

Review

# Shedding Light on the Interaction Between Rif1 and Telomeres in Ovarian Cancer

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**ABSTRACT:** Ovarian cancer, more precisely high-grade serous ovarian cancer, is one of the most lethal age-independent gynecologic malignancies in women worldwide, regardless of age. There is mounting evidence that there is a link between telomeres and the RIF1 protein and the proliferation of cancer cells. Telomeres are hexameric (TTAGGG) tandem repeats at the tip of chromosomes that shorten as somatic cells divide, limiting cell proliferation and serving as an important barrier in preventing cancer. RIF1 (Replication Time Regulation Factor 1) plays, among other factors, an important role in the regulation of telomere length. Interestingly, RIF1 appears to influence the DNA double-strand break (DSB) repair pathway. However, detailed knowledge regarding the interplay between RIF1 and telomeres and their degree of engagement in epithelial ovarian cancer (EOC) is still elusive, despite the fact that such knowledge could be of relevance in clinical practice to find novel biomarkers. In this review, we provide an update of recent literature to elucidate the relation between telomere biology and the RIF1 protein during the development of ovarian cancer in women.

**Key words:** ovarian cancer, telomere, TERT, TERC, RIF1, cancerogenesis

## Introduction

Owing to its prevalence, neoplasia of the ovary is one of the most common gynecologic, age-independent pathologies in women worldwide [1]. More precisely, a large number of women suffer from epithelial ovarian cancer (EOC), which is estimated to have the lowest survival rate [2]. Although the search for new therapeutic strategies has yielded reliable results, the overall prognosis and survival rate for patients with EOC remains poor [3]. The survival rate of women with ovarian tumors depends, among other things, on the stage of the tumor (Fig. 1), i.e., the higher the stage, the lower the chance of survival. Therefore, elucidating the molecular mechanisms of EOC tumorigenesis and progression is a prerequisite for exploring new therapeutic targets and treating the occurrence of ovarian cancer in women. In

this context, telomeres and the RIF1 protein could be very important players. Telomeres are hexameric (TTAGGG) tandem repeats at the tip of chromosomes that shorten as somatic cells divide, limiting cell proliferation, and serving as an important barrier in preventing cancer [4]. Moreover, there is the shelterin complex at the telomeric region and the enzyme telomerase, which consists of two components, the RNA template (TERC), and the catalytic subunit, the telomerase reverse transcriptase (TERT) [5]. Among other species, the human TERT (hTERT) is responsible for telomerase activity, and therefore, for transcription, too. Interestingly, hTERT is upregulated in most ovarian tumors [6] and has been shown to be a central regulator of many cancer traits, like proliferation, survival, and characteristics of cancer stem cells [7-9]. Noteworthy, EOC stem cells do reflect these latter-

