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Bi-directional routing of DNA mismatch repair protein human exonuclease 1 to replication foci and DNA double strand breaks

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Abstract

Human exonuclease 1 (hEXO1) is implicated in DNA metabolism, including replication, recombination and repair, substantiated by its interactions with PCNA, DNA helicases BLM and WRN, and several DNA mismatch repair (MMR) proteins. We investigated the subnuclear localization of hEXO1 during S-phase progression and in response to laser-induced DNA double strand breaks (DSBs). We show that hEXO1 and PCNA co-localize in replication foci. This apparent interaction is sustained throughout S-phase. We also demonstrate that hEXO1 is rapidly recruited to DNA DSBs. We have identified a PCNA interacting protein (PIP-box) region on hEXO1 located in its COOH-terminal (⁷⁸⁸QIKLNELW⁷⁹⁵). This motif is essential for PCNA binding and co-localization during S-phase. Recruitment of hEXO1 to DNA DSB sites is dependent on the MMR protein hMLH1. We show that two distinct hMLH1 interaction regions of hEXO1 (residues 390–490 and 787–846) are required to direct the protein to the DNA damage site. Our results reveal that protein domains in hEXO1 in conjunction with specific protein interactions control bi-directional routing of hEXO1 between on-going DNA replication and repair processes in living cells.

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Conflict of interest statement

There are no conflicts of interest.

Keywords

hEXO1; PCNA; Replication foci; Double strand break; DNA mismatch repair

1. Introduction

The DNA mismatch repair (MMR) system is responsible for post-replicative repair of DNA mismatches arising during replication and plays an important role in safeguarding genetic integrity [1–3]. The initial recognition step of MMR is carried out by hMutS α (hMSH2-hMSH6) or hMutS β (hMSH2-hMSH3) that acts together with hMutL α (hMLH1-hPMS2) to regulate a downstream repair process that involves nucleolytic excision of a stretch of DNA containing the mismatched base. To this date, the 5' $\rightarrow$ 3' directed human exonuclease 1 (hEXO1) is the only exonuclease identified as part of the eukaryotic MMR system [4–10]. Exonuclease 1 (EXO1) belongs to class III of the RAD2 family of endonucleases and exonucleases and it possesses, in addition to its primary 5' $\rightarrow$ 3' exonuclease activity, both 5'-flap endonuclease activity and RNaseH activity. Several studies describe a role for EXO1 in various DNA metabolic pathways such as replication, repair, and recombination [4,9–14]. Studies from yeast imply that EXO1 may function as a backup protein for RAD27 (FEN1) in primer removal and Okazaki fragment maturation during DNA replication [10,14]. In accordance, human EXO1 localizes in specific nuclear foci in mammalian cells [15], supporting a role for EXO1 during DNA replication. Recent work indicates that hEXO1 is involved in DNA damage signaling upon replication fork stalling and it has been suggested that regulation of hEXO1 activity is critical for the maintenance of stalled replication forks [16,17]. The 5' $\rightarrow$ 3' DNA resection of DSB ends to produce 3' single stranded DNA (ssDNA) tails is a critical step in the repair of DSBs by homologous recombination (HR) [18]. Yeast studies suggest that EXO1 activity can substitute or complement the MRX (MRN) complex during DNA resection of DSBs in mitotic cells [19–22]. Moreover, it appears that hEXO1 is required for normal HR repair of DSBs in human cells, based on recent findings that cells depleted of hEXO1 display chromosomal instability and hypersensitivity to ionizing radiation (IR) [23]. Finally, hEXO1 interacts with different helicases involved in HR, including WRN, BLM and RecQ1, and these interactions stimulate the endonucleolytic and exonucleolytic cleavage activities of hEXO1 [24–26].

Proliferating cell nuclear antigen (PCNA) acts as processivity factor for DNA polymerases δ and ϵ during DNA replication, and is also important for several steps of MMR [27–30]. PCNA interacts with MSH3, MSH6 and MLH1 in yeast as well as in human cells [27,30–33] through a conserved binding motif, known as the PCNA Interacting Protein (PIP-box) motif [32,34–36]. MMR protein complexes hMutS α , hMutS β and hMutL α are recruited to newly replicated DNA through interactions with PCNA in an *in vitro* system [29]. Likewise, recombinant PCNA and hEXO1 interact *in vitro* and co-localize in discrete nuclear foci *in vivo* when transiently expressed [5,15]. Unlike in MLH1, MSH6 and MSH3, a consensus PIP-box motif has until now never been identified in hEXO1. Importantly, certain MMR proteins are recruited to various types of DNA damage in human cells, and this recruitment is, to some extent, dependent on interactions between MMR proteins and PCNA [37]. Here, we show that specific co-localization of hEXO1 and PCNA in nuclear foci is sustained

during S-phase. We have identified a PIP-box motif in hEXO1 (⁷⁸⁸QIKLNELW⁷⁹⁵), which is necessary for this sub-nuclear formation and co-localization with PCNA and provided data showing direct and specific binding between this peptide sequence and PCNA. In parallel, we showed that hEXO1 is rapidly recruited to laser induced DNA DSBs, supporting a role for its involvement in double strand break repair (DSBR). The protein domain required for hEXO1 recruitment to the damage site includes the bi-partite hMLH1 interaction domain. Apparently, localization of hEXO1 to replication foci versus DNA DSB sites is dependent on distinct regions within the protein and on specific interaction with either PCNA or hMLH1, respectively.

Our results suggest that hEXO1 is a multifunctional protein that plays roles in DNA replication, MMR and DSBR. We propose that hEXO1 is a central player in these processes and may be switched to function between these various pathways. This switch is seemingly regulated through alternating protein interactions; binding of hEXO1 to PCNA place the protein at replication forks, whereas interaction with hMLH1 directs hEXO1 to DNA DSBs.

2. Materials and methods

2.1. Bioinformatic analysis

To identify possible PIP-box like regions in hEXO1 the following approach was taken. First the amino acid sequence of hEXO1 was searched to identify glutamine (Q) residues in hydrophobic regions. Upon identification of hydrophobic regions containing a Q residue, alignments of the hEXO1 protein sequence with EXO1 sequences of higher eukaryotes, were used to select highly conserved hydrophobic regions for further analysis. Since the PIP-box regions of several human proteins have been shown to depend on two COOH-terminal phenylalanine (F) residues (FF-tail) [38], we included the only FF tail found in hEXO1 (F506 and F507) in the study. Finally, the secondary structure of hEXO1 was predicted with web server software programs Jpred3 and PredictProtein to identify regions that would form either α -helices or β -sheets and that are expected to be exposed in the predicted protein structure. Based on these analyses, four suggested PIP-box like regions were chosen for further experimental analysis.

