Experimental Hematology

Experimental Hematology 2020;90:12–17

INVITED REVIEW

Role of Rap1 in DNA damage response: implications in stem cell homeostasis and cancer

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(Received 7 May 2020; revised 17 August 2020; accepted 21 August 2020)

Mammalian Rap1 is a part of the telomere binding complex named shelterin and is one of the most conserved telomeric proteins. With its essential requirement in lower species to its becoming necessary in higher species, it appears to have gained and lost several functions simultaneously evolving with telomeres. Mammalian Rap1 has been reported to play a role in inflammation, metabolism, and oxidative stress. Mammalian Rap1 has also been found to regulate DNA damage response from telomeres in senescent cells. Recently our group uncovered its novel role in stem cell maintenance, and modulation of the chemotherapeutic response. Mechanistically it was found to function as an adaptor via protein–protein interactions and to modulate the response to DNA damage. In the current review we highlight newly identified functions of Rap1 in regulating telomeric and general DNA damage response with its impact at the cellular and organismal levels. **© 2020 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.**

Telomeres are the ends of linear chromosomes composed of tandem TTAGGG repeats in mammals, including humans. With every cell division, the telomere shortens because of the inability of canonical DNA replication to duplicate the ends of linear DNA. To overcome this end replication problem, eukaryotes employ a special enzyme named telomerase, which utilizes its reverse transcriptase activity to lengthen the telomeres [1].

Structurally, telomeres appear as double-strand breaks and can potentially activate DNA damage signaling and DNA repair reactions. The major enzymes involved in this DNA damage signaling include ataxia telangiectasia-mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) kinases, DNA-dependent protein kinases (DNA-PKs), and poly(ADP-ribose) polymerase 1 (PARP1) [2–5]. The DNA repair reactions involve three pathways, namely, classic nonhomologous end joining (c-NHEJ), alternative (alt)-NHEJ, and homology-directed repair (HDR) [6]. To overcome this, telomeres fold into special conformations referred to as t-loops [7]. However, activation of these signaling pathways and repair cascade can result in cell cycle arrest, telomere fusions, t-loop cleavage, telomere sister chromatid exchanges (T-SCE), hyper-resection at telomeres, or telomere loss subsequently resulting in genome instability [8]. This is also referred to as an end protection problem encountered by telomeres. To avert this situation and maintain genome homeostasis, telomeres are associated with multiple copies of a six-membered complex named shelterin. Individual components of shelterin function in managing different aspects of these DNA damage signaling and repair reactions. Shelterin components have also been found to regulate telomere length. The shelterin complex comprises telomeric repeat-binding factor 1 (TRF1), telomeric repeat-binding factor 2 (TRF2), protection of telomeres 1 (POT1), TRF1-interacting nuclear protein 2 (TIN2), TIN2- and POT1-organizing protein (TPP1), and repressor-activator protein 1 (Rap1).

Human Rap1 was identified as an orthologue of yeast telomere-binding protein, namely, scRap1p [9].

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https://doi.org/10.1016/j.exphem.2020.08.009

The major difference between them is that yeast RAP1 can directly associate with telomeric DNA; however, mammalian Rap1 is recruited to telomeres via TRF2. While deletion of all shelterin components has been found to be embryonic lethal in mouse, *Rap1* is an exception [6]. Deletion of *Rap1* in the mouse has no effect on development and normal functioning of the mouse [10]. Additionally, in human cell lines, *Rap1* deletion has no apparent phenotype of telomere dysfunction [11].

Interestingly, Rap1 is an active player in regulating the DNA damage response under specific conditions, and we discuss this function of Rap1 in the light of already known data with recent findings. We have organized the review into four sections. In the first section we describe the structure of human Rap1 protein as it is important to understand and connect functions of Rap1 with its structural domains. In the second and third sections, we present the current understanding of the role of Rap1 in the DNA damage response at telomeres and away from telomeres, respectively. In the last section, we state our conclusions and future perspectives.

