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14-3-3 checkpoint regulatory proteins interact specifically with DNA repair protein human exonuclease 1 (hEXO1) *via* a semi-conserved motif

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Abstract

Human exonuclease 1 (hEXO1) acts directly in diverse DNA processing events, including replication, mismatch repair (MMR), and double strand break repair (DSBR), and it was also recently described to function as damage sensor and apoptosis inducer following DNA damage. In contrast, 14-3-3 proteins are regulatory phosphoserine/threonine binding proteins involved in the control of diverse cellular events, including cell cycle checkpoint and apoptosis signaling. hEXO1 is regulated by post-translation Ser/Thr phosphorylation in a yet not fully clarified manner, but evidently three phosphorylation sites are specifically induced by replication inhibition leading to protein ubiquitination and degradation. We demonstrate direct and robust interaction between hEXO1 and six of the seven 14-3-3 isoforms *in vitro*, suggestive of a novel protein interaction network between DNA repair and cell cycle control. Binding experiments reveal weak affinity of the more selective isoform 14-3-3 σ but both 14-3-3 isoforms η and σ significantly stimulate hEXO1 activity, indicating that these regulatory proteins exert a common regulation mode on hEXO1. Results demonstrate that binding involves the phosphorable amino acid S746 in hEXO1 and most likely a second unidentified binding motif. 14-3-3 associations do not appear to directly influence hEXO1 *in vitro* nuclease activity or *in vitro* DNA replication initiation. Moreover, specific phosphorylation variants, including hEXO1 S746A, are efficiently imported to the nucleus; to associate with PCNA in distinct replication foci and respond to DNA double strand

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2011.11.007.

breaks (DSBs), indicating that 14-3-3 binding does not involve regulating the subcellular distribution of hEXO1. Altogether, these results suggest that association may be related to regulation of hEXO1 availability during the DNA damage response to plausibly prevent extensive DNA resection at the damage site, as supported by recent studies.

Keywords

hEXO1; 14-3-3; DNA repair; Cell cycle control; DNA damage; Protein phosphorylation

1. Introduction

Human exonuclease 1 (hEXO1) is a 5' → 3' directed DNA nuclease playing significant roles in diverse DNA metabolic processes, including DNA replication, DNA mismatch repair (MMR) and DNA double strand break repair (DSBR) by homologous recombination (HR) [1–7]. Moreover, studies indicate a prominent function for hEXO1 and its mouse and yeast homologs in various cellular processes, including gene conversion, class switch recombination (CSR), somatic hypermutation (SH), telomere processing, post-replication repair (PRR) and apoptosis signaling following DNA damage [8–15]. Together, these studies provide a distinct picture of a multi-functional protein involved in biological events monitoring and preserving genomic fitness.

The catalytic domain of hEXO1 can degrade ssDNA exonucleolytically but shows a preference for dsDNA substrates [16]. In addition, it displays endonucleolytic incision activity on flap structure specific 5' overhangs. The crystal structure of the catalytic domain was recently solved and revealed that hEXO1 employs a common nuclease mechanism to recognize and process DNA, involving bending the DNA strand at nicks or gaps [17]. The functional characteristics of hEXO1 help to explain its involvement in different DNA processing events. The nucleolytic activity of hEXO1 is often regulated by other proteins, either stimulating or inhibiting hEXO1 activity by DNA loading or displacement. Specifically, *in vitro* MMR assays show that hEXO1 removes a DNA stretch containing the misincorporated base by exonucleolytic DNA degradation, a task which is tightly regulated by MMR components MutSα and MutLα *via* direct protein–protein interaction [18,19]. Examination of protein structure suggests that regulation is allosteric and that the carboxyl terminal of hEXO1 functions as an autoinhibitory element which is relieved upon protein association [17]. The role of hEXO1 in DNA replication is not well defined, but the recombinant protein does not discriminate between RNA and DNA substrates *in vitro* and ectopically expressed hEXO1 co-localizes with PCNA in replication foci throughout S-phase, indicating that it might function as a backup protein for FEN1 in primer removal [1,3]. hEXO1 is regulated by phosphorylation upon replication inhibition, leading to protein ubiquitination and degradation, respectively [20]. Different studies support a functional role for EXO1 during HR of DSBs either in resolving intermediate recombination structures or during the initiating 5' resection step. hEXO1 interacts with helicases WRN and RecQ1 and both proteins stimulate its enzymatic activity *in vitro* [21,22], a scenario presumed to relate to role for hEXO1 in resolution of recombination repair intermediates. Conversely, it was recently found that recruitment of hEXO1 to DNA DSBs is dependent on CtIP and MRN