2.2. DNA plasmids

Plasmids pECFP-C1-PCNA, pECFP-C1-hMSH2, pEYFP-C1-hMSH2, pEYFP-C1-hMSH6, pECFP-C2-hMLH1, pEYFP-C2-hMLH1 used for expression of fusion proteins CFP-PCNA, CFP-hMSH2, YFP-hMSH2, YFP-hMSH6, CFP-hMLH1 and YFP-hMLH1 have been described previously [15,39]. Plasmid pLJR115 expressing NH₂-terminal tagged YFP-hEXO1 fusion protein [40] was used as template to construct a series of YFP-hEXO1 mutant proteins. Plasmid pSDA43 also expressing NH₂-terminal tagged YFP-hEXO1 fusion protein was constructed by subcloning *hEXO1* sequence from pcDNA 3.1/His C/hEXO1 [17] into pEYFP CI between *Bgl*II and *Ap*aI sites using *Bam*HI and *Ap*aI digestion. This construct differs in linker length compared to pLJR115, but otherwise no functional distinctions were observed between the two. The hEXO1 allele contained in both pLJR115 and pSDA43 has previously been described (Genbank: AAD13754) [9,15,40–42]. Mutations in hEXO1 were introduced using QuikChange site-directed mutagenesis according to

manufacturer's guidelines (Stratagene, La Jolla, CA) employing specifically designed oligonucleotide primers (Invitrogen) (Supplementary data Table 1). Coding regions of the resulting constructs were verified by DNA sequencing (Macrogen).

For *in vitro* transcription translation (IVTT) of hEXO1, plasmid pcDNA3.1A/myc-His(-)A-hEXO1 expressing COOH-terminal his₆ and myc epitope tagged hEXO1, was used as a template to construct various pcDNA3.1A/myc-His(-)A-hEXO1 variants. Mutations, truncations and deletions were introduced using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), using oligonucleotide primers listed in Supplementary data Table S1. Plasmid pSEL54 (pcDNA3.1A/myc-His(-)A-hEXO1-Q154A;Y157A) was constructed by subcloning from pSEL43 (pEYFP-C1-hEXO1-Q154A;Y157A) achieved by *Sall* and *BamHI* digestion and ligation into pcDNA3.1A/myc-His(-)A vector restriction sites *XhoI* and *BamHI*, creating non-tagged hEXO1 protein variants. Coding regions in the resulting constructs were verified by DNA sequencing (Macrogen). Plasmids for expression of GST-hMSH2 and GST-hMLH1 fusion proteins (pGEX-4T-1-hMSH2 and pGEX-4T-1-hMLH1) have been described elsewhere [15,40].

2.3. Western blot analysis of YFP-hEXO1 mutant proteins

Expression of full-length fusion proteins in NIH3T3 cells was verified by western blot analysis of whole cell extracts from transfected cells. 8×10^5 exponentially growing NIH3T3 cells were seeded one day prior to transfection in 92×17 mm dishes (Nunc). Cells were transfected with lipofectAMINE2000 (Life Technologies Inc., USA) according to manufacturer's instructions using 4 μ g of the relevant plasmid. Cells were incubated for 24 h in a humidified 95% air–5% CO₂ atmosphere. Cells were rinsed in PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (PBS, pH 7.4 (9.1 mM NaHPO₄, 1.7 mM NaH₂PO₄, 150 mM NaCl), 1% Igepal CA630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS)) containing freshly added protease inhibitors (Complete, Roche), by passing suspensions 10 times through a 21 $\times$ g syringe. Lysates were incubated on ice for 60 min, centrifuged at 20,000 g for 20 min at 4 °C. Supernatants were transferred to new tubes and protein concentrations determined using the BioRad Protein Assay (BioRad) according to manufacturer's instructions. Protein samples (40 μ g per lane) were separated on 7.5% SDS polyacrylamide gels (Lonza), and transferred to nitrocellulose membranes (BioRad), which were probed with rabbit anti-GFP antibody (ref 632377, dilution 1:500, CLONTECH) and secondary antibody anti-rabbit (dilution 1:10000, Pierce IL). Immunostained bands were visualized with SuperSignal Western blotting detection system (Pierce, IL).

2.4. Confocal laser-scanning microscopy

Murine NIH-3T3 cells were maintained as monolayer cultures in DMEM (Gibco, Life Technologies), supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 μ g/ml) (Gibco BRL, Life technologies). Cell lines were grown at 37 °C in a humidified atmosphere containing 10% CO₂. Transient transfections were performed with ExGen500 (Fermentas) according to manufacturer's instructions. Briefly, 2×10^5 cells/dish were seeded on glassbottom dishes (WillCo Dish, HBSt 5040) 24 h prior to transfection and incubated overnight at 37 °C in a humidified 95% air–10% CO₂ atmosphere. Cells were transfected with a total of 3 μ g of the relevant DNA plasmids and incubated for 24–48 h at

which point expression and subcellular localization of CFP and YFP fusion proteins were examined with a confocal Zeiss LSM510 microscope. Co-localization was quantified with the LSM510 software (Supplementary data Fig. S6).

2.5. MicroPoint laser irradiation

HeLa cells were maintained in DMEM (Gibco) supplemented with 10% FBS and Hygromycin (1:250) and grown at 37 °C in a humidified atmosphere containing 5% CO₂. Approximately 1*10⁵ cells were seeded one day prior to transfection in 15 mm dishes containing thin glass plates with grits at the bottom (Mat-Tek). Cells were transfected with lipofectAMINE2000 (Life Technologies Inc., USA) according to manufacturer's instructions using 4 µg of the relevant plasmid. Briefly, seeded cells were washed once in PBS and incubated for 6 h in fresh media lacking FBS and antibiotics, but containing a mixture of plasmid and lipofectamine, prepared by preincubation for 20 min before addition. The media was then changed to DMEM containing 10% FBS and Hygromycin (1:250) and the cells were incubated over night in a humidified 95% air–5% CO₂ atmosphere for fluorescent protein expression. Targeted DNA damage was introduced in thick track lines, using the MicroPoint® Ablation Laser System from Photonic Instruments equipped with a nitrogen laser (NL100, Stanford Research Systems) producing a 435 nm beam when passed through a dye cell. The laser was coupled to a Nikon eclipse 2000E confocal microscope with five imaging modules and a CCD camera (Hamamatsu), for targeting cells and detection of fluorescence migration. Velocity software 4.3.1 was used for attenuation of laser power in terms of percent intensity and for data analysis (build 6). The region of interest (ROI) was targeted to the nucleic of cells via a 40X oil objective lens. Fluorescent protein recruitment to the irradiated sites was monitored in real time for 2–4 min with time lapse acquisition starting 2 s before irradiation and carried out every second, except during laser firing. Following irradiation, the cells were fixed and stained with antibodies against γH2AX and XRCC1. Briefly, cells were washed twice in PBS, incubated for 10 min at RT in 3.7% formaldehyde (Polyscience), washed 5 times in PBS, incubated for 5 min at RT in 0.2% Triton-X-100, followed by 5 times washing in PBS. Cells were then blocked in 5% FBS over night at 4 °C or for 1 h at 37 °C, stained with primary antibody for 1 h at 37 °C, washed 5 times in PBS, stained with secondary antibody at RT for 30 min, washed thoroughly in PBS, dried and mounted in mounting media containing DAPI (Vectashield). Mounted plates were stored in the dark at 4 °C and irradiated cells were identified and imaged in the confocal microscope. All dilutions were in PBS. Applied primary antibodies were rabbit polyclonal Anti XRCC1 (H-300) (Sc-11429, dilution 1:250, Santa Cruz) and mouse monoclonal Anti Phosphohistone H2AX (Ser 139) (05-636, dilution 1:250, Upstate). Applied secondary antibodies were Alexa Flour 647 Goat Anti-Mouse (A-21240, dilution 1:1000, Invitrogen), Alexa Flour 647 Goat Anti-Rabbit (A21244, dilution 1:1000, Invitrogen), Alexa Flour 594 Donkey Anti-Mouse (A-31571, dilution 1:1000, Invitrogen) and Alexa Flour 594 Donkey Anti-Rabbit (A-21207, dilution 1:1000, Invitrogen).