Structure of Rap1 protein

Human Rap1 is encoded by a gene named *TERF2IP* and was identified with a yeast two-hybrid screen of Hela cells (human cervical cancer cell line) cDNA using TRF2 as a bait protein [9]. Rap1 is a 399–amino acid–long protein with a molecular weight of about 47 kDa. Rap1 protein possesses three major domains: a breast cancer susceptibility protein that appears on its C-terminus (BRCT) in the N-terminus, one central Myb motif, and a Rap1-specific protein-interaction domain at the C-terminus (RCT). Between the Myb and RCT domains lies an acidic region also called the coil–coil region whose functional significance is unclear and is more specific to humans [9]. Figure 1 is a schematic of the structure of the Rap1 protein.

The BRCT domain is normally present in various DNA damage response proteins with function in cell cycle checkpoint; in general, it interacts with phosphorylated peptides [12,13]. Deletion of this domain from human Rap1 has been reported to reduce telomere length heterogeneity [14]. Each Rap1 molecule associates with each TRF2 molecule and, in a shelterin complex such as TRF2, is present as a dimer, two molecules of Rap1 in tandem. By use of a small-angle X-ray scattering technique, it has been observed that two Rap1 BRCT domains coming from two molecules of Rap1 are closely packed in the TRF2–Rap1 complex [15]. Thus, some phospho-proteins are expected to interact with this structurally tandem BRCT domains. However, its exact function is unclear and interacting proteins have not been reported.

Figure 1. Schematic of the structure of the human Rap1 protein. The domains of Rap1 with amino acid (aa) positions are indicated: breast cancer susceptibility protein that appears on the C-terminus (BRCT), Myb motif, acidic coil–coil domain, and Rap1-specific protein-interaction domain (RCT).

The canonical Myb domain is a DNA binding motif with three alpha helices arranged in an orthogonal bundle [16]. Rap1 possesses one Myb domain which has been shown to lack DNA binding activity due to lack of significant positively charged residues on its surface. It could possibly interact with DNA with the help of another protein which can provide this positive charge or it is presumed to function in protein-protein interactions [17]. However, its binding partners have not been reported.

At C-terminus Rap1 contains RCT domain, which represents a well-known protein–protein interacting domain. Rap1 interacts with TRF2 through this domain, which thus tethers it to the telomeres [18].

With several interaction domains, Rap1 appears to be functioning more as an adaptor introducing various factors into functional complexes at or away from telomeres [19,20]. The first mass spectrometry (MS) analysis to determine the interacting partners of human Rap1 was performed by O'Connor et al. [21], who reported that Rap1 could associate with various DNA damage response proteins. Interestingly, they found that Rap1 could associate with Rad50/Mre-11 and Ku86 independent of TRF2, while PARP1 interaction with Rap1 required TRF2. The second MS analysis of human Rap1 was reported by our group, who also observed that Rap1 could associate with DNA damage response proteins and found that some of the associations of Rap1 with these proteins were independent of the TRF2 binding requirement [22]. These observations also suggest that Rap1 has a role in the general DNA damage response.

Rap1 in modulation of telomeric DNA damage response

Rap1 is recruited to telomeres via TRF2, and as TRF2 has been found to have an essential role in repressing ATM signaling and the NHEJ pathway, it was initially thought that Rap1 might also contribute to this activity. However various studies have found that Rap1 makes no contribution in repressing NHEJ at telomeres or inhibiting ATM kinase signaling. The first such evidence was provided by studies in mouse embryonic fibroblasts (MEFs) with *TRF2* or *Rap1* deletion (–/–). *TRF2*–/– MEFs exhibited accumulation of proteins such as 53BP1, MDC1, and γ-H2AX at chromosomal

ends (referred to as telomere dysfunction-induced foci [TIF]), activation of ATM pathway, and telomeric fusions. However, *Rap1*−/− MEFs did not exhibit any such TIFs or activation of ATM kinase signaling [10]. Furthermore, when the TRF2 mutant (which cannot interact with Rap1) was introduced into *Rap1*−/− MEFs and *TRF2*−/− MEFs, no telomere fusions or ATM kinase activation was observed, suggesting that TRF2 alone was necessary and sufficient to repress the NHEJ pathway and ATM kinase activity at telomeres. This is further supported by the finding that *Rap1*−/− mice are viable and fertile and display no lethal telomere dysfunction.

