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Role of SIRT1 in homologous recombination

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ABSTRACT

The class III histone deacetylase (HDAC) SIRT1 plays a role in the metabolism, aging, and carcinogenesis of organisms and regulates senescence and apoptosis in cells. Recent reports revealed that SIRT1 also deacetylates several DNA double-strand break (DSB) repair proteins. However, its exact functions in DNA repair remained elusive. Using nuclear foci analysis and fluorescence-based, chromosomal DSB repair reporter, we find that SIRT1 activity promotes homologous recombination (HR) in human cells. Importantly, this effect is unrelated to functions of poly(ADP-ribose) polymerase 1 (PARP1), another NAD(+)-catabolic protein, and does not correlate with cell cycle changes or apoptosis. Interestingly, we demonstrate that inactivation of Rad51 does not eliminate the effect of SIRT1 on HR. By epistasis-like analysis through knockdown and use of mutant cells of distinct SIRT1 target proteins, we show that the non-homologous end joining (NHEJ) factor Ku70 as well as the Nijmegen Breakage Syndrome protein (nibrin) are not needed for this SIRT1-mediated effect, even though a partial contribution of nibrin cannot be excluded. Strikingly however, the Werner helicase (WRN), which in its mutated form causes premature aging and cancer and which was linked to the Rad51-independent single-strand annealing (SSA) DSB repair pathway, is required for SIRT1-mediated HR. These results provide first evidence that links SIRT1's functions to HR with possible implications for genomic stability during aging and tumorigenesis.
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1. Introduction

Sirtuins, also referred to as class III HDACs, are a class of NAD^+-dependent protein deacetylases which are found in organisms ranging from bacteria to humans [1]. Sirtuins are involved in such diverse biological processes as metabolism, inflammation, rDNA transcription, insulin secretion, senescence, apoptosis, and DNA repair. In mammalian cells, there are seven homologues (SIRT1-7), of which SIRT1 is the closest counterpart of the prototype Sir2 from budding yeast. Sir2 and SIRT1 have met with growing interest, after it had been discovered that Sir2 promotes longevity and DNA stability and that these functions can be manipulated by small molecule pharmacological compounds [2,3].

Accumulating evidence has connected SIRT1 to the stress response. First, DNA damage by etoposide treatment causes up-regulation of *SIRT1* at the transcriptional level [4]. Second, SIRT1-depleted cells are highly sensitive towards etoposide and ionizing radiation (IR) treatment [4,5]. Remarkably, SIRT1 deacetylates and negatively regulates p53 and Forkhead transcription factors, which is thought to represent the mechanism underlying SIRT1-mediated reduction of apoptosis in response to various types of stress ranging from nutritional depletion to oxidative and genotoxic stress [6–8]. A genome stabilizing function was recognized already in the early work on Sir2 [3], so that subsequent studies have also searched for potential links between Sir2/SIRT1 and DNA repair. Indeed suggesting an involvement in DNA repair, Sir2 and SIRT1 were shown to be recruited to the chromatin upon different types of DNA damage in a manner depending on the signaling kinase ATM [9,10]. Given that Sir2 and SIRT1 modify the chromatin and silence transcription, the possibility was considered that genomic instability is suppressed by chromatin changes affecting the accessibility of the damaged sites for repair enzymes [10,11]. More recently, evidence for a more direct involvement of SIRT1 in DSB repair came from reports demonstrating deacetylation of the DSB repair and recombination proteins Ku70, nibrin, and WRN [5,12,13]. However, conflicting results were obtained regarding the

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role of Sir2/SIRT1 in these repair processes. Thus, Howitz et al. [3] observed that Sir2 suppresses recombination at ribosomal DNA (rDNA) repeats, whereas Oberdoerffer et al. [10] found that SIRT1 is required for efficient repair of DSBs by HR.

Independently of the underlying mechanism, it is clear that SIRT1 deficiency leads to the formation of a variety of chromosomal aberrations and aneuploidies [10,14]. This genome stabilizing function is compatible with SIRT1's role in protecting against aging [11]. However, SIRT1's role in carcinogenesis appears to be more complex. On the one hand, SIRT1 was found to be up-regulated in various cancer cells, suggesting a role as a tumor promoter, which is consistent with its anti-apoptotic activities via p53 and FOXO transcription factors in response to stress [15]. On the other hand, a recent study revealed decreased SIRT1 levels in breast cancer associated with *BRCA1* mutations, which indicates a role as a tumor suppressor. This anti-cancerogenic role could be explained by SIRT1-mediated suppression of the expression of the anti-apoptotic gene *survivin* and is also compatible with SIRT1's genome stabilizing functions [16]. Interestingly, *SIRT1* turned out to be transcriptionally up-regulated by BRCA1, which is best known for its central role as a surveillance factor in DSB repair [17]. Importantly, as a consequence of this dual role of SIRT1 in stressed versus non-stressed cells, SIRT1 may represent a target for selective killing of cancer versus non-cancer cells [8], and a recent drug screening approach has led to the identification of a potent SIRT1/2 inhibitory substance with potential use in cancer therapy [18].

To gain a deeper understanding of SIRT1's functions in stabilizing the integrity of the human genome, we analyzed the role of SIRT1 in the repair of DSBs in the light of the different mechanisms and molecular relationships proposed. Through examination of different DSB repair factors downstream of SIRT1, we found that WRN, a helicase of the RecQ family, which is mutated in patients with Werner syndrome (WS), serves as a downstream mediator of SIRT1 function in regulating HR [19].