2.6. GST-fusion interaction (pull-down) assay

Escherichia coli strain BL21 was transformed with plasmids expressing GST-hMSH2 and GST-hMLH1 and the fusion proteins were purified as described previously [40]. 1 µg GST proteins (hMSH2 or hMLH1) were bound to 20 µl GST beads (Glutathione Sepharose™

4B, Amersham Biosciences) prepared in a 50% slurry with binding buffer (20 mM Tris–HCl, pH 7.5, 10% glycerol, 300 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Tween 20 (poly-oxyethylenesorbitan monolaurate), 0.75 mg/ml BSA and protease inhibitors (complete, Roche Diagnostics)) by incubation for 1 to 2 h at 4 °C on a rocking platform. The protein-bound beads were washed three times with 500 µl binding buffer (4 °C). Samples were diluted with binding buffer to 83 µl GST beads/ml and incubated for 30 min at 4 °C on a rocking platform. All *in vitro* transcribed and translated (IVTT) protein reactions were carried out using the TNT-coupled reticulocyte lysate system (Promega) and proteins were labeled with ³⁵S-cysteine (PerkinElmer). IVTT protein products were quantified using software VisionWorksLS (UVP) or Carestream molecular imaging and volumes corresponding to equal IVTT product were added to the GST bound beads and incubated for 1–2 h at 4 °C on a rocking platform. Samples were washed three times with binding buffer (4 °C), resolved on 7.5% SDS polyacrylamide gels (Lonza) and bound proteins visualized using PhosphorImaging system (STORM 840, Amersham Pharmacia Biotech).

2.7. PCNA purification and hEXO1 peptide preparation

The sequence coding for PCNA was cloned into vector proEX-HTb (Invitrogen) containing a 6xHis tag and TEV site in NH₂-terminal and expressed in *Escherichia coli* BL21. Cells were grown at 37 °C overnight and induced with 0.5 mM IPTG during 3 h at 30 °C. Cells were lysed by sonication in PBS buffer containing 0.5 mg/ml lysozyme, 0.1 mg/ml Dnase I and 5 mM MgCl₂. After centrifugation, the supernatant fraction was applied to NiNTA (Qiagen) equilibrated with lysis buffer containing 20 mM imidazole and 5 mM β-mercaptoethanol. PCNA was eluted with Tris 50 mM, pH 7.5, NaCl 150 mM and 200 mM imidazole. The 6xHis tag was removed by TEV digestion in Tris buffer (50 mM, pH 8, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT and 10% Glycerol). Digested PCNA was incubated with NiNTA to remove the tag and PCNA was then dialyzed against 20 mM Tris buffer, pH 7.5, containing 50 mM NaCl and 20 mM β-mercaptoethanol. The synthetic peptides of hEXO1 p22 (aa 783–804) and p16 (aa 498–513) used in this study were purchased from Genecust at 95% purity and the concentrations of the stock peptide solutions were determined by amino acid composition.

2.8. Isothermal titration calorimetry

Binding between PCNA and hEXO1 peptides p22 (aa 783–804) and p16 (aa 498–513) was determined using a VP-ITC calorimeter (Microcal, Northampton, MA). Prior to measurements, all solutions were degassed under vacuum. The reaction cell was loaded with 18 µM PCNA solution and the syringe contained either 200 µM hEXO1 p22 solution or 173 µM hEXO1 p16 solution. To correct for heat effects of dilution, control experiments were performed with peptide solutions injected into pure buffer. Thermodynamic parameters H , N , and K_a were obtained by nonlinear least-squares fitting of the experimental data using the single set of independent binding sites model of the Origin software provided with the instrument. The free energy of binding (G) and the entropy (S) were determined using the classical thermodynamic formulas: $G = -RT \ln(K_a)$ and $G = H - T S$. All binding experiments were performed at 30 °C in 20 mM Tris buffer, pH 7.5, containing 50 mM NaCl and 20 mM β-mercaptoethanol. A control experiment at 10 °C, was performed with

peptide hEXO1 p16 in which 24 injections of 10 μ l peptide solution (173 μ M) was injected into the calorimeter cell containing a 15 μ M PCNA solution.

3. Results

3.1. Sub-nuclear localization of human mismatch repair proteins

The sub-cellular, and in particular the sub-nuclear, localization of co-expressed proteins can provide information on protein interactions within biological processes. To improve our understanding about the nature of these interactions and the protein recruitment occurring during DNA replication and MMR, we studied the co-localization of PCNA and core MMR proteins. When transiently expressed in mammalian cells, YFP-hEXO1 displays two different localization patterns; localization in discrete foci or diffuse nuclear staining [15,43]. To evaluate the nuclear distribution pattern of MMR proteins, murine NIH3T3 cells were transiently co-transfected with plasmids expressing CFP-PCNA and YFP-hEXO1, YFP-hMLH1, YFP-hMSH2, or YFP-hMSH6, and cellular distribution of fluorescent fusion proteins were analyzed with confocal laser scanning microscopy (Fig. 1).

We observe that YFP-hEXO1 and CFP-PCNA co-localize in discrete nuclear foci. The foci distribution pattern changes through S-phase in a manner characteristic for PCNA replication foci as described in the literature [44–46]. In early S-phase, numerous randomly distributed small foci are seen. In mid S-phase the number of foci becomes fewer, their size increase and some align at the periphery of the nucleus. In late S-phase cells even fewer and larger foci are seen. The strong co-localization between PCNA and hEXO1 in both early, mid and late S-phase (Fig. 1A) suggests that the two proteins are involved in common events during S-phase, such as DNA replication and repair. Similarly, both YFP-hMSH2 (Fig. 1C) and YFP-hMSH6 (Fig. 1D) form PCNA co-localizing foci throughout S-phase, whereas we were unable to observe CFP-PCNA co-localized YFP-hMLH1 foci (Fig. 1B). The role of hMSH2/6 recruitment to PCNA foci is expected to be related to post-replicative mismatch recognition events occurring in the vicinity of the replication fork, while recruitment of hEXO1 could be related either to its role in MMR, replication fork maintenance, primer processing, or a combination of all. Taken together, we show that specific MMR proteins; hEXO1, hMSH2, hMSH6, but not hMLH1 co-localize with PCNA in replication foci throughout S-phase.