Martinez et al. [23] reported a protective role for Rap1 on mouse telomeres in *Terc*−/− mice. This was an interesting observation as it revealed that simultaneous deletion of *Terc* (which causes telomere shortening) and Rap1 led to telomere deprotection as reflected by increased multiple telomere signals, T-SCEs, and end-to-end chromosome fusions. The authors suggested that in studies where loss of Rap1 did not reveal any telomere deprotection, the reason could be the absence of critically short telomeres. In agreement with this finding, recently Lototska et al. [24] reported that human Rap1 specifically prevents DNA damage by inhibiting the NHEJ pathway at critically short telomeres such as those found in senescent cells and cancer cells. Mechanistically, they described that Rap1 prevents accumulation of 53BP1 at telomeres and does not let the telomeres fuse. However, the role of TRF2 in this function of Rap1 requires further clarification.

Additionally, in vivo studies in *Rap1*−/− mice have suggested that Rap1 is critical for repressing HDR at telomeres [10]. HDR at telomeres can result in T-SCEs, which if unequal could result in telomeric loss and, thus, could be deleterious to cells. It has been reported that loss of Rap1 in the absence of Ku70/80 (because it represses HDR at telomeres as well as at the genomic DSBs) in mice resulted in HDR activation at telomeres, leading to telomere recombination, and almost 10% of chromosomal ends had T-SCEs in metaphase MEFs [10]. Molecularly it was observed that Rap1, along with TRF2, represses PARP1 and SLX4 association with telomeres, which is the major factor contributing to HDR at telomeres [25]. Their accumulation at telomeres in the absence of Rap1 and TRF2 resulted in rapid telomere resection and generation of telomere-free chromosomal fusions. The Myb domain of Rap1 has been found to be important in this activity. A more detailed understanding of this molecular mechanism is particularly important because alternative lengthening of telomeres (which is observed in almost 15% of cancers) utilizes HDR for telomere maintenance, and it would be interesting to investigate how Rap1 activity is regulated in this situation [26].

Rap1 in modulation of nontelomeric DNA damage response

Interaction of Rap1 with various DNA damage response proteins in the absence of TRF2 (as discussed under Structure of the Rap1 protein) suggests that it might have a role in regulating the nontelomeric DNA damage response. Recently, our group reported a novel function of Rap1 in regulating the survival of hematopoietic stem cells (HSCs), as well as in affecting oncogenesis and chemotherapeutic response [22]. Rap1 was found to serve as an adaptor in the NHEJ, pathway and this activity did not require its association with TRF2, suggesting that it was nontelomeric.

The hematopoietic system is essential to the formation of blood throughout life. Hematopoietic stem and early progenitor cells (HSPCs) divide to produce lineage-committed progenitors, which further differentiate into mature blood cells of various types [27]. The highly proliferative nature of the hematopoietic system makes it susceptible to DNA damage signals, and therefore, the DNA damage response becomes critical in regulating the survival and function of hematopoietic cells. Exposure of the body to irradiation and DNA-damaging chemotherapeutic drugs has been implicated in the development of bone marrow failure, myelodysplastic syndrome, and therapy-induced leukemia [28,29]. Irradiation and chemotherapeutic drugs generally result in DSBs that can be repaired via either the NHEJ or the HDR pathway [30]. These pathways are very different in terms of the regulation, outcome, and molecular mechanisms. The HDR pathway is homology dependent; thus, it is error free and is mostly active in the S phase of the cell cycle. The NHEJ pathway is not dependent on homology; thus, it is considered error prone and is active throughout the cell cycle. During steady state, HSPCs are mostly quiescent and thus depend on the NHEJ pathway for repair of any DNA damage that occurs while the downstream progenitors are mostly actively dividing, and they utilize the HDR pathway to repair any DNA damage encountered [31]. Consequently, HSPCs have been found to be more prone to exogenous DNA damage resulting in bone marrow failure and leukemia. Thus, an understanding of the underlying factors regulating the DNA damage response in HSPCs becomes important.