[6], pointing to a role for hEXO1 in early resection of the DSB, as demonstrated for the yeast protein [23,24]. Reconstitution of the resection reaction with yeast proteins demonstrates that MRX and Sae2 stimulates EXO1 5' resection, by promoting its binding to DNA ends and performing initial 5' strand processing [25]. In comparison, resection reactions with recombinant CtIP shows that it interacts directly with hEXO1 but appears to inhibit its nuclease activity *in vitro*, possibly to prevent excessive DNA degradation [6]. Other studies with human cell lines show that BLM and hEXO1 work in parallel pathways to promote DNA resection at the DSB site, to induce DSB signaling and to confer resistance to DSB inducing agents [5]. hEXO1 excision activity is stimulated by direct association with BLM *in vitro* [26] indicating that the proteins may function in conjunction for optimal DSB resection. In fact, current models based on reconstituted *in vitro* DNA resection reactions suggest that processing of DNA ends may occur by two routes; either *via* DNA unwinding by BLM followed by DNA2 resection or *via* hEXO1 resection which is specifically stimulated by BLM enhancing its affinity for DNA ends and by MRN recruitment [27].

In our search to unravel more details on the cellular role and function of hEXO1, we looked for new interaction partners for hEXO1 and we report here the specific association of hEXO1 with checkpoint regulatory proteins 14-3-3. The 14-3-3 proteins comprise a large family of highly conserved dimeric regulatory proteins that specifically recognize and bind to phosphorylated Ser or Thr residues enclosed in a sequence specific context in the ligand: RSx(pS/T)xP (mode 1) or Rxxx(pS/T)xP (mode 2) [28–30]. Upon ligand binding 14-3-3 proteins usually modulate the function or cellular compartmentalization of the associated protein or they may mediate protein interaction between proteins *via* molecular scaffolding. The 7 human isoforms: β , γ , ϵ , ζ , η , τ and σ , display high sequence homology enabling them to form both homo- and heterodimers. The dimers form a U-shaped complex structure enclosing two oppositely oriented amphipathic grooves at the inside bottom, each able to coordinate binding of an extended phosphopeptide [31]. More than 200 proteins have been identified as 14-3-3 ligands, including protein kinases, receptor proteins, enzymes, cell structural proteins and G proteins. Multiple 14-3-3 ligands are involved in various cellular processes entailing DNA damage signaling, transcriptional control, cell cycle regulation and apoptosis. Some ligands bind to all isoforms while others display association with a few or only a single isoform. Direct binding to 14-3-3 is usually regulated by kinases and phosphatases that determine ligand phosphorylation status at the phosphoserine/threonine binding motif. hEXO1 is modified by posttranslational phosphorylation of 10 Ser and 2 Thr residues located primarily in its serine rich carboxyl terminus [32] (Fig. 4A). Three of these residues are phosphorylated in response to replication inhibition by hydroxyurea (HU), while the remaining appears to be phosphorylated under basal cellular conditions. The significance of these phosphorylation events remains to be established.

A recent study reported positive interaction between three isoforms of 14-3-3 and an NH₂-terminus truncated form of hEXO1 in a yeast two-hybrid (YTH) screen, while ectopically expressed Omni-hEXO1 was recovered from immunoprecipitations of 14-3-3 using a pan 14-3-3 antibody, both suggestive of protein complex formation *in vivo* [33]. We now expand on these results and provide evidence of a specific interaction between hEXO1 and all 7 isoforms of 14-3-3, as demonstrated by *in vitro* GST pulldown assays. This interaction

