized by predisposition to cancer and premature aging, emphasizing the importance of studying the RecQ proteins and their cellular activities. Interestingly, three autosomal recessive disorders have been associated with mutations in the *RECQL4* gene: Rothmund-Thomson, RAPADILINO, and Baller-Gerold syndromes, thus making RECQL4 unique within the RecQ family of DNA helicases. To date, however, the molecular function of RECQL4 and the possible cellular pathways in which it is involved remain poorly understood. Here, we present an overview of recent findings in connection with RECQL4 and try to highlight different directions the field could head, helping to clarify the role of RECQL4 in preventing tumorigenesis and maintenance of genome integrity in humans.

Keywords. Genome stability, cancer, RecQ helicases, RECQL4, Rothmund-Thomson syndrome, RAPADILINO syndrome, Baller-Gerold syndrome.

Introduction

The maintenance of the genetic material (so called 'genome stability') is an essential process in every living organism, and the failure of this process can lead to the development and progression of cancer. If we are to understand the causes of genome instability in humans, we must first understand the function of proteins that normally act to keep the genetic material intact. One family of proteins required to maintain genome stability is the RecQ helicase family [1]. Five human RecQ homologues, called RECQL1, BLM, WRN, RECQL4, and RECQL5, have been identified so far, and three of them have been shown to be associated with five autosomal recessive disorders characterized by genomic instability and cancer predisposition. Werner syndrome (WS), linked to a defect in the WRN protein, is characterized by the appearance of unusually accelerated aging (progeria) [2]. Bloom syndrome (BS), which is associated with a defect in the BLM protein, is characterized by stunted

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growth, developmental problems, immunodeficiency, male infertility and, at the cellular level, a slowing of S-phase progression and a dramatic increase in sister chromatid exchange and genomic instability [3]. Interestingly, three different human disorders have been associated with mutations in the *RECQL4* gene: Rothmund-Thomson (RTS), RAPADILINO and Baller-Gerold (BGS) syndromes [4–6]. Below, we review the recent findings and highlight the importance of studying RECQL4 in order to understand its role in the maintenance of genome stability in humans.

RECQL4 and genetic diseases

RTS was originally described in 1868 by the German ophthalmologist Rothmund [7] and subsequently confirmed by the English dermatologist Thomson in 1936 [8]. It is an unusual autosomal recessive condition associating poikiloderma, growth deficiency, juvenile cataracts, premature aging and a predisposition to malignant tumours, particularly osteosarcomas [9]. Despite its long history, only about 300 cases of RTS have been reported in the scientific literature thus far [9, 10]. Interestingly, mutations in the *RECQL4* gene cause only 60% of all RTS cases [11]. Accordingly, RTS seems to be a heterogeneous disease and mutations in other, yet unidentified, gene(s) seem to be responsible for the phenotype of the remaining 40% of RTS patients. The number of mutations found in *RECQL4* is quite low, ranging from nonsense, frameshift and splice site mutations to intronic insertions and deletions [5]. Most of these mutations result in premature termination of protein translation and truncated RECQL4 proteins that often lack a large part of the helicase domain [12]. Cells derived from RTS patients show genomic instability, including trisomy, aneuploidy and chromosomal rearrangements [9, 13]. Additionally, RTS cells are sensitive to ionizing radiation and oxidative stress/damage [14, 15].

Another autosomal recessive disease associated with mutations in the *RECQL4* gene is RAPADILINO syndrome [5]. The syndrome was originally described in 14 patients from Finland. To date, three additional cases of RAPADILINO syndrome have been reported [5]. The acronym stands for the characteristic clinical features: RAdial hypo-/aplasia, Patellae hypo-/aplasia and cleft or highly arched PAlate, DIarrhoea and DIslocated joints, LIttle size and LImb malformation, NOse slender and NOrmal intelligence. The most common mutations of the *RECQL4* gene in RAPADILINO patients represent in-frame deletions of exon 7 which do not affect the helicase domain of RECQL4 protein [5]. Although RAPADILINO patients share some clinical features with RTS patients, like photosensitivity with extra pigmentation of skin, or growth deficiency, there are unique diagnostic findings such as joint dislocations and patellar hypo/aplasia. In contrast to RTS, RAPADILINO is more common in females, and only 7% of current RAPADILINO patients carry malignant tumors, mainly osteosarcomas [16].

