Exonuclease-1 interacts with the transcriptional co-repressor KAP1

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Abstract

Error-free repair of DNA double-strand break is orchestrated by homologous recombination pathways and requires the concerted action of several factors. Among these, EXO1 and DNA2/BLM are responsible for extensive resection of DNA ends to produce 3'-overhangs that are key intermediates for downstream steps of Homologous Directed Repair (HDR). To help shed light on regulatory aspects of DNA repair and DNA remodeling pathways in which EXO1 participates, and to help reveal novel cellular processes in which EXO1 is involved, we set out to identify proteins interacting with EXO1. Combined immunoprecipitation and mass spectrometry led to the identification of the non-sense RNA mediated decay protein UPF1 and the DNA damage response protein KAP1/TIF1-β/TRIM28/RNF96, among others, as EXO1 partners. Follow-up cellular and biochemical studies with recombinant proteins allowed validation of the direct interaction between EXO1 and KAP1.

As a whole, these results provide the basis for future in-depth studies on novel mechanisms controlling EXO1 and affecting genome stability.

Introduction

The human genome is continuously challenged by different types of insults, with thousands of lesions occurring to DNA every day in each cell [1]. DNA damage either arises as a byproduct of normal cellular metabolism and DNA replication or is induced by external factors [2]. DNA double-strand breaks (DSBs) are the most dangerous lesions, generated by ionizing radiation (IR), certain chemotherapeutic drugs, collapse of stalled DNA replication forks, or caused by endogenous metabolic processes, such as meiotic recombination or immunoglobulin diversity generation [3] [4] [5] [6]. DSBs are estimated to occur at a rate of ten per cell per day in primary human or mouse fibroblasts [7]. Inappropriate repair of DSBs interferes with DNA replication and transcription and may cause gross chromosomal aberrations [8], resulting in developmental defects, neurodegeneration, aging, immunodeficiency, radiosensitivity and sterility [9], or facilitating the development of cancer through activation of oncogenes or inactivation of tumor suppressor genes [10]. DNA damage response (DDR) pathways have evolved as surveillance and protection mechanisms to counteract the adverse consequences of DNA lesions and to prevent their transmission to daughter cells [11]. Exonuclease-1 was originally identified in S. pombe (Exo1) [12], and subsequently in humans (EXO1) [13], as an enzyme belonging to the Rad2 family of DNA repair nucleases, able to remove mononucleotides from the 5' end of the DNA duplex [14]. EXO1 is implicated in several DNA repair pathways, including mismatch repair, post-replication repair, meiotic and mitotic recombination and double-strand break repair [15] [16] [17] [18] [19]. Human and yeast EXO1 are tightly regulated by interaction with CtIP/RBBP8 and 14-3-3 proteins, at DSBs and stalled forks, respectively [8] [20] [21]. Additionally, human EXO1 is controlled by post-translational modifications (PTMs), with Ataxia-Telangiectasia and Rad3-related (ATR)-dependent phosphorylation targeting it to ubiquitin-mediated degradation upon replication fork stalling [22] [23], Ataxia-Telangiectasia-Mutated (ATM)-dependent phosphorylation restraining its activity during homologous recombination or at uncapped telomeres [24] [25], and CDK-dependent phosphorylation affecting the pathway choice of DSB repair [26]. Adding a further layer of complexity, a recent report demonstrated that EXO1 resection activity is controlled by sumoylation [27]. In this study, we report the identification of novel EXO1 interaction partners and biochemically characterize the interaction with
Objective

Considering the multifaceted role of EXO1 in various DNA repair processes, the identification of factors cooperating with EXO1 at sites of damage is expected to shed further light on the function of this important nuclease.

Figure Legend

Figure 1. EXO1 interacts with KAP1.