3.2. Recruitment of human mismatch repair proteins to DNA double strand breaks

MMR proteins are involved in DSB where they act to suppress recombination between divergent sequences, promote removal of nonhomologous DNA at DSB ends and correct mismatches in heteroduplex DNA [47]. Nucleolytic degradation at the ends of DSBs to create ssDNA tails is a critical step for initiating DSB. Owing to its polarity and processivity EXO1 makes a good candidate for a nuclease acting in the early steps of DSB. To characterize the involvement of hEXO1 in repair of DSBs, we studied the recruitment of hEXO1 to laser induced DSBs. We also examined DSB recruitment of PCNA, hMSH2, hMSH6 and hMLH1 to explore whether hEXO1 recruitment to DSBs could be MMR protein dependent. We used a 435 nm micropoint laser to evaluate protein recruitment of transiently expressed CFP or YFP tagged MMR proteins to DNA DSBs. This laser system

directly induces single strand breaks (SSBs) and DSBs in the DNA of mammalian cells. The intensity of the laser determines the nature of the DNA lesions induced; 2% only generates SSBs, while 8–14% produces both SSBs and an increasing amount of DSBs. The differentiation between SSB and DSB induction at varying laser intensities was validated by parallel immunostaining for the following markers: SSB marker; XRCC1 and the DSB marker; γ H2AX [48].

We found that MMR proteins PCNA, hMSH2, hMSH6 and hMLH1 are recruited to laser induced DNA damage in HeLa cells (Fig. 2B–E) and we show that hEXO1 is recruited directly to DNA damage induced by laser micro-irradiation (Fig. 2A). The figures represent protein recruitment at different laser intensities of 8%, 11% and 14%, and all proteins were recruited at all three laser intensities. Both DNA SSBs and DSBs are produced at 8–14% laser intensities as seen by immunostaining for XRCC1 and γ H2AX (Fig. 2A–E). We find that the major target for MMR protein accumulation at the irradiated site is DNA DSBs, as protein recruitment was not observed at 2% laser intensity for any of the investigated fusion proteins (data not shown). Protein recruitment appears fast for all MMR proteins, as the fluorescent proteins are apparent at damage sites in less than 1 min in the majority of targeted cells. Our data show that hEXO1, like other MMR proteins, is recruited to laser induced DSBs, substantiating a role for this nuclease in the repair or signaling of such DNA damage.

3.3. The region required for nuclear foci formation and co-localization with PCNA is located in the COOH-terminal of hEXO1

To understand the nature of the hEXO1/PCNA foci, we wanted to identify the region(s) on hEXO1 required for PCNA co-localization and formation of S-phase foci. We additionally wanted to evaluate if PCNA is involved in recruitment of hEXO1 to DSBs, or alternatively, if hEXO1 DSB recruitment is dependent on MMR protein interactions. The study was initiated by functional screening of three COOH-terminally truncated versions of YFP-hEXO1; YFP-hEXO1-C508X; YFP-hEXO1-S598X and YFP-hEXO1-S702X (Fig. 3B and Supplementary data Fig. S3). The sub-cellular localization of all three truncated fusion protein variants is strictly nuclear (Fig. 3C and Supplementary data Fig. S3), which was expected given that all three protein versions maintain the reported NLS sequence ⁴¹⁸KRPR⁴²¹ [43]. However, none of the variant proteins were able to form foci or co-localize with PCNA during S-phase (Fig. 3C and Supplementary data Fig. S3). The phenotypes of YFP-hEXO1-C508X, YFP-hEXO1-S598X, and YFP-hEXO1-S702X are very manifest, observed in nearly 100% of transfected cells. In contrast to the abrogated replication foci formation none of the three truncation variants appear impaired in their recruitment to micropoint laser induced DNA DSBs (Fig. 3D and Supplementary data Fig. S3), suggesting that hEXO1 is recruited to DSBs through factors not involved in its recruitment to DNA replication foci.

3.4. Sequence search for putative PIP-box motifs in hEXO1

Recombinant hEXO1 has been demonstrated to interact with PCNA *in vitro* [5] and accordingly, the observed co-localization of hEXO1 and PCNA *in vivo* (Fig. 1A) very likely represents a direct interaction between the two proteins. Most PCNA interacting protein

partners contain a conserved PCNA binding motif; the PIP box [49–51]. To identify the specific region on hEXO1 responsible for co-localization with PCNA in replication foci, the protein sequence of hEXO1 was analyzed for PIP-box-like regions. Putative PIP-box like regions identified on hEXO1 are indicated in Fig. 3A. All four suggested peptide sequences are conserved among EXO1 proteins of higher organisms, suggesting a significant biological role. From sequence analysis and secondary structure predictions, the likelihood of each putative PIP-box region could be ranked as follows; ⁷⁸⁸QIKLNELW⁷⁹⁵ is the most likely PIP-box candidate, second is ⁴⁹⁷VVVPGTRSRFF⁵⁰⁷, third is ¹⁵⁴QLAYLNKA¹⁶¹, while the least likely PIP-box candidate is ²⁸³LYQLVFDP²⁹⁰. To investigate whether these motifs have an impact on the sub-nuclear localization of hEXO1, we introduced specific amino acid substitutions in the various putative PIP-boxes to interfere with PCNA interaction in case of a functional PIP-box motif (Fig. 3B). Mutant fusion proteins; YFP-hEXO1-Q154A;Y157A, YFP-hEXO1-Q285A;F288A, hEXO1-F506A;F507A, and YFP-hEXO1-Q788A;L791A were examined to investigate the sub-cellular distribution and nuclear foci formation ability.

3.5. Substitution of amino acids within the putative PIP-box region ⁷⁸⁸QIKLNELW⁷⁹⁵ abolishes formation of hEXO1 replication foci

In order to obtain a quantitative measurement of the foci formation abilities of the different YFP-hEXO1 variants, co-transfected cells were counted and categorized in the following groups:

- “No foci” – CFP-PCNA fusion protein seen as diffuse whole cell staining and YFP-hEXO1 fusion proteins as diffuse nuclear staining.
- “Small foci” – CFP-PCNA fusion protein and YFP-hEXO1 fusion proteins displaying numerous small nuclear foci.
- “Mid foci” – CFP-PCNA fusion protein and YFP-hEXO1 fusion proteins seen in small foci, some aligned at the periphery of the nucleus.
- “Large foci” – CFP-PCNA fusion protein and YFP-hEXO1 fusion proteins seen in fewer and larger foci.

The functionality of the most likely PIP-box sequence at ⁷⁸⁸QIKLNELW⁷⁹⁵ was analyzed by substituting two amino acids constructing the variant YFP-hEXO1-Q788A;L791A (Fig. 3B). This variant is unable to form nuclear foci and does not co-localize with PCNA, at any time point during S-phase (Figs. 4A and 5B). This abrogate foci formation and non-co-localizing phenotype is robust, as 100% of the analyzed cells show diffuse nuclear staining in comparison to 40% of WT YFP-hEXO1 (Figs. 4A and 5B, and Table 1). Mutations in ⁷⁸⁸QIKLNELW⁷⁹⁵ completely impair hEXO1 recruitment to PCNA replication foci, with no adverse effects on S-phase distribution, as CFP-PCNA foci are consistently observed in both early, mid and late S-phase cells concomitantly with diffuse YFP-hEXO1 staining (Fig. 5B and Table 1).