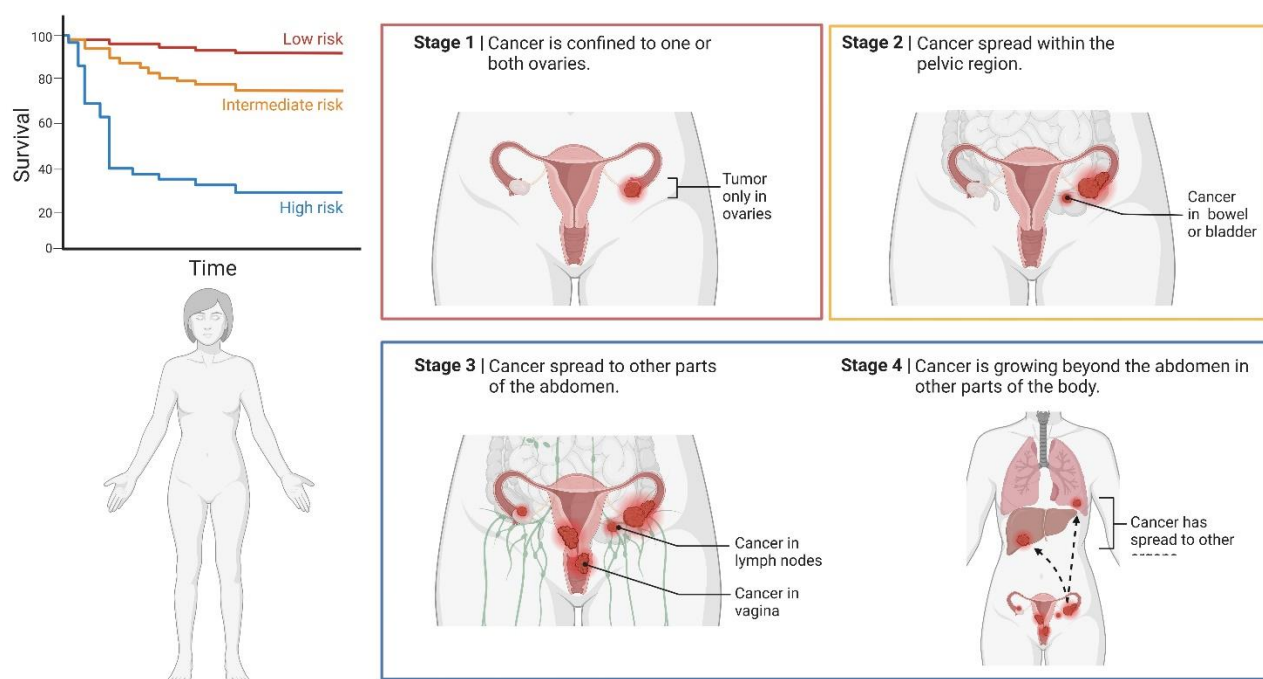
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mentioned properties, too [10-12]. Although hTERT appears to be a reliable biomarker and therapeutic target for EOCs, its relevance in neoplastic cells remains elusive. Numerous cancer types show typical maintenance of telomere length in their cells since among other mechanisms the catalytic subunit is responsible for adding the hexameric sequence [13]. It is interesting though that the Replication Time Regulation Factor 1 (RIF1) binds directly to the promoter for hTERT enabling its expression, and in consequence, it plays an essential role in the regulation of telomere length [14]. Besides, RIF1 impacts the selection of DNA double-strand break (DSB) repair pathway and the regulation of replication timing [15-18], and is highly expressed in mouse

embryonic stem cells, too [19-21]. Upregulation of RIF1 in breast cancer tissues has been reported and the knockdown of RIF1 reduced cell growth and increased susceptibility of uterine cervical cancer cells to cisplatin [22-23]. However, the specific role of RIF1 in EOC needs further elucidation.

In this review, we aim to shed light on the importance of the relationship between telomere biology and the RIF1 protein during cancerogenesis, especially during the development of ovarian cancer in women. Moreover, we intend to answer the question of whether TERT or RIF1 could be established as new biomarkers for the early detection of ovarian cancer regardless of the patient's age.



**Figure 1.** Scheme showing the different stages of ovarian cancer and their characteristics with regard to localisation and expansion in regard to their potential chance of survival.

### The relationship between *Rif1* and telomeres in ovarian cancer

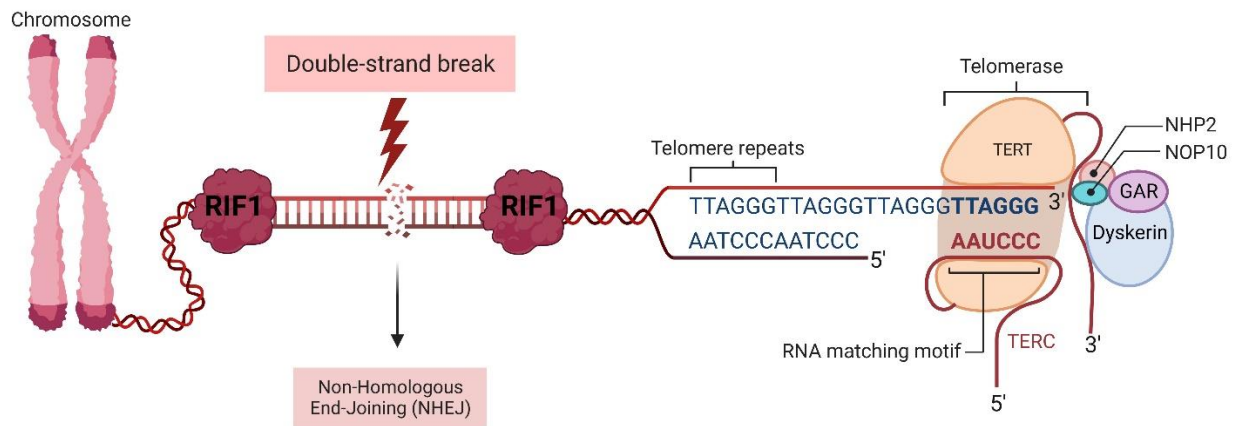
Telomere shortening reflects a so-called mitotic clock in somatic cells since by every cell division and with advancing age, the chromosome's tip gets shorter and therefore not only determines the life span of somatic cells but also serves as an intrinsic barrier for oncogenic transformation [24]. The latter-mentioned physiologic event is enabled due to specific pathways, such as the amplification of the gene encoding for TERT [25], the influence on transcriptional activators of TERT [26], and the cytosine methylation at CpG islands close to the TERT promoter [27]. Telomere length in specific cancer can be