(A) Endogenous EXO1 was immunoprecipitated from HEK293T whole cell extract (WCE, 20 mg) and proteins were resolved on an 8% SDS-PAGE gel. Silver stained bands were excised and analyzed by tandem LC-MS/MS. Lane 1: molecular weight markers; lane 2: untreated cells immunoprecipitated with antibody F15; lane 3: untreated cells immunoprecipitated with pre-immune (PI) serum; lane 4: cells treated with HU (2 mM, 16 h) and immunoprecipitated with antibody F15.

(B) OMNI-EXO1 and FLAG-KAP1 were co-expressed in HEK293T cells. KAP1 was immunoprecipitated from WCE (2 mg) using an antibody to the FLAG and proteins were revealed with monoclonal antibodies to EXO1 and the FLAG.

(C) FLAG-KAP1 was expressed in HEK293T cells that were either left untreated or treated with HU (2 mM, 16 h). MG-132 (10 mM) was added for the same period of time to rescue degradation of EXO1. WCE (5 mg) were precipitated with either pre-immune (PI) or rabbit polyclonal antibody F15 (IP: EXO1). Proteins were revealed with monoclonal antibodies to EXO1 and the FLAG tag.

(D) Far-Western blot analysis. Upper panel: purified recombinant EXO1 (500 ng) and MutS-α (MSH2/MSH6, 500 ng) were resolved by SDS-PAGE, transferred to PVDF and stained with Ponceau-red (PR). Lower panels: the membrane was blocked in 5% milk, incubated in the presence or the absence of purified recombinant FLAG-KAP1 and probed with monoclonal antibodies to the FLAG (center), EXO1 (left) or MSH2/MSH6 (right).
HEK293T cells expressing OMNI-EXO1 were either left untreated or treated with HU and MG-132. OMNI-EXO1 was immunoprecipitated from WCE (2 mg) using an antibody to the OMNI tag. Proteins were revealed with monoclonal antibodies to MDM2 and EXO1.

HEK293T cells were either left untreated or treated with HU and MG-132. Endogenous EXO1 was immunoprecipitated from WCEs (5 mg) with antibody F15 (IP: EXO1). Pre-immune serum (PI) was used as control. Proteins were revealed with monoclonal antibodies to MDM2 and EXO1.

OMNI-EXO1 and HA-MDMX were co-expressed in HEK293T cells. EXO1 was immunoprecipitated from WCE (2 mg) using an antibody to the OMNI tag and proteins were revealed with monoclonal antibodies to the HA-tag and EXO1.

HA-MDMX was expressed in HEK293T cells that were either left untreated or treated with HU and MG-132. WCE were precipitated with either pre-immune serum (PI) or rabbit polyclonal antibody F15 (IP: EXO1). Proteins were revealed with monoclonal antibodies to the HA-tag and EXO1.

Far-Western blot analysis. Top panels: 500 ng of purified recombinant MutS-α (MSH2/MSH6), KAP1 or EXO1 were resolved by SDS-PAGE, transferred to PVDF and incubated in the presence or the absence of purified recombinant MDM2 as indicated. Bottom panels: 500 ng of purified recombinant EXO1 or MutS-α (MSH2/MSH6) was resolved as described above and incubated in the presence or the absence of purified recombinant MDM2+KAP1 or KAP1 alone, as indicated. All membranes were revealed with a monoclonal antibody to MDM2.

Whole cell extracts from HEK-293T cells stably transfected with empty vector (shEV) or KAP1 (shKAP1) shRNA constructs (left panels) were immunoprecipitated with either rabbit pre-immune serum (PI) or an antibody to EXO1 (right panels) and proteins were revealed with the indicated antibodies.

Whole cell extracts from HEK-293T cells stably transfected with empty vector (shEV), KAP1 (shKAP1) or MDM2 (shMDM2) shRNA constructs and treated in the presence or the absence of HU (2 mM, 16 h) were immunoprecipitated as described above and EXO1 was revealed with a mouse monoclonal antibody.

Schematic model of the interaction between EXO1 and the KAP1/MDM2/MDMX complex.