scheme indicates a common regulation mode of hEXO1, which is confirmed by demonstration of weak binding affinity towards the more selective isoform 14-3-3 σ . Specific binding by 14-3-3 involves amino acid S746 in hEXO1, which is reportedly phosphorylated under basal cellular conditions and surrounded by a motif that resembles the generally defined 14-3-3 binding motifs. 14-3-3 association does in some cases influence the nuclease activity of hEXO1 but does not directly influence *in vitro* DNA replication initiation, and results using specific phosphorylation variants indicate that the subcellular distribution of hEXO1 is not regulated by 14-3-3 binding. We propose three scenarios that could account for the functional outcome of 14-3-3 regulation of hEXO1 involving its specific regulation during the DNA damage response. These include indirect inhibition of activity by either degradation or phosphorylation or interference with the apoptotic role of hEXO1.

2. Materials and methods

2.1. DNA cloning

The hEXO1b allele contained in applied constructs has previously been described (Genbank: AAD13754 [36]). Single amino acid substitutions were introduced into pcDNA 3.1/His C hEXO1 by site directed mutagenesis and all constructs were checked by DNA sequencing. hEXO1 wild type (WT) and variants were sub-cloned from pcDNA 3.1/His C into pEYFP C1 by restriction enzyme digestion (*Bam*HI and *Apa*I) and ligation (*Bg*III and *Apa*I).

2.2. Protein purification

Recombinant GST-MLH1 and GST-MSH2 were purified as described in [34]. Briefly, non-induced cell cultures were grown in 2X YT for time periods of 5–6 h. Pelleted cells were resuspended in PBS containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche) and lysed according to protocol (Pharmacia Biotech). Cellular lysates were bound to GST beads (Glutathione Sepharose™ 4B, Amersham Biosciences) for 2 h at 4 °C using a rocking platform. Beads were washed in PBS and fusion proteins eluted with Glutathione Elution Buffer. Likewise, recombinant GST-14-3-3 isoforms were purified as described in [35] and GST-Importin β and His-Importin α 3 were purified as described in [36]. His-14-3-3 ζ and His-14-3-3 ζ K49E were purified as described in [37]. hEXO1 and His-hEXO1 was expressed and purified from SF9 insect cells using the Bac-to-Bac baculovirus system (Invitrogen) according to manufacturer's guidelines. Briefly, 400 ml insect cells were cultured at 27 °C in serum-free HyQ-SFX medium (HyClone, Inc.) containing 50 μ g/ml gentamicin. At a density of $1\text{--}1.5 \times 10^6$ cells/ml the culture was infected with baculovirus expressing hEXO1 or His-hEXO1 at a MOI of approximately 10 and incubation was continued for 60 h. Infected cells were collected by centrifugation, the supernatant was discarded and the cell pellet resuspended in icecold phosphate buffer (2–3 g/ml) containing salt (NaCl or KCl) and glycerol. Pellets were snap-frozen in liquid nitrogen.

hEXO1 was purified as described in [4], with the exception of NaCl replacing KCl in all buffers. Briefly, the cell suspension was dissolved in phosphate buffer containing 150 mM NaCl, glycerol, EDTA and protease inhibitors as described. Cellular debris were removed by centrifugation and the cleared supernatant was first loaded onto a 5 ml pre-equilibrated

HiTrap Q FF anion-exchange column (Amersham Biosciences), which was developed with a 40 ml linear NaCl gradient (145–640 mM). hEXO1 containing fractions were combined and diluted to a conductivity equivalent to ~200 mM NaCl. The solution was then loaded onto a pre-equilibrated 1 ml HiTrap Heparin HP column (Amersham Biosciences) developed with a 20 ml linear NaCl gradient (190–550 mM). hEXO1 containing fractions were again combined and diluted to a conductivity equivalent to ~200 mM NaCl and now loaded onto a pre-equilibrated 1 ml FPLC Mono S column (HR 5/5, Amersham Biosciences) developed with a 20 ml linear NaCl gradient (190–640 mM). Peak fractions (95% pure) were pooled and distributed into 50 µl aliquots, which were snap frozen in liquid nitrogen. All collected fractions were supplemented with protease inhibitors as described in [4].