To confirm the identification of a COOH-terminal PIP-box in hEXO1 and to evaluate the strength of interaction between PCNA and this motif, we characterized, by microcalorimetry, the affinity and stoichiometry of the interaction between purified PCNA

and a synthetic peptide hEXO1 p22 (Fig. 6E) containing the PIP box motif ⁷⁸⁸QIKLNELW⁷⁹⁵. The binding reaction between hEXO1 p22 and PCNA (Fig. 6B) gives an exothermic heat exchange and we observe no dilution effects upon injection of peptide into pure buffer (Fig. 6A). The integrated thermogram has been fitted to a one-site binding model providing a dissociation constant K_d of 8.3 μ M (Fig. 6E). This K_d is in the range of affinities measured for other PIP-box containing peptides like the p21 CDK inhibitor (K_d 82 nM) and a FEN1 PIP-box peptide (K_d 59 μ M) (Fig. 6E). Calorimetry studies hereby confirm a role for this motif as an essential component for the interaction between hEXO1 and PCNA as suggested by co-localization studies. In contrast, we observe no interaction between PCNA and hEXO1 p16 peptide (Fig. 6E) containing the putative PIP-box motif at ⁴⁹⁸VVPGTRSRFF⁵⁰⁷ (Fig. 6C), validating the specificity of hEXO1 p22. To rule out the possibility that interaction between PCNA and hEXO1 p16 was not registered due to zero enthalpy change at the employed temperature, we investigated binding between p16 and PCNA at a second temperature, 10 °C (Fig. 6D) and found that lack of binding is temperature independent. Instead, hEXO1 p16 peptide interacts specifically with the COOH-terminal of hMLH1 [52], which together with our results demonstrates this sequences function as a MIP-box motif and not a PIP-box motif.

3.6. Expression of hEXO1 variants Q154A;Y157A, Q285A;F288A, or F506A;F507A interferes with S-phase distribution

We were unable to detect replication foci in late S-phase of cells expressing the NH₂-terminal variants; YFP-hEXO1-Q154A;Y157A and YFP-hEXO1-Q285A;F288A (Fig. 4C and D). When further analyzed we noticed that none late S-phase cells expressed these variants; however all detected late S-phase cells only expressed CFP-PCNA. This strongly infers that cells expressing these hEXO1 variants do not progress onto late S-phase (Fig. 5C and D). The total transfected cell population displayed cells in all stages of S-phase, determined by the variation of PCNA replication foci pattern (Supplementary data Table S2), demonstrating that the lack of late S-phase cells co-expressing CFP-PCNA and YFP-hEXO1 is directly related to the expression of these particular hEXO1 protein variants. Expression of YFP-hEXO1-F506A;F507A was also observed to interfere with S-phase distribution, as only very few S-phase cells, co-expressing this variant and PCNA were observed (Fig. 5E). In the few early S-phase cells observed co-expressing YFP-hEXO1-F506A;F507A and CFP-PCNA, the mutant protein was unable to associate with PCNA in replication foci (Fig. 4D). In addition, YFP-hEXO1-F506A;F507A displays a higher degree of cytoplasmic distribution (Fig. 4D and Table 1) compared to WT YFP-hEXO1, which is only rarely observed in the cytoplasm (Table 1) [15,43]. Distribution of PCNA and hEXO1 foci in co-expressing cells is presented in Fig. 5. This quantitative analysis of transfected cells shows a clear deviation between the examined hEXO1 variants and WT hEXO1 in the distribution of PCNA co-localizing S-phase foci. The motifs mutated in YFP-hEXO1-Q154A;Y157A and YFP-hEXO1-Q285A;F288A are located in the nuclease domain and the hMSH3 interaction domain of hEXO1 and might affect both protein activity and interaction, and could explain the observed phenotypes. It seems unlikely that either of these motifs constitutes the PIP-box region on hEXO1 as mutant proteins to some extent are able to form S-phase foci in co-localization with PCNA (Fig. 4). In addition, the inability of YFP-hEXO1-F506A;F507A to form nuclear foci cannot be explained by impaired PCNA

recruitment, since CFP-PCNA foci are not observed along with expression of hEXO1-F506A;F507A, as would be expected if protein interaction was disrupted. It must be considered that these fusion protein variants are constitutively expressed, leaving proteins both over-expressed and potentially deregulated, which could lead to excessive DNA degradation and formation of large ssDNA regions in principle halting S-phase progression and resulting in the diminished number of observed S-phase cells expressing these variant proteins.

3.7. Mapping of region on hEXO1 required for recruitment to DNA double strand breaks

We observe that hEXO1 is rapidly recruited to laser induced DNA DSBs, where it most likely is involved in the processing of breaks to create ssDNA tails required for initiation of HR repair. Identification of the specific region on hEXO1 required for recruitment of the protein to DSBs can provide insight into the mechanism and interactions involved in DSB recruitment and repair. To identify the specific region on hEXO1 responsible for recruitment to damage sites a panel of hEXO1 protein deletion variants was constructed (Fig. 7A). We observed that the variant YFP-hEXO1-C-term, lacking the NH₂-terminal domain (amino acids 1–405) is recruited to DSBs (Fig. 7B (IV)), indicating that protein recruitment is independent of hMSH3 interaction as well as catalytic activity. This result is confirmed by observations with smaller deletion variants; hEXO1- N, hEXO1- I, hEXO1- NI, and hEXO1- 290–383 (Supplementary data Fig. S3). All of these variants are recruited to laser induced DSBs despite their partial or complete lack of catalytic domains or hMSH3 interaction region. In addition, variants lacking the PIP-box motif (hEXO1-S702X (Fig. 7B (III))) and the hMSH2 interaction region (hEXO1-S598X (Supplementary data Fig. S3)) are efficiently recruited to DSBs, further indicating that hEXO1 recruitment is not dependent on hMSH2 or PCNA interactions. Interestingly, one deletion variant was not recruited to induced DSBs; hEXO1-S702X; 423–507 (Fig. 7B (VII)). This variant is truncated from amino acid 702 and, in addition, contains an internal deletion between amino acids 423 and 507, leaving the variant protein depleted of its two hMLH1 interaction regions. This result strongly suggests that interactions between hMLH1 and hEXO1 are required for recruitment of hEXO1 to DSBs. Both variants; hEXO1-S702X (Fig. 7B (III)) and hEXO1- 423–507 (Fig. 7B (VI)) are recruited to DSBs and so is a 102 amino acid peptide that contain the hEXO1 region 405–507 fused to YFP (Fig. 7B (VIII)). These three constructs contain either the COOH-terminal or internal hMLH1 interaction region, suggesting that partial hMLH1 interaction is sufficient to sustain DSB recruitment of hEXO1.