maintained via a telomerase-independent pathway, meaning an alternative elongation of telomeres [28], based on homologous recombination [29]. As previously mentioned, an elevated expression of hTERT and therefore, its positive effect on telomere length is crucial for the process of tumour development. Previous investigations provided evidence that a plausible reason for this elevated transcription is mutations at the promoter of hTERT [30, 31]. In the study of Wu and co-workers [30] the before-mentioned mutations were described in 15.9% of patients suffering from clear cell carcinomas of the ovary. Worth mentioning that the mutation of the hTERT promoter seems not to be present at the beginning of oncogenesis and appears to be linked with the lack or

downregulation of ARID1A, a tumour suppressor gene, in ovarian clear cell carcinomas (OCC) [30]. Previous research provided evidence that the mutations are positioned at two loci [32,33]. Based on other studies and the study of Wu et al. [30] it appears that the pathogenetic mechanisms between OCC and ovarian endometrioid carcinoma are divergent. Other studies have also confirmed among others the mutation of the gene encoding for ARID1A mutation as a property during the early onset of OCC development but does also occur in endometriotic cysts [34,35]. Additionally, genome-wide analysis has also confirmed that ARID1A mutations have been detected in ovarian clear cell carcinomas [36,37]. According to the so-called telomere crisis theory, which is relevant for tumorigenesis, the accumulated telomere

attritions cause senescence and/or harmful genomic instability [38].

As a logical consequence, precancer cells aim to escape from the latter-mentioned telomere crisis to survive, and during the phase of telomere crisis, only those cells with adequate maintenance of telomere length will progress due to the selection pressure [30]. However, the data about TERT promoter mutations in the gynecological tract are rare [39,40]. Interestingly, it has been revealed that in patients diagnosed with OCC (FIGO stages I and II), mutation of the TERT promoter appeared to be an independent prognostic factor in combination with significantly shorter overall survival. Besides, in patients with recurrent OCC (early FIGO stage), mutation of the TERT promoter was significantly correlated with a relapse within six months [41].



**Figure 2. Scheme showing directed Rif1-DNA interactions required to prevent telomerase and inadvertent activation of checkpoints at chromosome ends.** In the event of a double strand break, the Rif1 seals the broken ends and opens access to tip resection machinery. As a result, the double-strand breaks ends are stabilized, promoting their retrocession by non-homologous end-joining (NHEJ).

### Relevance of RIF1 in cancerogenesis and different cancer types

As previously mentioned, understanding the mechanisms and regulators controlling cancerogenesis has become a priority for specialists worldwide. Given that Rif1 is involved in telomeric regulation (Fig. 2), the search for its role in tumorigenesis has begun. In one study, it was shown that as telomere length decreases, the amount of free Rif1 increases due to the loss of its specific association with Rap1 and thus association with telomere ends. The abnormally increased amount of free Rif1 promotes tumorigenesis due to its impact on genomic instability and rearrangement of the chromosomes [42]. Furthermore, Rif1 may act as an anti-checkpoint shield in repairing defective double-strand breaks (DSB) of DNA in breast cancer, and inhibition of its expression sensitizes cancer cells to drugs [42,43]. A direct mechanism in

which Rif1 promotes tumour growth has also been revealed. In human er EOC cells, there is a close interaction between Rif1 and hTERT. As it turns out, Rif1 binds directly to the promoter for hTERT allowing its expression, while Rif1 knockout inhibited ovarian tumour growth [42-44]. Furthermore, Rif1 knockout inhibits EOC cell migration and markers of the epithelial-mesenchymal transition leading to apoptosis and G2 cell cycle arrest of EOC cells [43]. Interestingly, also in EOC, Rif1 gene knockout sensitized its cells to drugs (cisplatin) and also platinum-based chemotherapy through inhibition of NER proteins in cancer cells [44]. A positive correlation between Rif1 and lung carcinoma cells was detected in another study. Importantly, a significant correlation was found between Rif1 and the regulators of proliferation signalling and maintenance of cancer stem cell characteristics, Wnt/ $\beta$ -catenin. As in previous studies, inhibition of Rif1 expression limited tumour growth,