**Chemicals and antibodies**

Hydroxyurea (HU) was dissolved in ddH$_2$O at 1 M stock concentration and filter-sterilized. MG-132 (Calbiochem) was prepared as 10 mM stock solution in DMSO and added to cells at 10 mM final concentration 30min before additional treatments. The antibodies to EXO1 (rabbit polyclonal F15) and MSH6 (mouse monoclonal 66H6) were previously described [8] [23] [47]. Additional antibodies used in this study were purchased from Santa Cruz Biotechnology: goat polyclonal to OMNI (sc-499G), mouse monoclonal to MDM2 (sc-965), Sigma: mouse monoclonals to Flag (F3165) and HA (12CA5); NeoMarkers: mouse monoclonal to EXO1 (ab4, clone 266). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were from GE-Healthcare.

**Recombinant proteins expression and purification**

Recombinant EXO1 and MutSα (MSH2/MSH6) were expressed and purified as described in [22] and [48] respectively. Plasmids pET28a+-Flag-KAP1 and pET28a+-His-MDM2 were introduced by electroporation into the *E. coli* strain BL21 (DE3). Protein expression was induced by 1 mM IPTG at 37℃ for 4 h. The cell pellet (20 g) was resuspended in 50 ml of ice-cold buffer A (50 mM Hepes, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.1% Triton X-100, protease complete-inhibitor cocktail (Roche), 0.1 mM phenylmethylsulfonyl fluoride, PMSF), sonicated, and cleared by ultracentrifugation at 4℃. The resulting supernatant was applied onto a 1 ml Ni-NTA column (GE Healthcare). The column was developed with 50 ml gradient of 0–500 mM NaCl in buffer C (20 mM triethanolamine pH 7.4, 1 mM EDTA, 50 mM NaF, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 0.1% Triton X-100). The column was developed with 30 ml gradient of 0–500 mM NaCl in buffer C. Fractions were analyzed by SDS-PAGE, divided in small aliquots, snap-frozen in liquid
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Cell culture, transfections and treatments
HEK-293T human embryonic kidney cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (Gibco) and penicillin/streptomycin (100 U/ml; Gibco). For DNA transfection experiments, cells were seeded in 10 cm dishes, allowed to adhere overnight, and then transiently transfected with constructs of interest using 1 μg DNA and 4 μl of the transfecting reagent Metafectene (Biontex, Germany) according to the manufacturer instructions. Cells were harvested 48 h after transfection. For RNA interference experiments, shEV (empty vector pRS), shKAP1 (OriGene Technology) or shMDM2 [49] were transfected in HEK-293T cells to generate stable cell lines, as described [23].

Western blot, Far-Western blot and Immunoprecipitation
Mammalian cell proteins were extracted using ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-pyrophosphate, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM PMSF and 1% Nonidet P-40) [8]. Protein concentration was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad). Proteins were separated on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) (GE-Healthcare), and probed with appropriate antibodies. Immune-complexes were revealed using the enhanced chemiluminescence (ECL) system (GE-Healthcare). Far-Western blotting was performed according to established protocols [50]. Immunoprecipitation and immunoblot analysis were performed as described previously [8] [22]. To ensure that the observed interactions were not DNA-mediated, ethidium bromide was included in all samples during immunoprecipitation.

Silver staining
All reagents were prepared in MilliQ-ddH₂O. Polyacrylamide gels were fixed in 50% methanol, 5% acetic acid in ddH₂O for 20 min, washed with 50% methanol in ddH₂O, then additionally for 10 min in ddH₂O. The gel was soaked in 0.02% sodium thiosulfate for 1 min, washed twice with ddH₂O for 1 min, soaked in cold 0.1% silver nitrate solution for 20 min at 4°C in the dark. The gel was then washed twice with ddH₂O for 1 min and developed in 0.04% formaldehyde in 2% sodium carbonate with continuous, gentle shaking. Development was terminated by addition of a 5% acetic acid solution.