To further understand the mechanism of hEXO1 recruitment to DSBs, we analyzed recruitment of variants containing amino acids substitutions of significant amino acids, including the ability of PIP-box variants to recruit to laser induced DSBs (Fig. 8B). All substitution variants were recruited to DSBs within minutes of local laser micro-irradiation, supporting the model that hEXO1 DSB recruitment is independent of specific interaction with PCNA. Furthermore, variants of putative PIP-boxes; hEXO1-Q154A;Y157A; hEXO1-Q285A;F288A, and hEXO1-F506A;F507A were investigated as well and all three variants are recruited to laser induced DSBs (Fig. 8B (I), (III), and (V)). Mutations in the MIP-box (hEXO1-F506A;F507A), have been demonstrated to influence hEXO1 hMLH1 interactions [52], but this variant was recruited to DSBs (Fig. 8B (V)), supporting the view that hEXO1

and hMLH1 interactions must be completely disrupted for DSB recruitment to be affected. We additionally confirmed DSB recruitment of the catalytically inactive variant hEXO1-D173A (Fig. 8B (II)). Finally, we observed that although the NLS mutant hEXO1-K418A, described in [43], is excluded from the nucleus in untreated cells, this mutant protein is rapidly recruited to sites of DSBs following laser irradiation (Fig. 8B (IV)), indicating that recruitment is specific and likely mediated by particular protein interactions, which, in addition, facilitates nuclear translocation of hEXO1.

4. Discussion

Through this study we have functionally characterized two distinct regions in hEXO1 required for its recruitment to separate cellular events such as DNA replication and DSB repair. We demonstrate that hEXO1 contains a PIP-box motif located at ⁷⁸⁸QIKLNELW⁷⁹⁵ and that hEXO1 is recruited to replicating DNA through direct interaction with PCNA involving this motif. Yeast EXO1 has been suggested to perform overlapping functions with RAD27 (FEN1) in primer removal and Okazaki fragment maturation [10,14]. FEN1 also co-localizes with PCNA in replication foci [53] and involvement of hEXO1 in Okazaki fragment maturation could explain the strong co-localization of PCNA and hEXO1 in S-phase cells. Generation of ssDNA is a common intermediate in excision repair pathways, recombination repair, and processing of collapsed replication forks and is believed to play a key function in DNA damage signaling. Yeast EXO1 appears to be directly involved in the processing of stalled replication forks by generating ssDNA intermediates that counteract fork regression [54]. A similar role for hEXO1 could explain the observed co-localization of hEXO1 with PCNA replication foci, as this would place hEXO1 physically adjacent to potentially stalled replication forks.

We observe a fast recruitment of PCNA, hMSH2, hMSH6, hMLH1, and hEXO1 to laser induced DNA DSBs (Fig. 2A–E), indicating a direct involvement in the immediate cellular response to this kind of DNA damage. A role of MMR proteins at DSB sites could include suppression of recombination between divergent sequences, thus promoting removal of nonhomologous DNA at the ends or correcting mismatches in heteroduplex DNA. Furthermore, MMR proteins have been suggested to be involved in the initial damage response following DSB induction [55–57]. Along these lines, it has previously been shown that both Pms2- and Msh2-deficient cells are more resistant to IR. These cells are Mlh1-proficient and, therefore, presumably proficient in Mlh1 mediated recruitment of Exo1 to DSBs. Also, low doses of IR have been shown to induce oxidative DNA damage that is recognized by MMR resulting in apoptosis. These results suggest that the MMR complex MutLα (Mlh1-Pms2) is not involved in the Mlh1-dependent recruitment of EXO1 to DNA DSBs [58–60]. Interestingly, Davis et al. [61] demonstrated that hMLH1-deficient cells are more sensitive to IR than the hMLH1-proficient cells. Furthermore, the hMLH1-deficient cells are deficient in G2/M cell cycle arrest following IR but not involved in G1 arrest. These results support our finding that hMLH1 is involved in DSB signaling and/or repair. Recruitment of hEXO1 by hMLH1 to sites of DNA damage particularly following the G1 cell cycle checkpoint, may serve to ensure that hEXO1 excision is able to initiate repair of the DSB by HR using the sister chromatide as template and leading to error-free repair.

In order to map the region on hEXO1 required for DSB recruitment, we investigated DSB recruitment of YFP-hEXO1 COOH-terminal truncation variants, YFP-hEXO1 NH₂-terminal or internal deletion variants as well as specific amino acid substituted variants (Figs. 7 and 8 and Supplementary data Fig. S3). We found the truncation variants (C508X, S598X and S702X) and the PIP-box mutant (Q788A;L791A) to be recruited to DSBs similar to WT YFP-hEXO1, clearly implying that hEXO1 recruitment is independent of direct PCNA or hMSH2 interaction. Furthermore, our data supports the notion that hEXO1 recruitment to DSBs is independent of its catalytic activity as well as hMSH3 interaction. Interestingly, we recorded that the variant hEXO1-S702X; 423–507, incapable of hMLH1 interaction (Supplemental data Fig. S1), is not recruited to laser induced DSBs. In addition, variants; hEXO1-S702X and hEXO1- 423–507 which sustain partial hMLH1 interaction are recruited to DSBs. These results demonstrate that recruitment of hEXO1 to DSBs is highly dependent on interaction with hMLH1, as even partial hMLH1 interaction is sufficient for recruitment. Investigation of hEXO1 recruitment in the hMLH1 deficient cell line; HCT116 substantiates the proposed mechanism for hEXO1 DSB recruitment. hEXO1 is recruited less efficient to laser induced DSBs in HCT116 when compared with DSB recruitment in the hMLH1 reconstituted cell line; HCT116 + Chr.3 (Supplementary data Fig. S5). Furthermore, hEXO1 co-localize with PCNA in replication foci in the HCT116 cell line (Supplementary data Fig. S5), confirming that hMLH1 is specifically required for DSB recruitment, whereas other factors influence localization of hEXO1 at replication foci. DSB recruitment of nuclear localization defective variants; hEXO1-K418A and hEXO1- 406–507 shows that these variants are translocated from the cytoplasm into the nucleus in response to laser induced DNA damage, underscoring that recruitment of hEXO1 to DSBs is specific and not just a result of a general damage response. We propose that this specificity is maintained through interactions between hEXO1 and hMLH1.

BLM helicase stimulates hEXO1 DNA resection via direct protein interaction and these resected ends are used by human RAD51 to promote homologous DNA pairing [25], providing evidence for a role of hEXO1 in recombination repair at DNA DSBs. We observed recruitment of hEXO1 to DSBs in fibroblast derived from both BLM and WRN patients (Supplemental data Fig. S4), suggesting that hEXO1 recruitment to DSBs is independent of these DSB-associated RecQ helicases. Furthermore, it was recently reported that recruitment of hEXO1 to DSBs is required for normal repair of breaks through the HR pathway, and that phosphorylation of specific residues on hEXO1 is involved in regulation of hEXO1 during DSB repair [23]. However, we observed that hEXO1 phosphorylation status does not influence the initial recruitment of the protein to DSBs (Andersen and Rasmussen, unpublished).