In our study, we observed that under steady-state conditions, loss of Rap1 had no significant effect on hematopoietic cell populations in spleen or bone marrow in mice. However, on irradiation at lethal dose or treatment with the DNA-damaging drug 5-fluorouracil, survival of Rap1−/− mice was significantly reduced compared with that of wild-type mice. At a sublethal dose of irradiation, it was observed that HSPCs in Rap1−/− mice were not able to proliferate and repopulate, resulting in bone marrow failure when compared

with the wild-type mice. HSPCs from Rap1−/− mice exhibited increased levels of the DNA damage marker γH2AX, which was persistent over 2 weeks, suggesting loss of Rap1 results in genomic instability. This further implied that because of loss of Rap1, HSPCs were unable to repair the DNA damage. Whether the same phenotype is observed in other stem cells in Rap1−/− mice requires further investigation. Furthermore, using cancer cell line-based studies, we observed that depletion of Rap1 led to increased DNA damage on treatment with chemotherapeutic drugs that caused DNA DSBs. The DNA damage-induced foci did not overlap with telomeric signal, suggesting a telomere-independent role for Rap1 in mediating the DNA damage response. We, however, were not able to detect the presence of Rap1 directly at DNA damage foci.

As the NHEJ pathway is the major repair pathway in HSPCs, we investigated the role of Rap1 in the NHEJ pathway. Using a cell-based double-vector system [32], we found that reducing Rap1 levels resulted in reduced NHEJ pathway efficiency in human cancer cells. As the NHEJ pathway is also physiologically relevant in B-cell class switching, we investigated the role of Rap1 in this process and observed that Rap1−/− B cells exhibited significantly reduced IgG1 class switching.

Genome instability is one of the hallmarks of cancer, and NHEJ pathway proteins play an important role in maintaining genome stability [33,34]. One of the outcomes of NHEJ pathway activity is chromosomal translocations, which also are a characteristic feature of hematological malignancies and are also known as initiating events in the process of transformation [28,35]. Mutations in these proteins are known to result in increased susceptibility to cancer. For example, Ku70-deleted mice have a higher incidence of thymic lymphomas [36]. Similarly, in mice with an SCID mutant background, deletion of DNA-PKs along with p53 resulted in a rapid onset of lymphomas and leukemias [37]. Similar to this, we also observed that deletion of Rap1 resulted in increased susceptibility of mice to cancer when crossed with cancer mouse models such as Eμ-Myc-induced lymphomagenesis and PyMT-driven breast cancer. Interestingly, we also observed that loss of *Rap1* and reduction in Rap1 levels sensitized the mouse and human cancer cells, respectively, to various chemotherapeutic agents. Similar observations in human cancer cells have been reported previously [38]. Rap1 levels have been found to increase in response to DNA-damaging agents, supporting our finding on the role of Rap1 as an adaptor in DNA repair [39]. Recently it was reported that depletion of Rap1 led to more DNA damage upon irradiation in colorectal cancer cells, and higher Rap1 levels are associated with a poor prognosis in colorectal cancers [40]. It has also been reported that mutations in *Rap1* are associated with familial melanoma [41]. Mutations in *Rap1* have also been found to be present in various other cancer types; however, their functional significance needs to be investigated. As the NHEJ pathway is important in hematological malignancies such as leukemia, investigating the functional significance of Rap1 in human leukemia might be insightful. It would also be interesting to investigate the susceptibility of *Rap1*−/− tumors to chemotherapeutic agents and how mutations in Rap1 affect its function and promote cancer.

