Final report: K119361 Biochemical analysis of the replication of stable secondary structure-forming DNA sequences

Our final goal was to understand better, how DNA replication machinery is going through the G-quadruplex DNA sequences. The continuous movement of the replication fork is often stalled by various obstacles like stable secondary DNA structures (*1*). Rescue of the stalled replication fork at damage sites is widely examined, but replication at stable secondary structure-forming DNA templates is not well understood, yet (*2, 3*). The G-quadruplex (G4) structure is an abundant and well-characterized type of replication-blocking, stable, alternative DNA or RNA structure. Four guanines form a G-quartet, a planar structure stabilized via Hoogsteen base pairing. Stacking of G-quartets leads to a higher ordered structure that is stabilized by a monovalent cation, most frequently potassium. They can be found at telomeres as well as at many endogenous sites (intrachromosomally) of eukaryotic chromosomes. G4 structures can be classified by several characteristics: based on the number of participating nucleic acid strand(s) (uni-, bi- or tetramolecular) or the direction of the nucleic acid strands in the G4 (parallel, anti-parallel or hybrid) (*4, 5*). Genomic regions with a high potential to fold into G4 structures can be determined experimentally (*6*) as well as computationally (*4, 7*). Searching for potential G4-forming sequences in the human genome showed more than 360 000 potential G4 structures (*4*). Additionally, an experimental approach suggested around 750 000 G4 structures in the human genome (this represents more than 4% of the human genome) (*6*).

If the replication machinery encounters a G4 structure, a DNA helicase is needed to unwind it. Several G4-unwinding DNA helicases have been identified (*8, 9*). In *Saccharomyces cerevisiae* at least three DNA helicases can unwind G4 structures *in vitro* (Pif1, Sgs1 and Hrq1) and have been implicated to function at G4 regions *in vivo* (*9*). Pif1 seems to be the primary G4-unwinding helicase in yeast cells, which is a highly conserved 5'-3' DNA helicase (*10-12*). Surprisingly the function of Piof1 in human is not well understood. In humans, several other G4 unwinding helicases were described (FANCJ, WRN, BLM) (*8*). Although lot of G4 unwinding DNA halicases were described, the mechanism of the replication of G4 prone regions in not clarified. One of the most interesting questions is in this field: How coordinated the action of DNA helicases and the replicative DNA polymerase complex.

The yeast Mgs1 (Maintenance of genome stability 1) protein belongs to a highly-conserved AAA+ ATPase family (*13*) and it is homologous to the Escherichia coli RarA (also known as MgsA) and human WRNIP1 (Werner Interacting Protein 1) (*14, 15*). Mgs1, as well as its human homolog, plays a crucial role in genome maintenance (*13, 15*), but the exact function of them is not known. The synthetic lethal phenotype of *mgs1*Δ*rad6*Δ double deletion containing yeast strain suggests its possible regulatory function over an alternative replication fork rescue pathway (*16*).

In the first part of our research we focused on the function of yeast Mgs1 protein. We demonstrate that Mgs1 is a novel G4-binding protein supporting genome stability, based on these facts:

- Biochemical analyses allowed us to identify Mgs1 as a novel binder of G4 structures.
- *In vivo* experiments confirm these *in vitro* observations, by showing that Mgs1 binds to G4 motifs in yeast.
- Treatment with the G4 stabilizing agent PhenDC3 resulted in Gross chromosomal rearrangements in *mgs*1Δ yeast cells.
- Mgs1 has some functional connection with Pif1 DNA helicase (which is the major G4 unwinding DNA helicase in yeast)

These findings resulted in two publicatins: (Zacheja T, Toth A, Harami GM, Yang Q, Schwindt E, Kovács M, Paeschke K, Burkovics P. FASEB J. 2020 Sep;34(9):12646-12662) (Paeschke K, Burkovics P Curr Genet. 2021 Apr;67(2):225-230)

Since the failure of G4 replication could lead to carcinogenesis, a regulator of G4 replication could be very interesting for several reasons. Therefore we compared that the human homologue of Mgs1 called WRNIP1 is also contributes in G4 replication, and its function could be similar to yeast Mgs1 or not.