Mass spectrometry
Silver stained protein bands were excised from the gel, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and cleaved with porcine trypsin (Promega, Madison, USA) in 50 mM ammonium bicarbonate (pH 8.0) at 37°C overnight. The extracted peptides were analyzed by capillary liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Magic C18 100 μm × 10 cm HPLC column (Swiss BioAnalytics, Switzerland) connected on line to a 4000 Q Trap (MDS Sciex, Concord, Ontario, Canada) as described earlier [29]. Peptides were quantified with the software Progenesis LC (Non-linear Dynamics, Durham, NC, USA).

Results & Discussion
Identification of EXO1 interacting proteins
Exponentially growing HEK-293T cells were either left untreated or treated for 16 h with 2 mM hydroxyurea (HU) [22] [23] [27], an inhibitor of the enzyme ribonucleotide reductase (RNR) causing depletion of the pool of dNTPs and early S-phase arrest [28]. Whole cell extracts (WCE, 20 mg) were immunoprecipitated with a rabbit polyclonal antibody to EXO1 and resolved on an 8% SDS-polyacrylamide gel (Fig. 1A). Upon silver staining, the major bands were excised from the gel, submitted to proteolytic digestion and analyzed by a 4000 Q-Trap mass spectrometer [29]. The data show that, in addition to EXO1, unique peptides for established (MSH2) [30] and novel partners could be specifically identified (Fig. 1A and Table S1). Genuine novel EXO1 interactors consisted of the GTPase-activating protein TBC1D4, the ATP-dependent helicase RENT1 (an essential factor of the nonsense-mediated decay (NMD) pathway deputed to the degradation of mRNAs containing premature stop codons), the leucine zipper protein 1 LUZP1, KAP1, a co-repressor of transcription and SUMO E3-ligase with established roles in the DNA damage response, the AMP deaminase AMPD2, and the polyadenylate-binding protein 1 PABP1 that regulates translational initiation [31] [32] [33] [34]. Interacting proteins
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commonly identified in mass spectrometry studies, such as NCL and HNRNPM, and listed at the Contaminant Repository for Affinity Purification site (www.crapome.org) were not considered further.

Analysis of the EXO1-KAP1 interaction

To validate the findings of mass spectrometry studies using independent methods, we focused on KAP1. Ectopic expression of OMNI-EXO1 and FLAG-KAP1 in HEK293T cells followed by immunoprecipitation with an anti-FLAG monoclonal antibody confirmed the interaction between the two proteins (Fig. 1B). Immunoprecipitation of the low abundance EXO1 protein [8] [22] [23] from untreated cells or cells undergoing replication stress upon treatment with hydroxyurea (HU), revealed constitutive interaction with ectopically expressed FLAG-KAP1 (Fig. 1C). To assess whether the observed protein-protein interaction is direct or is mediated by unknown bridging proteins, we performed Far-Western blot analysis. To this end, purified recombinant EXO1 or the MutSα complex (MSH2/MSH6)- used as control were resolved by SDS-PAGE and transferred to PVDF. Upon visualization of the proteins by Ponceau-red (Fig. 1D, upper panel), the membrane was overlaid with purified recombinant FLAG-KAP1 and subsequently probed with a monoclonal antibody to the FLAG or control antibodies to EXO1, MSH6 and MSH2. The data showed a specific signal for the FLAG in the EXO1 lane only (Fig. 1D, lower panels), confirming that the observed interaction between EXO1 and KAP1 is direct. Since KAP1 was reported to bind MDM2 and contribute to the functional regulation of p53 [35], we decided to examine whether MDM2 is also part of the EXO1-KAP1 complex. To this end, we immunoprecipitated OMNI-EXO1 from extracts of transiently transfected HEK-293T cells. The data showed that endogenous MDM2 could be found as a constitutive partner of ectopically expressed EXO1 (Fig. 1E). Immunoprecipitation of endogenous EXO1 confirmed interaction with endogenous MDM2 (Fig. 1F), strengthening the validity of this observation. The RING-domain MDM2 ubiquitin E3-ligase interacts with MDMX, which contains a non-functional RING-domain [36]. To examine whether MDMX was also part of the protein complex, we ectopically expressed OMNI-EXO1 and HA-MDMX in HEK293T cells. Immunoprecipitation of OMNI-EXO1 from cells treated in the presence or the absence of HU showed constitutive interaction with MDMX (Fig. 1G). More importantly, we confirmed that endogenous EXO1 was able to interact with HA-MDMX (Fig. 1H). Finally, we performed Far-Western blot analysis with purified, recombinant proteins to explore molecular interactions. The data showed that MDM2 directly interacted with KAP1 but not with EXO1 (Fig. 1I, top panels, lanes 2 and 3) and that the MDM2-EXO1 interaction was only detectable upon bridging by KAP1 (Fig. 1I, bottom panels, lane 1). Co-immunoprecipitation experiments confirmed that in cells where KAP1 expression was lowered by RNA interference, interaction between EXO1 and MDM2 proteins was decreased (Fig. 1J).