BGS, originally discovered in 1950 [17], is the third recently reported autosomal recessive disorder linked to mutations in the *RECQL4* gene [6]. Approximately 20 cases of BGS have been reported in the scientific literature thus far [6]. The clinical hallmarks of BGS are radial aplasia/hypoplasia and craniosynostosis. To date, most mutations of *RECQL4* found in BGS patients represent an R1021W missense mutation and a 2886 delta T frameshift mutation of exon 9. Surprisingly, none of the 24 BGS patients reported so far show any predisposition for cancer [6]. Thus, the cumulative set of data suggests that RECQL4 has roles independent of helicase activity. Whether the role of the RECQL4 N-terminal domain is independent of the helicase domain, or whether it is sufficient to perform a partial function remains to be elucidated in future experiments.

Further studies are needed to characterize different symptoms and evaluate whether the three *RECQL4* associated diseases are separate disorders or whether it would be more appropriate to combine them under a new name. Furthermore, the identification of *RECQL4* mutations and their effects on the *RECQL4* transcript is of particular importance. It will therefore be interesting to test whether certain mutations always lead to distinct phenotypes or whether the correlation is more complex. In addition, investigating the genetic causes of RTS, RAPADILINO and BGS that have arisen due to mutations in proteins other than RECQL4 might help to identify additional genes/proteins involved in the same pathway(s).

RECQL4 mouse models

In 2002, the Furuichi group created the first *RECQL4* knockout mouse model [18]. These mice were embryonic lethal, showing severe proliferation defects and therefore could not be used as a model for RTS. One year later, the Kito group generated another *RECQL4* knockout mouse model by deleting exon 13 of the *RECQL4* gene, which encodes part of the central RecQ-helicase domain. Exon 13 is a hot spot for mutations identified in RTS patients [19]. This *RECQL4*-deficient mouse showed severe growth retardation and several tissue abnormalities that

resemble those of RTS patients. Additionally, the proliferation rate of Mouse Embryonic Fibroblasts (MEFs) derived from this *RECQL4*-deficient mouse was decreased. Even though no poikiloderma was observed, other epidermal symptoms such as brittle skin, hair loss and hair color loss were noticed. These mice had hypoplasia of the epidermis, dermis and subcutaneous tissue. Only 5% of the mutant mice survived the first 14 days, and these mice failed to develop malignancies, a unique characteristic of RTS [19].

One year ago, the Luo group generated the third RECQL4 knockout mouse by deleting the *RECQL4* coding region, including exons 9–13 [20]. 84% of all homozygous *RECQL4*$^{-/-}$ progeny survived to adulthood, with some exhibiting typical clinical features of RTS, such as hypo-/hyperpigmented skin, skeletal limb defects and palatal patterning defects. Furthermore, chromosomal analysis using different cell types derived from this *RECQL4*-deficient mouse displayed an overall aneuploid phenotype and a significant increase in the frequency of premature centromere separation, suggesting that this *RECQL4*$^{-/-}$ mouse is a good model for human RTS [20]. In summary, deletion of exon 13 resulted in only 5% survival of the mice, while deletion of exons 9–13 resulted in 84% survival. It seems that the protein remaining after the 9–13 exon deletion appears to be more functional than the protein remaining after deletion of exon 13. This effect might be due to misfolding of the RECQL4 protein after deletion of exon 13, but not after exon 9–13 deletion, which may result in a more stable mutant RECQL4 protein.

Taken together, the results derived from experiments with various *RECQL4*$^{-/-}$ mice showed that these mice accumulate defects that clearly reflect the situation in humans, i.e. different mutations in the *RECQL4* gene lead to different phenotypes. It would therefore be of particular interest to investigate whether the genotype-phenotype correlations seen in humans could be observed in mice by mimicking mutations causing RAPADILINO and BGS syndromes and whether systematic mutation of particular regions of the *RECQL4* gene could be made in cell lines or in other mouse models.