while its overexpression promoted tumour growth through activation of the PP1-AXIN trail resulting in induction of Wnt/ $\beta$ -catenin pathways [45]. Research into the function of Rif1 in tumorigenesis has yielded new solutions and alternative treatment pathways for various tumour types. Solutions based on inhibition of the expression of this protein have proven beneficial in the negative regulation of tumour growth through the Rif1-hTERT or Rif1-PP1/AXIN- Wnt/ $\beta$ -catenin interaction pathways, and it is therefore believed that further research into Rif1 in tumorigenesis may provide many effective solutions for cancer therapy [45].

### Crosstalk between Rif1 and telomeres in other cells

Although Rif1 is conserved in cells from yeast to humans, the way it binds in organisms differs by interacting with different binding fractions. In budding yeast cells (*Sacharomyces Cervisiae* - scRif1), the connection of the Rif1 protein with the respective domain on its C-terminal is mediated by Rap1 [46]. A second Rif2 protein is attached to this complement, containing two binding sites on Rap1 which together form a complex involved in telomere end protection. As it turns out, the N-terminal domain of scRif1 allows direct attachment to telomere ends. This connection is made possible by the unique shape of the N-terminal domain which adopts the contour of a shepherd's stick [46,47]. Further studies have shown that this region forms a conserved HEAT domain that is also responsible for dimerisation of scRif1 structures [47]. While Rif1 in budding yeast (*Sacharomyces pombe* - spRif1) is also involved in telomere-end protection, its mode of binding is somewhat different. Although the rap1 protein has been shown to be present, the main binding protein for the connection between the telomere end and spRif1 is Taz1 [48]. In the methylotrophic yeast Rif1 (*Hansenula polymorpha* hpRif1), it has also been shown to bind to Rap1, but this is not the main mechanism recruiting this hpRif1. Firstly, the hpRap1 protein has two fractions, hpRap1A and hpRap1B of which the first has no binding sites on telomeres and is associated rather with subtelomeric regions while the latter is responsible for telomere binding to dsDNA [49]. Secondly, the same authors suggest that hpRif1 binding to the hpRap1B domain is mainly responsible for recombination and not telomere length [49]. Subsequent studies have shown that Rif1 interacts with the Cdc13 complex, which in turn obtains an association with Stn1 similar to that in *S. Cervisiae* [50]. Finally, it has been shown that the main hpRif1 binding fraction is the Ku80 heterodimer, which is able to bind telomere ends via Stn1 [51]. Although in mammals, including humans, the presence and ability of Rap1 protein to bind to telomere ends via the TRF1-TRF2 complex has been demonstrated, so its interaction with

hRif1 at this site has not been reported [46]. This is primarily related to the distinct function of hRif1, where it is involved in DNA damage repair mechanisms via crosstalk with 53BP1 by protecting non-homologous DNA ends [46]. Furthermore, in mouse Rif1 (muRif1) a compact structure can be formed which is able to bind several DNA G-quadruplexes (G-4 DNA) [52]. In turn, G-4DNA has been shown to be involved in binding to the telomere-binding factor (TRF) in humans [53], therefore, researchers point to a high role for G-4 DNA in telomere end protection [54]. The situation is different when it comes to the genome of cells of the genus *Drosophila*. The mechanism of telomere-end protection is based on the action of retrotransposons [55], and not telomerase as in most organisms known to date, hence binding complexes such as Rap1, Taz1 or TRF are not present in the telomere-end region. Although the presence of the binding factor Rif1 has been demonstrated, the knowledge of the main location of dRif1 is incompletely understood. Studies indicate that dRif1 is associated with the three most commonly described functions of this protein, namely control of replication time, being its main inhibitor through interaction with protein phosphatase and S-phase kinases [56,57]. Interestingly, studies have shown dRif1 does not localize to telomeres obtained from yeast while the hRif1 homologue is capable of this interaction, suggesting that it retains the ability to bind telomere ends [58]. The above data show that Rif1 orthologues exist from yeast to humans, but their localisation and binding factors are different. In addition, not all Rif1 orthologues retained the ability to bind telomeres which translates into their later functions, so understanding these orthologues turns out to be crucial for understanding the activity and localisation of this regulator.