To address the functional role of the EXO1-KAP1-MDM2 interaction, we attenuated expression of either KAP1 or MDM2 with specific shRNAs. The data showed that KAP1 depletion did not rescue EXO1 (Fig. 1K, top right panel, lane 2 vs. 4), the protein level of which is controlled by ubiquitylation-mediated degradation in response to HU [22]. Similar results were obtained upon MDM2 depletion (Fig. 1K, bottom right panel, lane 2 vs. 4), suggesting that neither KAP1 nor MDM2 is involved in the control of EXO1 protein level.

Dysfunction of the machinery that signals DNA damage and/or addresses DNA repair is associated with cancer development and resistance to therapy [10] [37], providing a direct demonstration of the link between genome instability and cancer [38]. Intense effort is currently being devoted to the identification of protein complexes addressing recognition and repair of various forms of DNA damage, as well as to the elucidation of pathways transducing signals to the cell cycle machinery [39] [40]. This knowledge, in turn, is expected to help the development of more efficient drugs that addresses the lack of specificity and side-effects of current chemotherapeutics. The study presented here, focused on an essential component of error-free DNA repair pathways, contributes to filling this gap through the identification of novel proteins interacting with EXO1, hence expanding our current knowledge [20] [8]. Our data show that EXO1 is part of a multi-protein complex comprising the co-repressor of transcription and E3 SUMO ligase KAP1 and the ubiquitin E3-ligase MDM2/MDMX (Fig. 1A-1J and 1L). Interestingly, KAP1 was
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reported to cooperate with MDM2 in promoting p53 inactivation [35]. However, functional studies that we conducted in cells depleted for KAP1 or MDM2 expression by RNA interference demonstrated that none of them contributes to modulating EXO1 protein level in response to stalled DNA replication (Fig. 1K). Hence, the fact that EXO1 is degraded in an ubiquitin-dependent manner upon stalled DNA replication [8] [22] [23] [27], but that KAP1 or MDM2 have no role in this process, indicates that other ubiquitin E3-ligases control EXO1 protein level and, in turn, the extent of DNA resection at sites of damage. A recent study reporting the ability of SCF-Cyclin F to control EXO1 stability upon UV-damage, but not in response to ionizing radiation [41], indicates that distinct ubiquitin-dependent pathways may be operative in different settings.