At the molecular level, the NHEJ pathway minimally requires seven core proteins for its activity [42]. DNA DSBs are recognized and bound by Ku70 and Ku86 proteins, which then recruit the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) followed by Artemis, which performs end processing. Lastly, X-ray cross-complementing 4 (XRCC4), XRCC4-like

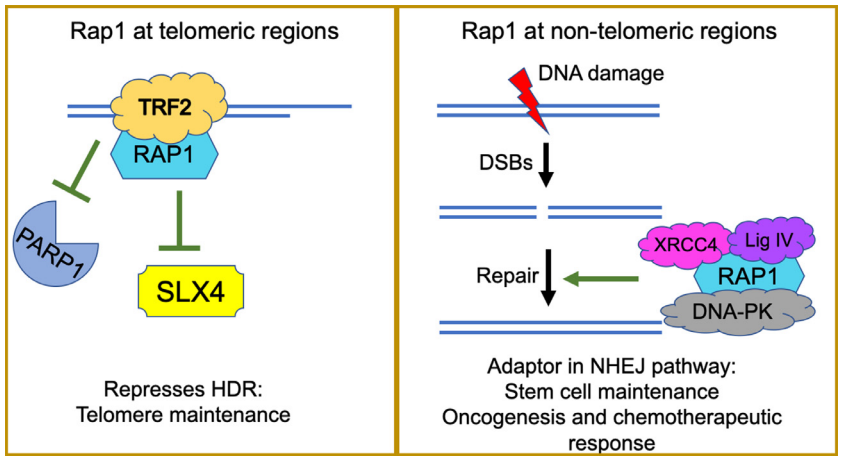

Figure 2. Role of Rap1 in DNA damage pathways at telomeric and nontelomeric regions. At telomeric regions, Rap1 associates with TRF2 protein. TRF2 and Rap1 inhibit PARp1 and SLX4, respectively, thus repressing homology-directed repair (HDR). At nontelomeric regions, DNA damage results in the formation of double-strand breaks (DSBs). This activates the nonhomologous end joining pathway (NHEJ), where Rap1 acts as an adaptor promoting association of XRCC4/ligase IV with DNA-PK, thus increasing its efficiency.

factor (XLF), and the DNA ligase IV (LIG IV) complex are recruited for ligation of DSBs. We observed that Rap1 serves as an adaptor in the NHEJ pathway by mediating the interaction between XRCC4/LIG IV and DNA-PKcs, which is followed by their reduced association with chromatin and, thus, impact on DNA repair. It would be interesting to further study the molecular details of this novel adaptor role of Rap1 in DNA repair. In vitro electron microscopy studies by Arat and Griffith [43] suggested that mammalian Rap1 can bind to DNA directly. They further observed that Rap1 associates directly with DNA in sequence-independent mode and has higher affinity for double-strand/single-strand DNA junctions. They proposed that this DNA binding activity of Rap1 might be via its BRCT domain and might be important for its extra-telomeric role. However, in vivo studies have not been able to find this direct DNA binding activity of Rap1 yet. Thus, further efforts are required to investigate this DNA binding activity in the light of our findings on its role in DNA repair at genomic regions. As Rap1 has also been found to regulate transcription at extratelomeric regions, it also might be interesting to investigate its role in regulating the DNA damage response via gene expression regulation [19].

Figure 2 summarizes role of Rap1 in the DNA damage response at telomeric and nontelomeric regions.

Conclusions and future directions

Mammalian Rap1 has been investigated mostly in the context of the shelterin component with function at telomeres. However, in the past decade, several studies have revealed its role as an adaptor in functional complexes formed under various physiological conditions. Its role in the DNA damage response at nongenomic regions is particularly important and interesting. DNA damage also controls stem cell survival, and Rap1 expression under those conditions becomes particularly important in maintaining their homeostasis. The molecular mechanisms underlying Rap1 regulation of various aspects of the DNA damage response and regulation of stem cell survival after induction of DNA damage merit further investigation. Additionally, the functional significance of genetic mutations in *Rap1* in humans and how it leads to cancer susceptibility should be explored.

Acknowledgments

EK is supported by research grants from the Department of Biotechnology (No. BT/RLF/Re-entry/06/2015) and Department of Science and Technology (No. ECR/2018/002117) and by an NMIMS Seed Grant (No. IO 401405).

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