RECQL4 in the context of DNA replication

In the past 2 years, analyses of RECQL4 in *Xenopus laevis* have indicated an interesting novel role for RECQL4 in the initiation of DNA replication. The *Xenopus laevis* RECQL4 protein (xRTS) is 60% identical to human RECQL4 and shares the central helicase domain common among all RecQ family members. The N-terminal region of xRTS shows 20% identity to two yeast proteins, Sld2 and Drc1, which are both required for the establishment of replication forks [21–23]. This feature makes xRTS unique among the members of the RecQ family. The Venkitaraman lab showed that immunodepletion of xRTS from *Xenopus laevis* egg extracts leads to reduction and delay of sperm chromatin replication, an effect that can be rescued by complementing the extracts with the purified recombinant wild-type human RECQL4 [24]. These findings were recently confirmed by the Takisawa group, showing that purified N-terminal fragments of xRTS were able to rescue the DNA replication activity of RECQL4-depleted *Xenopus laevis* egg extracts [25]. Accordingly, by knocking down the murine homologue of xRTS with small hairpin RNA (shRNA) in primary MEFs, the proliferation rate and DNA replication of these primary cells was highly perturbed. Together, these results suggest that RECQL4 has a role in DNA replication and that this function is conserved during evolution.

RECQL4-interacting partners

Several attempts have been made thus far to identify proteins that associate with RECQL4. Recent studies in the Wang group have revealed that RECQL4 isolated from HeLa cells is found in a complex with UBR1 and UBR2 [26]. These two 200-kDa proteins share high sequence similarities to each other and belong to the family of E3 ubiquitin ligases of the N-end rule pathway, which is part of the ubiquitin-proteasome system [27]. The N-end rule pathway exists in all organisms examined, from mammals to fungi and bacteria. In eukaryotes it is part of the ubiquitin system. By the action of the three ubiquitin-ligases (E1, E2 and E3), a substrate protein is polyubiquitylated and subsequently degraded by the 26S proteasome [27]. Surprisingly, the Wang group found that RECQL4 is not ubiquitylated *in vivo*. In addition, newly synthesized RECQL4 was shown to be a stable protein (half-life of 2 h), and the level of RECQL4 protein was not increased by inhibiting the proteasome with different drugs. Despite these discoveries, the functionality of the physical interaction between RECQL4 and UBR1/UBR2 remains unknown, and further investigations are needed to elucidate a novel function of ubiquitin ligases in the maintenance of genome stability. For example, it would be interesting to investigate RECQL4 function in cells derived from Johanson-Blizzard syndrome patients, which are deficient in UBR1 protein.

Our group found that RECQL4 is in a complex with Rad51 *in vivo* [28]. It has previously been reported

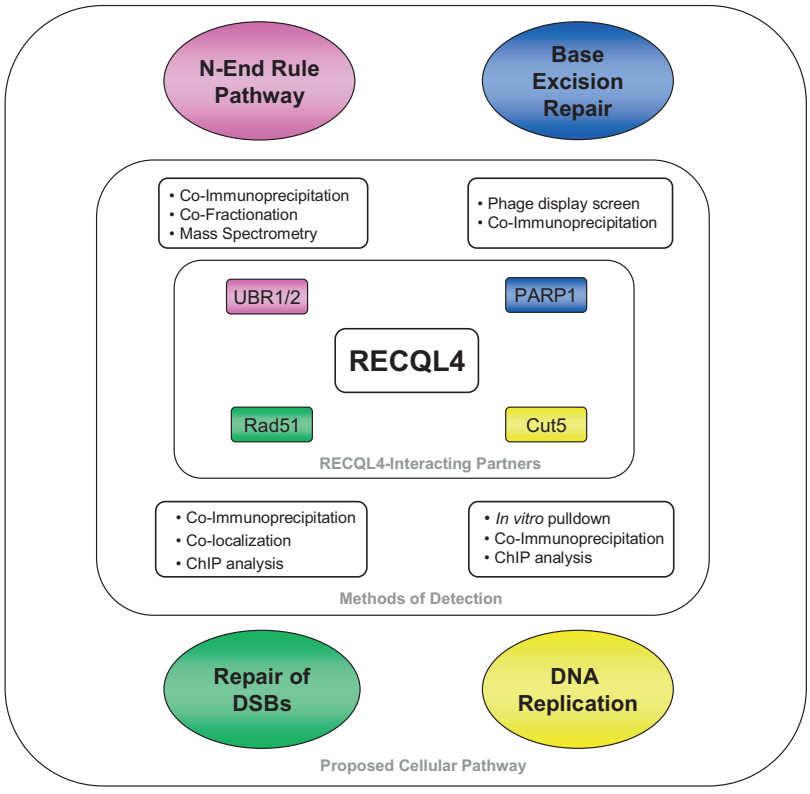

Figure 1. Schematic representation of cellular pathways RECQL4 may be involved in: different RECQL4-interacting partners and corresponding methods of detection.