### Rif1 functions in different species

Since the regulatory protein Rif1 was first discovered in budding yeast cells, interest in its function in relation to telomere end protection has increased significantly. Thanks to the binding factor scRif1, it is possible for it to bind at telomere ends and participate in telomere protection through the Rif1 recruitment factor – Rap1 [13]. The same function is attributed to spRif1 as well as hpRif1 but, as previously mentioned, binding takes place using other linking fractions [48,51]. A study using PAL cells, i.e. model yeast organisms lacking telomerase function and telomerase capacity, showed that the concentration of Rif1 at an appropriate, constant level enabled telomere ends to be protected from spontaneous senescence. Moreover, while Rif1 was overexpressed the initiation of cell ageing and also the accumulation of DNA DSBs occurred [42]. When one of the complexes required for telomere capping is lost, scRif1 takes over as the major

regulator of telomere function which only points to its critical role in telomere end protection [59,60]. However, despite its well-defined role in regulating telomere elongation, one study determining the effect of scRif1 telomere gene mutations on telomere origins firing showed that it was not associated with telomere length regulation suggesting that, despite origin gene mutations, the main function of scRif1 was still active [61]. In addition, much attention is focused on the degree of phosphorylation of the SCD domain of scRif1 since, according to the authors, it can positively as well as negatively regulate telomere length. One of the phosphorylation sites is Tel1, which is a homologue of the human ATM kinase. Synthetic telomeres lacking this kinase showed an increase in length, but there was no significant effect on chromosomal telomeres. The authors indicate that mutation of both the Tel1 fraction on the SCD domain and other telomeric DNA binding proteins could be used to study other functions of scRif1 in DNA repair and replication [62] was confirmed by one of the most recent studies analyzing mutation of the Rif1 gene and Tel1 simultaneously [63]. This situation is prevented due to its close crosstalk with protein phosphatase 1 (PP1), for the reason that by recruiting PP1 to scRif1, telomerase, in general, Tel1 effectively suppresses telomere end repeat elongation [64]. In addition to telomere end protection, Rif1 has been shown to be intimately involved in the process of non-homologous DNA end joining (NHEJ) taking part in DNA repair [47], which has also been demonstrated in mammalian cells where it is indicated that this is one of the main mechanisms regulated by hRif1, without showing a direct effect on telomere end protection [65]. The accumulation of scRif1 itself at DSB sites is made possible by the S-acylation of the N-terminal domain of scRif1 through the palmitoyl acyltransferase complex pfa4 [66]. In addition, scRif1 acts as a checkpoint inhibitor by inhibiting DNA damage response. This prevents mutation of certain DNA end-strand genes and excessive telomere shortening, which would lead to cell death [60,67]. Ortholog of Rif1 in another yeast species *Candida Glabrata* did not exhibit telomere-protective functions while it was involved in subtelomeric DNA silencing [68].

It is also worth focusing on the role of mammalian mRif1 because, as previously mentioned, it does not bind directly on telomeres preventing the positive or negative effects of restriction enzymes. Instead, a very interesting correlation between the ZSCAN4 gene of embryonic stem cells (ECs) and Rif1 was indicated. The latter-mentioned gene encodes for a specific protein that is responsible for the recombination-dependent telomere elongation mechanism and is required for normal, balanced cell growth [69]. In addition, the recombination-dependent telomere elongation mechanism through ZSCAN4 acts

autonomously from the other mechanism regulated by telomerase. This seems to be confirmed by a study conducted on mouse embryonic cells and human ALT tumour cells during telomerase-encoding gene knockout. It was shown that the protein which is mainly involved in the overriding function of telomere homeostasis was encoded by ZSCAN4 [70]. It was indicated that the hRif1 protein may be involved in limiting its overexpression, through a mechanism of subtelomeric silencing of ZSCAN4 thus leading to genomic stability of ECs [71]. The regulatory mechanism is based on a specific interaction at the promoter of the ZSCAN4 gene combining with histone H3K9 methyltransferases leading to subtelomeric silencing [21,71]. In the same study, knockout of hRif1 was performed in ECs cells leading to telomere hypercombination, elongation, and heterogeneity. Furthermore, knockout of both the gene encoding hRif1 and ZSCAN4 partially rescued defective embryogenesis by protecting telomere recombination phenotypes [21], thus indicating a key role for hRif1 in an indirect mechanism of telomere protection.