Our data shows that hEXO1 can be located at replication foci and recruited to laser induced DSBs within the same cell. How is the switch between activity at DNA replication foci and DSBs regulated? We suggest that alternating protein interactions are crucial for the localization/recruitment of hEXO1 to specific events. hEXO1 interacts with PCNA through its PIP-box (⁷⁸⁸QIKLNELW⁷⁹⁵), and this interaction assures localization of hEXO1 at the replication fork. On the contrary, direct PCNA interaction is not required for recruitment of hEXO1 to DNA DSBs, which instead is mediated by interaction with hMLH1. It could be speculated that the DSB damage response involving hMLH1 as an initial damage sensor, is

mediated through recruitment of hEXO1, which then creates ssDNA tails amplifying the actual damage response. The MMR systems role during HR is generally believed to involve modulation of HR by inhibition of recombination between genetically divergent sequences and correction of heteroduplex DNA generated during the process of recombination [3]. However, observations support a role for hMLH1 in the initial steps of HR during DSB repair as well as in damage signaling [55–57]. Interestingly, hEXO1 has been reported to be involved in the signaling of specific DNA damages [17,23,62,63] and thus it could be speculated that hMLH1-associated DSB damage signaling is mediated through recruitment and regulation of hEXO1 activity. This is supported by previously published data from our group showing that exonuclease activity in a hEXO1-hMLH1 complex is differentially regulated on a DNA substrate resembling a DSB [15]. We showed that hEXO1-hMLH1 interaction inhibits exonuclease activity on a heteroduplex substrate suggesting a biological role in MMR since stimulation of hEXO1 activity requires interaction with hMLH1 and hPMS2. We also showed that hEXO1-hMLH1 interaction did not inhibit exonuclease activity on a homoduplex substrate suggesting a potential biological role in HR repair.

In conclusion, our data provide insight into the mechanism of functional separation by hEXO1. We show that *in vivo* recruitment of hEXO1 to DSBs and to PCNA replication foci is mediated by different mechanisms and protein motifs. Complex formation with PCNA directs hEXO1 to DNA replication foci whereas hEXO1 recruitment to DSBs appears to be independent of interactions with PCNA and MSH2, but instead involves hMLH1 interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.dnarep.2010.09.023.

Fig. 1.

Sub-nuclear localization of human MMR proteins. NIH3T3 cells were transiently transfected with plasmid DNA and incubated for 24–48 h before fluorescent fusion proteins were visualized by confocal imaging; CFP (green), YFP (red), and co-localization (yellow). (A) CFP-PCNA and YFP-hEXO1, from the left; early S-phase cells are characterized by numerous small foci evenly distributed throughout the nucleus, mid S-phase cells are characterized by an enlargement of foci size, reduction in the number of foci and alignment of foci at the nuclear periphery, and finally late S-phase cells are characterized by few and larger foci. (B) CFP-PCNA and YFP-hMLH1; diffuse nuclear distribution of hMLH1 seen together with PCNA foci in all three stages of the cell cycle: early S-phase (left), mid S-phase (center), or late S-phase (right). (C) CFP-PCNA and YFP-hMSH2; hMSH2 forms nuclear foci co-localizing with PCNA in early (left), mid (center), and late (right) S-phase cells. (D) CFP-PCNA and YFP-hMSH6; hMSH6 forms nuclear foci co-localizing with PCNA in early (left), mid (center), and late (right) S-phase cells. The percentage of pixels overlapping (co-localizing proteins) in the presented images is displayed in the table.

Fig. 2.

Recruitment of MMR proteins to micropoint laser induced DSBs. HeLa cells were transiently transfected with plasmid DNA and incubated for 24 h for expression of fluorescent fusion proteins; CFP (cyan) or YFP (green). Expressing cells were irradiated using a 435 nm micropoint laser and real time protein recruitment monitored in the relevant channels. Extended focus view of fluorescing cells obtained by Z-stack imaging 2 min after micropoint laser irradiation. The pictures represent typical targeting of 1–3 cells in one round of laser firing. Following micro-irradiation, cells were fixed, blocked, and stained with primary antibodies against XRCC1 and γ H2AX combined with Alexa Fluor secondary

antibodies and detected with confocal fluorescence microscopy. (A) YFP-hEXO1 expressing cells targeted in a thick line using 11% laser intensity (B) CFP-PCNA expressing cells targeted in 1 or 2 thick lines using 8% laser intensity (C) CFP-hMSH2 expressing cells targeted in a thick line using 11% laser intensity (D) CFP-hMSH6 expressing cells targeted in a thick line using 11% laser intensity (E) YFP-hMLH1 expressing cells targeted in a thick line using 14% laser intensity.

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Fig. 3. Nuclear foci formation and DNA double strand break recruitment of YFP-hEXO1 COOH-terminal truncated variant. (A) Consensus PIP-box, (h) is a hydrophobic residue and (a) are aromatic residues. EXO1 sequences from the following species were aligned; human (NM_130398), chimpanzee (XM_514304), dog (XP_547491), mouse (NP_036142), and rat (XP_222932). Using homology search and bioinformatic tools Jpred3 and PredictProtein putative PIP-box motifs in conserved regions were identified and indicated. (B) hEXO1 protein domain structure, regions involved in protein interactions are specified; hMSH3 (green), hMSH2 (blue), and hMLH1 (pink), nuclease domains N and I, and the nuclear localization signal (NLS). Positions of putative PIP boxes and amino acid substitutions are indicated. Amino acid substitutions were introduced with site directed mutagenesis. (C) NIH3T3 cells were transiently transfected with plasmid DNA and incubated for 24–48 h before visualization of fusion proteins by confocal imaging; CFP (green), YFP (red), and co-localization (yellow). PCNA forms foci but the COOH-terminally truncated variant is unable to form foci as seen by the diffuse nuclear staining pattern. The percentage of pixels overlapping (co-localizing proteins) in the presented image is displayed in the table. (D) HeLa cells were transiently transfected with plasmid DNA and incubated for 24 h for expression of fluorescent fusion protein YFP-hEXO1-S702X (light green). Expressing cells were irradiated using a 435 nm micropoint laser at 14% laser intensity, and extended focus view of fluorescing cells obtained by Z-stack imaging 2 min after micropoint laser irradiation. Recruited protein fluorescence at the irradiated path line is indicated by an arrow for each cell for clarification. Following micro-irradiation, cells were fixed and stained with antibodies against XRCC1 and γ H2AX to confirm induction of SSBs and DSBs at the laser irradiated path line (not shown).

Fig. 4.

Sub-nuclear localization and co-localization of CFP-PCNA and YFP-hEXO1 putative PIP-box variants. NIH3T3 cells were transiently transfected with plasmid DNA and incubated for 24–48 h before fusion proteins were visualized by confocal imaging; CFP (green), YFP (red), and co-localization (yellow). (A) YFP-hEXO1-Q788A;L791A from the left; YFP-hEXO1-Q788A;L791A does not form foci in early, mid or late S-phase cells (B) YFP-hEXO1-Q154A;Y157A from the left; early S-phase cells showing co-localization of foci and mid S-phase cells with a less distinct co-localization (C) YFP-hEXO1-Q285A;F288A from the left; early S-phase cells showing co-localization of foci and mid S-phase cells showing a less specific co-localization (D) YFP-hEXO1-F506A;F507A from the left; early S-phase cells with no YFP-hEXO1-F506A;F507A foci and non S-phase cells displaying cytoplasmic localization of hEXO1-F506A;F507A (red). The percentage of pixels overlapping (co-localizing proteins) in the presented images is displayed in the table (n.d.: not determined).