that Rad51 foci, which are important for DNA repair by homologous recombination, accumulate at sites of single-stranded DNA after induction of DNA double strand breaks (DSBs) [29]. In response to the induction of DSBs by treatment with etoposide, a portion of RECQL4 and Rad51 nuclear foci colocalized, suggesting that RECQL4 plays a role in the repair of DSBs by homologous recombination [28]. The precise role of RECQL4 in DSB repair via its interaction with Rad51 is currently being investigated in our lab.

Recently, Cut5 was shown to interact with the *Xenopus laevis* homologue of RECQL4 both *in vitro* and *in vivo* [25]. Cut5, the metazoan homologue of *Saccharomyces cerevisiae* Dpb11, is required for loading DNA polymerases onto chromatin [30, 31]. As mentioned above, the N-terminus of RECQL4 has similarity to Sld2 ('synthetically lethal with *dpb11*'), one of six Sld family members found to interact with Dpb11 [32]. Thus, these findings provide additional evidence that RECQL4 functions during DNA replication.

The Frank group reported the physical interaction between RECQL4 and poly(ADP-ribose) polymerase-1 (PARP-1) [33]. PARP-1 is involved in different pathways of DNA metabolism, such as recombination, repair and transcriptional regulation [34]. Additionally, PARP-1 is part of the base excision repair (BER) pathway and is activated in response to DNA breaks [35]. The authors showed complex formation between RECQL4 and PARP-1 *in vitro* and *in vivo*. Additionally, PARP-1 was able to poly(ADP-ribosyl)ate RECQL4 *in vitro* [33]. Therefore, RECQL4 might be among a large spectrum of proteins whose deficiencies induce cellular sensitivity to the inhibition of poly(ADP-ribose) polymerase activity. However, the functional relevance of this posttranslational modification of RECQL4 has to be confirmed by further *in vitro* and *in vivo* experiments. For example, it would be interesting to test whether various RTS cells show deficiency in the PARP1 poly(ADP-ribosyl)ation pathway after exposure to DNA damaging agents like hydrogen peroxide (H_2O_2) or methyl methanesulfonate (MMS).

In summary, the current data from several different protein interaction assays indicate that RECQL4 is involved in DNA repair and replication processes (Fig. 1). However, further functional studies are needed to characterize in more detail the exact role of RECQL4 in these processes, as well as to identify additional RECQL4-interacting proteins in order to draw a more precise picture of the exact cellular pathways RECQL4 might be involved in. Finally, it will be important to determine whether RECQL4 interacts with multiple protein partners simultaneously or independently in different pathways.

The extended N- and C-terminal domains of all RecQ helicases studied so far show very little sequence identity and are therefore thought to confer specificity to these proteins by mediating protein-protein interactions [1]. For this reason, N- and C-terminal fragments of RECQL4 could be used as bait in a yeast two-hybrid screen in order to find novel RECQL4-interacting partners. On the other hand, different RECQL4-specific antibodies have been raised in the last couple of years that could be used to co-immunoprecipitate RECQL4-interacting partners from mammalian cells extracts, followed by mass spectrometry analysis.

Subcellular localization of RECQL4

Four different groups have examined the subcellular localization of RECQL4 in different mammalian cells, and their observations are contradictory. The Furuichi group overexpressed full-length FLAG-tagged RECQL4 in HeLa cells, and immunofluorescence analysis suggested that RECQL4 is localized exclusively to the nucleus [11]. In 2004, the Wang group extensively studied the subcellular localization of RECQL4 using different cell types and three polyclonal anti-RECQL4 antibodies [26]. Western blot analysis of HeLa, MCF7 and Jurkat cell extracts revealed that RECQL4 was found predominantly in the cytoplasm, while indirect immunofluorescence experiments using HeLa cells found RECQL4 localization to both the nucleus and the cytoplasm. In contrast, RECQL4 was largely present in the nuclear fraction of untransformed WI-38 fibroblasts.