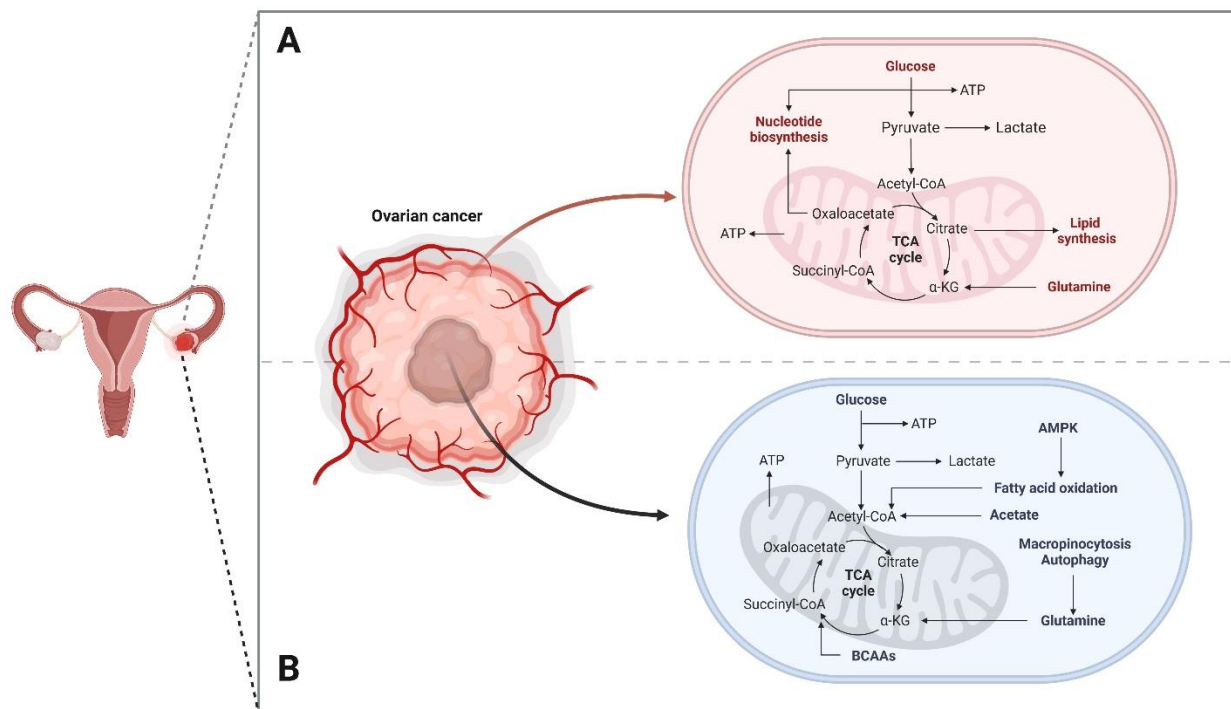
The above data report that Rif1 protein homologues in cells from yeast to humans play a very important role in maintaining genetic stability, being one of the critical regulators of telomere biology. However, the growing interest in this protein over the past few years has led scientists to search for further important functions of Rif1 given its highly conserved nature. Among others, it has been pointed out that it prevents too early activation of the onset of replication [72], DNA double-strand breaks repairs [73], or maintains genomic stability in mouse embryonic cells [74].

### Rif1 and tumor microenvironment in ovarian cancer

The cancer microenvironment is considered to be not only involving the transformation of tumor cells themselves, but also interactions between them and non-cancerous cells, as well as the conditions around the tumor cells. One of the most commonly mentioned facts in this regard is the poor oxygen availability in the tumor microenvironment leading to intense, disorganized angiogenesis within the tumor. This induction depends on a heterodimer formed of hypoxia-inducible factors HIF- $\alpha$  and HIF- $\beta$  which, under hypoxic conditions, migrate to the cell nucleus to induce VEGF and stimulate angiogenesis [75,76]. As a result of the developing hypoxia, cells reprogram the way they obtain energy from oxidative phosphorylation to anaerobic glycolysis. For this reason, there is an accumulation of lactic acid in the tumor microenvironment causing a drop in pH below 6.8. The acidified environment inhibits the response from the immune system and promotes malignancy and metastasis of tumor cells [77]. In addition, acidic pH influences