We analyzed the two most characteristic property of yeast Mgs1 in humans, using human WRNIP1. One of them was the G4 binding characteristic of the protein (*17*). The other was its effect on Homologous recombination mediated double strand break repair (*13*). The biochemical analyzes of WRNIP1 resulted in the finding that WRNIP1 as well as the Mgs1 is a G4 binder protein. But the effect of WRNIP1 on HR is completely the opposite then the effect of yeast Mgs1. Therefore we were concluded that over the similarities, WRNIP1 has completely unique human specific function.

First we analyzed the wrnip1 function during theG4 replication: We described that

- WRNIP1 bind specifically to G4 DNA structure (Figure1, Figure2 Table 1)
- WRNIP1 contributes in G4 replication in vivo (Figure3)
- The overexpression of WRNIP1 also resulted in G4 processing failure during the replication (Figure 4)

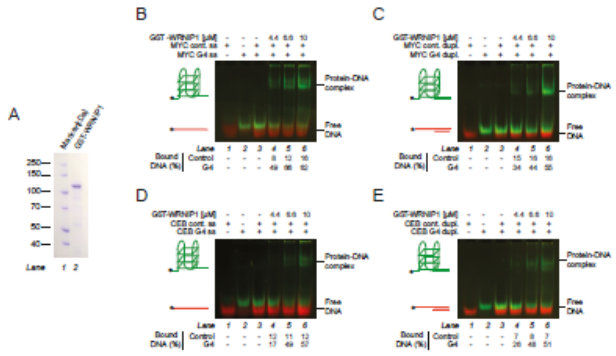

Figure 1. WRNIP1 protein preferentially binds G4 structures. **A.** Purified GST-WRNIP1 protein. **B-C.** Competitive EMSA experiment with (B) single-stranded or (C) partial duplex 5'-FITC labelled MYC G4 (green) and 5'-Cy3 labelled MYC control (red) substrates. **D-E.** Competitive EMSA experiment with (D) single-stranded or (E) partial duplex 5'-FITC labelled CEB G4 (green) and 5'-Cy3 labelled CEB control (red) substrates. The percentage of DNA substrate in protein-DNA complex is indicates in % below the corresponding lanes.

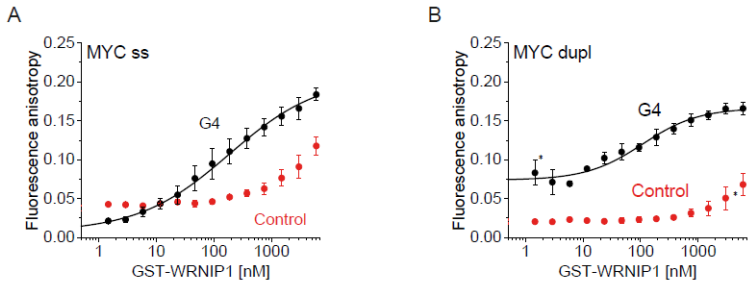

Figure 2. WRNIP1 protein specifically binds to G4 structures *in vitro*. **A-F.** Fluorescence anisotropy measurement performed with (A) single-stranded MYC G4 (black) and MYC control (red), (B) partial duplex MYC G4 (black) and MYC control (red), Error bars represent SEM of 3 biological replicates. Solid lines represent best fits based on the Hill-equation. Fitted parameters are shown in **Table 1**.