The low abundance of EXO1, both in yeast (estimated to ~800 molecules per cell in yeast, http://www.yeastgenome.org/) and in humans [22] [23] (www.proteinatlas.org/), compared to the high expression of KAP1 (www.proteinatlas.org/), suggests that only a sub-population of KAP1 will be engaged in a stoichiometric interaction with EXO1. While KAP1 has also important roles in transcription [42] [43], the subpopulation of KAP1 molecules modulating chromatins relaxation [44] in response to DNA damage [45], a function that is under the strict control of PTMs [46], will likely be engaged in a stoichiometric interaction with EXO1. We speculate that constitutive physical interaction with a chromatins remodeling factor is beneficial to the cell, as it ensures the presence of EXO1 in the vicinity of regions where damage may occur and where, under the control of CtIP [8] and PTMs such as phosphorylation, ubiquitylation and sumoylation [22] [23] [26] [27], it can be promptly engaged in the repair of DNA in an error-free manner.

Conclusions

The identification of novel EXO1 interacting proteins presented in this study, such as mRNA processing factors, DNA binding proteins, and components of chromatins remodeling factors, represents important additional information on the complexity of cellular responses to genotoxic damage and offers an opportunity for further in-depth studies on the regulation of the DNA damage response.

Additional Information

Methods
Chemicals and antibodies
Hydroxyurea (HU) was dissolved in ddH2O at 1 M stock concentration and filter-sterilized. MG-132 (Calbiochem) was prepared as 10 mM stock solution in DMSO and added to cells at 10 mM final concentration 30min before additional treatments. The antibodies to EXO1 (rabbit polyclonal F15) and MSH6 (mouse monoclonal 66H6) were previously described [8] [23] [47]. Additional antibodies used in this study were purchased from Santa Cruz Biotechnology: goat polyclonal to OMNI (sc-499G), mouse monoclonal to MDM2 (sc-965); Sigma: mouse monoclonals to Flag (F3165) and HA (12CA5); NeoMarkers: mouse monoclonal to EXO1 (ab4, clone 266). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were from GE-Healthcare.

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X-100). The column was developed with 30 ml gradient of 0–500 mM NaCl in buffer C. Fractions were analyzed by SDS-PAGE, divided in small aliquots, snap-frozen in liquid N₂, and stored at -80°C.

**Cell culture, transfections and treatments**

HEK-293T human embryonic kidney cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (Gibco) and penicillin/streptomycin (100 U/ml; Gibco). For DNA transfection experiments, cells were seeded in 10 cm dishes, allowed to adhere overnight, and transiently transfected with constructs of interest using 1 μg DNA and 4 μl of the transfecting reagent Metafectene (Biontex, Germany) according to the manufacturer instructions. Cells were harvested 48 h after transfection. For RNA interference experiments, shEV (empty vector pRS), shKAP1 (OriGene Technology) or shMDM2 [49] were transfected in HEK-293T cells to generate stable cell lines, as described [23].

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**Silver staining**

All reagents were prepared in MilliQ-ddH₂O. Polyacrylamide gels were fixed in 50% methanol, 5% acetic acid in ddH₂O for 20 min, washed with 50% methanol in ddH₂O, then additionally for 10 min in ddH₂O. The gel was soaked in 0.02% sodium thiosulfate for 1 min, washed twice with ddH₂O for 1 min, soaked in cold 0.1% silver nitrate solution for 20 min at 4°C in the dark. The gel was then washed twice with ddH₂O for 1 min and developed in 0.04% formaldehyde in 2% sodium carbonate with continuous, gentle shaking. Development was terminated by addition of a 5% acetic acid solution.

**Mass spectrometry**

Silver stained protein bands were excised from the gel, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and cleaved with porcine trypsin (Promega, Madison, USA) in 50 mM ammonium bicarbonate (pH 8.0) at 37°C overnight. The extracted peptides were analyzed by capillary liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Magic C18 100 μm × 10 cm HPLC column (Swiss BioAnalytics, Switzerland) connected on line to a 4000 Q Trap (MDS Sciex, Concord, Ontario, Canada) as described earlier [29]. Peptides were quantified with the software Progenesis LC (Non-linear Dynamics, Durham, NC, USA).

**Supplementary Material**

Please see https://sciencematters.io/articles/201606000006.

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