tumor metabolic remodeling, which in consequence impacts tumor cell growth due to the reorganization of metabolic pathways [78]. Interactions between tumor cells and other cell types have been reported [79], for instance, ovarian cancer cells which program the cellular targets of tumor stroma fibroblasts to maximize glutamine anabolism positively affecting tumor metabolism and infiltrative growth [80]. The lipid chaperone protein FABP4 of ovarian cancer cells enables growth, development and enhances metastasis in a lipid-rich environment, leading to intense proteo-metabolic changes manifested by intensification of lipid metabolism [81]. It seems that, in addition to intercellular interactions, the correlation between tumor cell organelles also exerts an influence on the corresponding adaptation relative to the tumor microenvironment. Thus, as a source of metabolism and energy acquisition, it is the mitochondria of tumor cells and their interactions mainly with the cell nucleus that enable adaptation to various conditions including oxidative or starvation stress [82,83]. The latter arises as a result of a nutrient-deprived condition thus activating modified energy acquisition pathways (Fig. 3), in addition to the natural derivation of pyruvate by

glycolysis, including through the activation of adenosine monophosphate-activated protein kinase (AMPK) as a result of a deficiency in intracellular glucose concentration [84], which are then involved in the production of acetyl-coenzyme A. Branched-chain amino acids (BCAAs) are also involved as a result of nutritional deficiencies, which, in addition to being incorporated into the tricarboxylic acid cycle (TCAC), also contribute to glutamine synthesis [80]. Correlations and information exchanges in the tumor microenvironment promote tumor aggression and infiltrative growth, but there are some pathways for cancerogenesis suppression induced by cellular stress. As critical regulators, it considers the p53 factor and p16ink4A which, upon DNA damage, telomere erosion, oncogene hyperactivation and inactivation of onco-suppressors, induce a program of cellular senescence and later cell necrosis to prevent neoplastic transformation (Fig. 4). On the other hand, despite the inhibition of proliferation of these cells, their metabolic activity is still active, and the released biomolecules can induce chronic inflammation and predispose to a pro-cancer microenvironment [85].



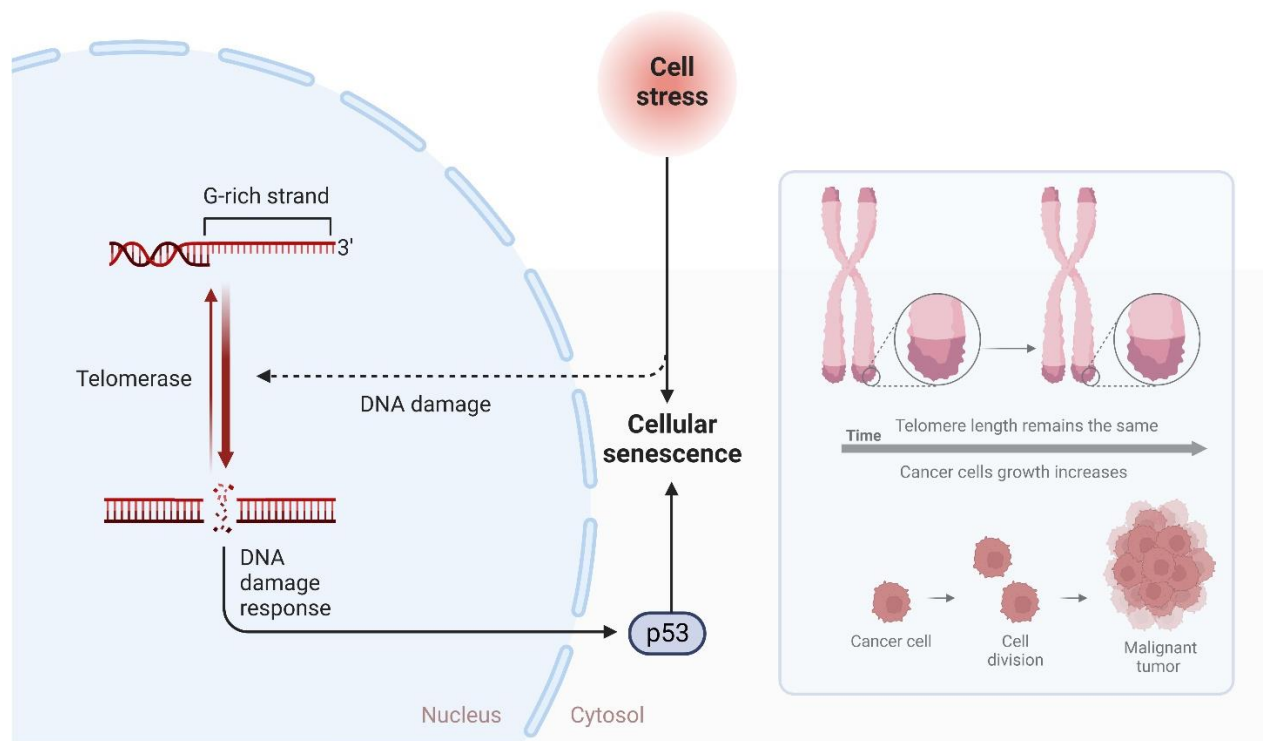
**Figure 3. Scheme showing the cancer cell metabolism in nutrient replete and nutrient deprived conditions. (A) Cancer cell metabolism in nutrient-replete conditions, (B) Cancer cell metabolism in nutrient-deprived conditions.**