Substrate			K_d ± fitting error [nM]	N (Hill-coefficient) ± fitting error	Number of biological replicates
MYC single-stranded	G4		184 ± 36	0.55 ± 0.053	3
	Control		> 5000	N/A	3
	GC-rich control		> 5000	N/A	3
	C-rich control		3496 ± 511	0.96 ± 0.176	3
MYC partial duplex	G4		108 ± 45	0.88 ± 0.16	3
	Control		> 5000	N/A	3
CEB single-stranded	G4		65 ± 6	0.95 ± 0.12	3
	Control		> 5000	N/A	3
CEB partial duplex	G4		221 ± 44	1.1 ± 0.15	3
	Control		> 5000	N/A	3
MYC single-stranded	ATP-	G4	175 ± 43	0.72 ± 0.11	3
		Control	> 3000	N/A	3
	ATP+	G4	183 ± 52	0.66 ± 0.10	3
		Control	> 3000	N/A	3

Table 1. K_d and Hill-coefficient values determined from Figure 2.

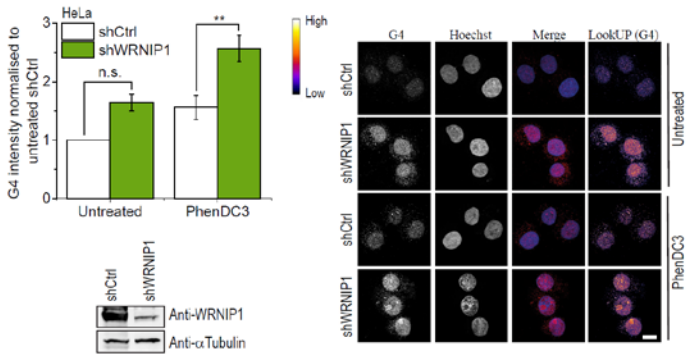

Figure 3. **WRNIP1 participates in the replication of G4-forming sequences.** Quantification of G4 intensity in shCtrl and shWRNIP1 carrying HeLa cells (See the representative images. Scale bars, 10 μm.). Where indicated, cells were treated with G4 ligand PhenDC3 (10 μM) for 1 hour. Cells were collected after 6 hours following PhenDC3-tratement. G4 intensity was measured in 100 nuclei per sample. The depletion of WRNIP1 was tested by Western blot analysis (bottom). Error bars represent SEM of 3 biological replicates on graph. p-values were obtained by ANOVA (Origin Pro) (*P<0.05, **P<0.01, and ***P<0.001).

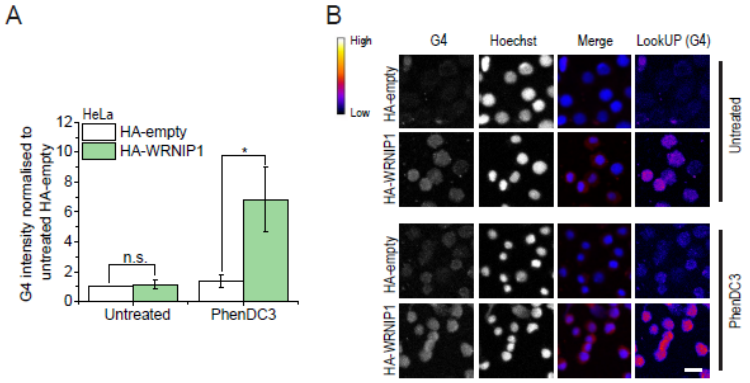

Figure 4. **Overexpression of WRNIP1 inhibits G4 resolution.** **A.** Quantification of G4 intensity in HA-empty and HA-WRNIP1 expressing HeLa cells. Where indicated, cells were treated with G4 ligand PhenDC3 (10 μM) for 1 hour. Cells were collected 6 hours after the treatment. G4 intensity was measured in 100 nuclei per sample. **B.** Representative images of untreated and PhenDC3 treated cell from (A). Scale bars, 20 μm. Error bars represent SEM of 3 biological replicates on graph. p-values were obtained by ANOVA (Origin Pro) (*P<0.05, **P<0.01, and ***P<0.001).

We concluded that WRNIP1 ha similar function during the G4 replication than the Mgs1.