Our group raised two antibodies, against the N- and C-termini of RECQL4, and used them in immunofluorescence experiments on several exponentially growing human cell lines. Although we found that the majority of RECQL4 protein is detected in the nucleus, a small amount of it was found in the cytosolic fraction of HeLa cell extracts [28]. Furthermore, we found that endogenous RECQL4 localized exclusively in discrete nuclear foci in HeLa, WI-38/VA13 and primary skin fibroblast cells, and that the number of these does not significantly change during the cell cycle or upon the induction of DNA DSBs. We found that RECQL4 foci partially coincide with those formed by promyelocytic leukemia (PML) bodies and Rad51 as well as with regions of single-stranded DNA (ssDNA) upon induction of DSBs. PML bodies and Rad51 foci have been shown to regulate the response to, and repair, of DSBs [36, 37]. These findings suggested a role for RECQL4 in the repair of DSBs by homologous recombination (HR) and indicated a completely novel function for RECQL4 in human cells [28]. The data from our group suggests that RECQL4 forms nuclear foci and that this is contradictory to the findings by Yin et al., who showed uniform distribution of RECQL4 in both the cytoplasm and the nucleus of human cells [26, 28]. These discrepancies can be explained by the use of different fixation methods and antibodies. The immunofluorescence method that we used results in the elimination of all RECQL4 that is not bound to the nuclear matrix, and this may not be the case for other techniques [28].

Very recently, the Frank group investigated the localization of RECQL4 in living cells by fusing EGFP to the N-terminus of the RECQL4 sequence [33]. RECQL4 displayed nucleoplasmic staining in most of the cells examined, and no significant cytoplasmic staining was observed. Using EGFP fused to different deletion mutants of RECQL4, the authors further characterized the RECQL4-domain structure. Their data suggest that RECQL4 contains at least two N-terminal nuclear localization signals (NLSs) and one nucleolar localization signal (NOS) spanning amino acids 376–386. Furthermore, RECQL4 nucleolar enrichment was observed after treating cells with H_2O_2 or streptonigrin, which leads to oxidative damage of DNA. Interestingly, RECQL4 localization did not change after treating cells with other DNA damage agents such as γ irradiation, etoposide, bleomycin, or UV irradiation [33].

Biochemical properties of the RECQL4 Protein

Compared with all other human RecQ family members, the biochemical properties of the RECQL4 protein are thus far only poorly understood. This is mainly due to the inability to purify the RECQL4 protein. Many groups, including ours, have attempted to purify full-length RECQL4 protein using different expression systems, but all efforts resulted in production of truncated RECQL4 protein. Consequently, the first enzymatic activity assays were performed by the Wang group, with immunoprecipitated RECQL4 protein from HeLa cell extracts [26]. RECQL4 coupled to protein-A sepharose beads showed DNA-dependent ATPase activity; however, RECQL4 failed to unwind any of the tested DNA substrates, although BLM helicase purified by the same way possessed DNA helicase and translocase activity.

One year ago, the Sung group purified full-length RECQL4 protein from *Escherichia coli* [38]. Although the yield of purified RECQL4 protein was low, the authors found that this RECQL4 has no detectable helicase activity, but possesses ssDNA-stimulated ATPase activity. In addition, the authors showed that

RECQL4 binds to ssDNA, an effect that could be inhibited by the ssDNA binding protein RPA [38]. Taken together, the current studies indicate that RECQL4 is not an active helicase; however, it still remains possible that RECQL4 needs a co-activator protein(s) and/or a particular post-translational modification(s) in order to accomplish a DNA unwinding function. To this end, it would be interesting to test whether RECQL4 purified from mammalian or insect cells possesses helicase activity.

Conclusions and future directions

The RecQ helicases have received a considerable amount of attention during the past 15 years, primarily owing to their link with premature aging and cancer susceptibility syndromes. While most of the studies involving human RecQ helicase members have thus far been performed on BLM and WRN helicases, only in the past 2 years have we seen increased interest in RECQL4. Although RTS, RAPADILINO and BGS syndromes caused by mutations in RECQL4 are rare (i.e. they currently affect ~400 individuals in total), it will be interesting to figure out how faulty RECQL4 hinders the normal process of DNA repair and replication, and how defects in these processes lead to such a broad spectrum of clinical features, as observed in RTS, RAPADILINO and BGS patients [39, 40]. There are many questions to be answered, and it is certain that new data regarding the identification of RECQL4-interacting partners, determination of RECQL4's post-translational modifications as well as its precise role during DNA metabolism will be forthcoming in the near future.

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