2. Materials and methods

2.1. Cells and cultivation

KMV clones derived from the human myeloid leukemia cell line K562 with stably integrated HR-EGFP/3′EGFP substrate (HR/3′) or stably integrated Δ-EGFP/3′EGFP substrate (Δ/3′) were cultivated in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine (Biochrom, Berlin, Germany) [20]. Lymphoblastoid cell lines AG03829 and AG07896 from WS patients [21] and the control lymphoblastoid cell line GM02253F [22] were cultivated in RPMI 1640 supplemented with 15% FCS, 3 mM L-glutamine. SaOS2 cells from a human primary osteogenic sarcoma cell line were cultivated in McCoy's Medium with 10% FCS and 2 mM L-glutamine. The cell cultures used in this work were free from mycoplasma contamination.

2.2. Cell cycle and apoptosis

For the analysis of cell cycle phases and apoptosis, cells were treated exactly under the conditions of the DSB repair assay and fixed at the time point of measurement. After propidium iodide staining cell cycle and apoptosis profiles were obtained by FACS analysis [23].

2.3. Recombination assay, drug treatment, and knockdown

To analyze HR, we used the established fluorescence-based test system described in Akyüz et al. [20]. The DSB repair plasmids utilized in this study were HR-EGFP/3′EGFP (HR/3′) and Δ-EGFP/3′EGFP (Δ/3′) stably integrated into the genome of KMV clones (Supplemental Fig. S1). To measure DSB repair, these cells were electroporated with pCMV-I-SceI together with pBS (control vector) or wild-type (wt) *EGFP* plasmid (for electroporation efficiency) to a total amount of 60–90 μg of plasmid DNA. When using lymphoblastoid cell lines for SSA measurements, the cells were electroporated with a mixture containing pCMV-I-SceI, substrate 5′EGFP/HR-EGFP, and pBS (control vector) or wt*EGFP* plasmid (for electroporation efficiency). Four hours after electroporation either nicotinamide (NM, Sigma, St. Louis, USA) or H_2O, NU1025 (8-hydroxy-2-methylqinazoline-4-one, Alexis Biochemicals, San Diego, USA), trichostatin A (TSA, Sigma), or DMSO were added to the medium as indicated. Cells were further cultivated for 20–24 h and, then, subjected to flow cytometry by use of a FACSCalibur FACSscan (Becton and Dickinson, Heidelberg, Germany) as described in Akyüz et al. [20]. To correct the calculation for electroporation efficiencies, the amount of green fluorescent cells in repair assays (with pBS) was individually normalized by use of the correspondingly determined electroporation efficiency (with wt*EGFP* plasmid). The statistical significance of differences was determined using the Student's *t*-test for unpaired samples (not significant: $p > 0.05$). To analyze DSB repair after caspase inhibition, cells were electroporated with pCMV-I-SceI and pBS or wt*EGFP* plasmid and grown in zVAD-fmk (50 μM) or DMSO containing medium.

In SIRT1 knockdown experiments 30 μg of pSuper, pSuper-SIRT1-S2, or pSuper-SIRT1-S3 plasmid was added to the electroporation mixture described above. After cultivating the cells for 24 h or the indicated times, FACS analysis was performed. We cloned the plasmids pSuper-SIRT1-S2 and pSuper-SIRT1-S3 for the synthesis of short hairpin RNAs (shRNAs) as described in [24], choosing the SIRT1 mRNA target sequences 5′-CCTTTGCCTCATCTGCATT-3′ (S2) and 5′-GGAGCAGATTAGTAGGCGG-3′ (S3), respectively. To down-regulate the expression of potential target proteins of SIRT1 during the repair measurements, we pre-electroporated KMV(HR-EGFP/3′EGFP) cells with 60 μg of the respective knockdown plasmid each: pSuper, pSuper-Ku70, or pSuper-WRN [23]; pRS-control or pRSNBS1-4/6 (Origene, Rockville, USA). The cells were cultivated for 48 h and, then, re-electroporated with the plasmid mixture described above containing pCMV-I-SceI and pBS (control vector) or wt*EGFP* plasmid (for electroporation efficiency) followed by addition of NM/H_2O and DSB repair measurements as before. To inactivate endogenous Rad51, cells were correspondingly pre-electroporated with pcDNA3.1 or pcDNA3.1-Rad51SM [25]. To inhibit endogenous PARP1 function, cells were co-eletroporated with pCMV-I-SceI, pBS/wt*EGFP* plasmid, and PARP-DBD expression vector pPARP6 or control plasmid [26].

Genomic PCR analysis of DSB repair products was performed as described in Akyüz et al. [20]. PCR products were generated by 35 (white cells) or 40 (green cells) PCR cycles from 1000 cells each and further applied to I-*Sce*I cleavage (white cells).

2.4. Western blotting and antibodies

For Western blot analysis cellular lysates were prepared at the time point of and following the same treatment as for DSB repair measurement. Cells were incubated in lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 2 mM EGTA; 2 mM EDTA; 25 mM NaF; 25 mM β- glycerophosphate; 0.1 mM NaV; proteinase-inhibitor, Roche, Penzberg, Germany). The protein concentration was determined with the BCA protein assay kit (Pierce/Perbio Science, Bonn, Germany). Fifty micrograms of total protein was loaded per lane in a sodium dodecyl sulphate gel (4–12% acrylamide) and transferred to Hybond-C Extra nitrocellulose (Amersham) or Immobilon-P (Millipore) membrane. The following antibodies were used for immunodetection: SIRT-1 antibody sc15404 rabbit (Santa Cruz, Heidelberg, Germany), Ku70 antibody S5C11 Ab 2172 mouse (Abcam, Cambridge, USA), anti-p53-Acetylated

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