We also found functional connection between WRNIP1 and Pif1 in humans, and we described one possible way, how the two protein acts together during the replication. We found that:

- Depletion of Pif1 suppresses the negative effect of WRNIP1 overexpression on G4 replication. (Figure 5)
- WRNIP1 regulates the targeting of Pif1 to the G4 structures (Figure 6)

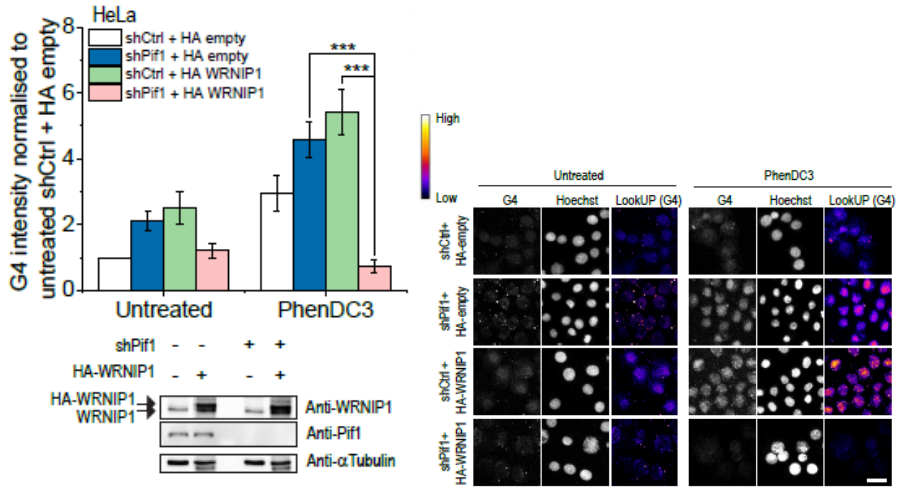

Figure 5. **WRNIP1 cooperates with Pif1 in replication of G4 forming regions.** Quantification of G4 intensity in shCtrl, shPif1, shCtrl/HA-WRNIP1 and shPif1/HA-WRNIP1 HeLa cells. (See representative images from Scale bars, 20 μm.) Where indicated, cells were treated with G4 ligand PhenDC3 (10 μM) for 1 hour. Cells were collected after 6 hours. G4 intensity was measured in 100 nuclei per sample. The depletion of Pif1 and expression of HA-WRNIP1 was tested by Western blot Error bars represent SEM of 3 biological replicates on graph. p-values were obtained by ANOVA (Origin Pro) (*P<0.05, **P<0.01, and ***P<0.001).

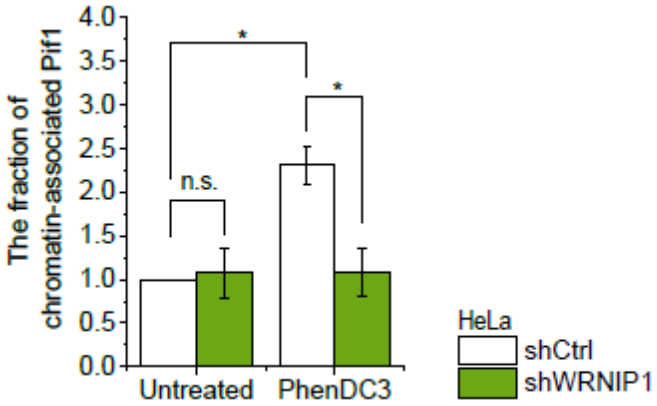

Figure 6. **WRNIP1 regulates the chromatin association of Pif1 onG4 forming regions.** Quantification of the chromatin fraction of WRNIP1 or Pif1 in shCtrl and shPif1 or WRNIP1 transfected HeLa cells. Where indicated, cells were treated with G4 ligand PhenDC3 (10 μM) for 1 hour and pre-extracted before immunostaining to get the chromatin associated fraction of WRNIP1 or Pif1. WRNIP1 or Pif1 intensities were measured in 100 nuclei per sample. Error bars represent SEM of 3 biological replicates on graph. p-values were obtained by ANOVA (Origin Pro) (*P<0.05, **P<0.01, and ***P<0.001).