Fig. 5.

Distribution of nuclear foci in cells co-expressing CFP-PCNA and YFP-hEXO1 variants. For each experiment 50 co-transfected cells were scored according to categories described herein. (A) Cells co-expressing hEXO1-WT and PCNA display co-localization throughout S-phase (B) Variant hEXO1-Q788A;L791A is unable to form replication foci, but expression does not affect the formation of replication foci by PCNA. (C) Cells expressing hEXO1-Q154A;Y157A display a higher proportion with no foci and early S phase foci, when compared to cells expressing hEXO1 WT, this applies for both hEXO1 and PCNA

replication foci. (D) Cells expressing hEXO1-Q285A;F288A display a higher proportion with no foci and early S phase foci, when compared to cells expressing hEXO1 WT, this applies for both hEXO1 and PCNA replication foci. (E) Cells expressing the variant hEXO1-F506A;F507A did not form replication foci by hEXO1 and only very few by PCNA.

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Fig. 6.

Binding of hEXO1 peptides to PCNA assessed by isothermal titration calorimetry. ITC measurements of the physical interaction between purified PCNA and hEXO1 peptides p22 (aa 783–804) and p16 (aa 498–513) containing the PIP-box (QIKLNELW) and MIP-box (RSRFF) sequences, respectively. (A) Thermogram of titration of hEXO1 p22 into pure buffer (B) Thermogram and binding isotherm of titration of hEXO1 p22 into PCNA solution at 30 °C (C) Thermogram of titration of hEXO1 p16 into PCNA solution at 30 °C (D) Thermogram of titration of hEXO1 p16 into PCNA solution at 10 °C (E) Table of thermodynamic parameters obtained for different PIP-box containing peptides for comparisons.

Fig. 7.

Mapping of region on hEXO1 required for recruitment to laser induced DSBs. (A) Map of hEXO1 indicating protein interaction regions and schematic overview of truncation and deletion variants (I) YFP, (II) YFP-hEXO1, (III) YFP-hEXO1-S702X, (IV) YFP-hEXO1-C-term, (V) YFP-hEXO1- 406–507, (VI) YFP-hEXO1- 423–507 (VII) YFP-hEXO1-S702X; 423–507, (VIII) YFP-405–507. (B) HeLa cells were transiently transfected with plasmid DNA and incubated for 24 h for expression of fluorescent fusion proteins; (II) YFP-hEXO1, (III) YFP-hEXO1-S702X, (IV) YFP-hEXO1-C-term, (V) YFP-hEXO1- 406–507, (VI) YFP-hEXO1- 423–507 (VII) YFP-hEXO1-S702X; 423–507, (VIII) YFP-405–507. Expressing cells were irradiated using a 435 nm micropoint laser at 8–11% laser intensity, and extended focus view of fluorescing cells obtained by Z-stack imaging 2 min after micropoint irradiation. Recruited protein fluorescence at the irradiated path line is indicated by an arrow for each cell for clarification. The pictures represent typical targeting of 1–3 cells in one round of laser firing. Following micro-irradiation, cells were fixed and stained with antibodies against XRCC1 and γ H2AX to confirm induction of SSBs and DSBs at the laser irradiated path line (not shown).

Fig. 8.

Recruitment of hEXO1 variants to laser induced DSBs. (A) Map of hEXO1 indicating protein interaction regions and sites of amino acid variant introduction (I) YFP-hEXO1-Q154A;Y157A, (II) YFP-hEXO1-D173A, (III) YFP-hEXO1-Q285A;F288A, (IV) YFP-hEXO1-K418A, (V) YFP-hEXO1-F506A;F507A, (VI) YFP-hEXO1-Q788A;L791A (B) HeLa cells were transiently transfected with plasmid DNA and incubated for 24 h for expression of fluorescent fusion proteins; (I) YFP-hEXO1-Q154A;Y157A, (II) YFP-hEXO1-D173A, (III) YFP-hEXO1-Q285A;F288A, (IV) YFP-hEXO1-K418A, (V) YFP-hEXO1-F506A;F507A, (VI) YFP-hEXO1-Q788A;L791A, (VII) YFP-hEXO1-Q788A;L791A;L794A;W795A A;L794A;W795A. Expressing cells were irradiated using a 435 nm micropoint laser at 14% laser intensity, and extended focus view of fluorescing cells obtained by Z-stack imaging 2 min after micropoint irradiation. Recruited protein fluorescence at the irradiated path line is indicated by an arrow for each cell for clarification. The pictures represent typical targeting of 1–3 cells in one round of laser firing. Following micro-irradiation, cells were fixed and stained with antibodies against XRCC1 and γ H2AX to confirm induction of SSBs and DSBs at the laser irradiated path line (not shown).

Distribution of nuclear foci in CFP-PCNA and YFP-hEXO1 co-transfected NIH-3T3 cells. For each transfection 50 co-transfected cells were categorized in the following categories; “No foci” refers to CFP-PCNA displaying diffuse cytoplasmic staining and YFP-hEXO1 (or variants) displaying a diffuse nuclear staining, “small foci” refers to CFP-PCNA or YFP-hEXO1 (or variants) appearing in characteristic early S-phase foci (many small foci), “mid foci” refers to CFP-PCNA or YFP-hEXO1 (or variants) appearing in characteristic mid S-phase foci (small foci and foci alignment at the periphery of the nucleus), “large foci” refers to CFP-PCNA or YFP-hEXO1 (or variants) observed in characteristic late S-phase foci (fewer and larger foci) and “Cytoplasmic” refers to whole cell distribution and is only recorded for YFP-hEXO1 (or variants) as cytoplasmic distribution of CFP-PCNA is included in the “No foci” category.

Table 1

Distribution of nuclear foci in co-transfected NIH-3T3 cells (%)							
Proteins expressed	No foci	Small foci	Mid Foci	Large foci	Cytoplasmic	Total cells	
CFP-PCNA and YFP-hEXO1	CFP	46	28	14	12	0	50
	YFP	40	28	14	12	6	
CFP-PCNA and YFP-hEXO1-S702X	CFP	54	18	20	8	0	50
	YFP	100	0	0	0	0	
CFP-PCNA and YFP-hEXO1-Q154A;Y157A	CFP	68	28	4	0	0	50
	YFP	66	28	4	0	2	
CFP-PCNA and YFP-hEXO1-Q285A;F288A	CFP	66	34	0	0	0	50
	YFP	58	34	0	0	8	
CFP-PCNA and YFP-hEXO1-F506A;F507A	CFP	96	4	0	0	0	50
	YFP	76	0	0	0	24	
CFP-PCNA and YFP-hEXO1-Q788A;L791A	CFP	52	22	18	8	0	50
	YFP	98	0	0	0	2	