The above data show that there are a number of endogenous factors that promote tumor cell aggression and proliferation. The tumor microenvironment, intercellular, and inter-organelle interactions induce

mechanisms of tumor survival and malignancy. Therefore, in the context of combating carcinogenesis, targeted therapy on the pathways of inhibition of the above interactions seems to be most beneficial which has

already been documented in many studies [86,87]. There are also defense pathways on the part of the organism, including through regulators of p53 and p16ink4A,

however, it needs to be determined under what conditions cellular senescence produces positive and under what conditions negative effects.



**Figure 4.** Scheme showing the pathway of cell stress inducing cellular senescence.

### Conclusions and potential clinical implications

In summary, there are many regulatory proteins that maintain genomic stability. However, some of them are critical for cell survival. Rif1 protein, as a highly conserved protein in cells from yeast to humans, unsurprisingly has very similar functions, but using different mechanisms to achieve its target. Nevertheless, it can be pointed out that Rif1 is an overarching regulator in the cells of many organisms, including humans, preventing the replicative senescence mechanism from occurring too quickly by excessively shortening telomeres. Thus, the telomeric function of the Rif1 protein has found application in cancer therapy with satisfactory results, but further research is needed to fully define the function of this protein in tumorigenesis mechanisms. It is also important to be aware of the other functions of Rif1 and of the series of correlations with various regulators that together maintain genomic stability in the cells of many organisms. For this reason, the Rif1 protein can be considered one of the main factors for cell survival not only humans. In summary, it can be recommended that ovarian cancer patients should be diagnosed for TERT promoter mutations and should be additionally under

adequate follow-up care in the months after chemotherapy. With regards to ovarian clear cell carcinomas, the screening for mutations in the genes of ARID1A, PIK3CA, and ZNF217, can be judged as an inadequate prognostic marker [88-91]. However, the detection of mutation in the TERT promoter in early FIGO stages of ovarian cancer patients appears to be a reliable prognostic marker. Importantly though, regular screening for TERT promoter mutations could be used to develop novel therapeutic strategies, to avoid the development of chemoresistance [41]. Nevertheless, there are very limited data about the impact of these observations in the specific subtypes of ovarian cancer, including low and high-grade tumors and the specific histological type such as clear cell cancers. Further investigations should also focus on the impact of Rif1 and telomeres on the survival rate and specific ovarian cancer subtypes and different FIGO stages since these are still research gaps that require further elucidation. As it has been shown in a recent study, short-time suppression of TERT reduces cell growth [31]. Based on this finding new antineoplastic compounds could be established which temporarily inhibit TERT. All in all, there is no doubt that both RIF1 and telomeres do play a critical role during the

pathogenesis of ovarian cancer in women regardless of age, therefore, they can be used in clinical practice as potential diagnostic and prognostic biomarkers. Our review aimed to shed light on the importance of the latter-mentioned factors, and due to the limited number of studies on the crosstalk and its relevance for ovarian cancer patients, we aimed to stimulate researchers to perform new experiments on this interesting topic.

### Competing interests

The authors declare no competing interests.

### Authors' contributions

S.G. writing of the article; P.K. conceptual idea, visualization, writing, and funding acquisition; S.M. and J.S. revised the manuscript.

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