We found that WRNIP1 and Mgs1 has opposing function during homologous recombination, because deletion of Mgs1 resulted in elevated rate of mitotic recombination (*13*), which means that Mgs1 inhibit HR events. Contrarily depletion of WRNP1 decreases the frequency of recombination. We found in this topic that:

- WRNIP1 depletion resulted in decreased frequency of HR (Figure6)
- WRNIP1 overexpression resulted in increased frequency of HR (Figure 6)
- Wrnip1 contributes in the end resection step of HR together with the BRCA1 and Pif1. (Figure 7)

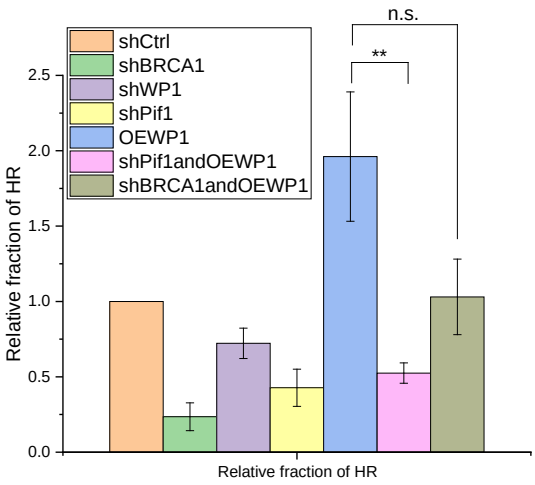

Figure 7. **Effec of WRNIP1 on Hr frequency.** Dr-GFP reporter cell line was used as indicated on the graph. GFP positive cells were measured in 10000 nuclei per sample. Error bars represent SEM of 3 biological replicates on graph. p-values were obtained by ANOVA (Origin Pro) (*P<0.05, **P<0.01, and ***P<0.001).

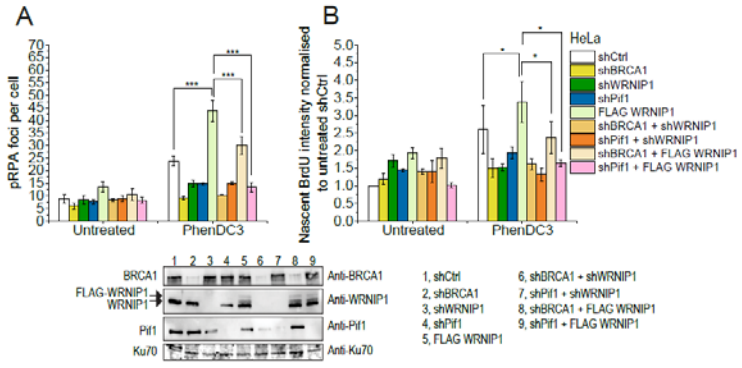

Figure 7. WRNIP1 promotes DNA-end resection at DNA breaks. A. pRPA foci number in shCtrl, shWRNIP1, shPif1, shBRCA, Flag-WRNIP1, shPif1/shWRNIP1, shBRCA/shWRNIP1, shBRCA1/Flag-WRNIP1 and shPif1/Flag-WRNIP1 HeLa cells. **B.** Quantification of nascent BrdU intensity in shCtrl, shWRNIP1, shPif1, shBRCA, Flag-WRNIP1, shPif1/shWRNIP1, shBRCA/shWRNIP1, shBRCA1/Flag-WRNIP1 and shPif1/Flag-WRNIP1 HeLa cells in the absence of PhenDC3 treatment. The downregulation of indicated genes and expression of FLAG-WRNIP1 were tested by Western blot analysis (bottom).

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