For purification of His-hEXO1 the cell suspension was dissolved in 15 ml phosphate buffer containing 200 mM NaCl, glycerol and protease inhibitors. Cellular debris were removed by centrifugation and the cleared supernatant was loaded onto a pre-equilibrated 1 ml HisTrap™ HP Ni²⁺ column (Amersham Biosciences) which was developed with a 20 ml linear imidazole gradient (50–300 mM). Collected fractions were supplement with EDTA and protease inhibitors. His-hEXO1 containing fractions were pooled and loaded onto a pre-equilibrated 1 ml FPLC Mono S column (HR 5/5, Amersham Biosciences), which was developed with a 10 ml linear NaCl gradient (190–640 mM). Peak fractions (95% pure) supplemented with protease inhibitors were pooled and distributed into 50 µl aliquots that were snap frozen in liquid nitrogen.

2.3. Cells

Murine NIH-3T3 and HeLa 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 µg/ml) (Gibco BRL, Life technologies) or hygromycin (1:250). All cell lines were grown at 37 °C in a humidified atmosphere containing 5–10% CO₂.

2.4. GST pull-down assay

Aliquots of 25 µl GST-beads were prepared in 50% slurry in binding buffer (20 mM Tris–HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.1% Tween 20 (Sigma), 0.75 mg/ml BSA) containing protease inhibitors (Roche). 1 µg GST or GST fusion protein (MSH2, MLH1, 14-3-3 or Importin β) was bound to each bead aliquot by incubation for 2 h at 4 °C on a rocking platform. Protein-bound beads were washed three times with binding buffer, diluted to approximately 100 µl GST-beads/ml and incubated for 30 min at 4°C. Recombinant His tagged Importin α3 was added and incubated along with the binding buffer in molar amounts comparable to Importin β for the import factor binding studies. R18 peptide was similarly added and incubated along with the binding buffer in excess amounts for the GST-14-3-3β and GST-MLH1 binding studies. *In vitro* transcribed translated (IVTT) proteins were prepared in advance using the TNT Coupled Reticulocyte Lysate Systems (version L4610 or L1170, Promega), appropriate expression vectors and [³⁵S]-Cysteine or [³⁵S]-Methionine (PerkinElmer) according to manufacturer's instructions. Labeled proteins were visualized by denaturing PAGE combined with phosphorimaging using a STORM 840 scanner (Amersham Pharmacia Biotech). Comparable IVTT protein amounts were added to each GST bead aliquot and beads were incubated for another 2 h at 4°C. Beads were then

washed three times, resolved by SDS-PAGE and bound proteins visualized by phosphorimaging. For pull-downs with recombinant hEXO1, GST-14-3-3 bound beads were diluted to 250 μ l GST-beads/ml and incubated for 30 min at 4°C. Approximately 400 ng recombinant hEXO1 was added and beads were incubated for 2 h at 4°C. Beads were washed three times and resolved by SDS-PAGE. Bound hEXO1 was visualized by Western Blotting using primary antibody Exo1 Ab-4 (Thermo Scientific, dilution 1:500) against hEXO1. Immunostained bands were visualized with SuperSignal Western blotting detection system (Pierce, IL).

2.5. Exonuclease assay

Excision assay was carried out by radiolabeled oligo A9 (5'-CCAGTGAATTCGAGCTCGGTACCCGCTAGCGGGGATCCTCTA), 5' end labeled with T4 PNK (NEB) and [γ -³²P]-ATP (PerkinElmer). Labeling reactions were incubated for 60 min at 37 °C. Non-incorporated [γ -³²P]-ATP was removed by illustra MicroSpin column G-50 (GE-Healthcare). Radiolabeled oligo was annealed with oligo A2 (5'-TAGAGGATCCCCGCTAGCGGGTACCGAGCTCGAATTCACTGG) in presents 100 mM KCl final concentration and incubated 10 min at 90 °C followed by cooling to RT in the heating block. 10 μ l exonuclease reactions contained 0.06 pmol radioactive labeled substrate and around 1 ng hEXO1 in 20 mM Hepes buffer containing 50 mM KCl, 0.5 mM DTT, 5 mM MgCl₂, 0.05% Triton X-100, 5% glycerol and 100 μ g/ml BSA. Reactions were incubated for 30 min at 37 °C and stopped by addition of 3.5 μ l formamide buffer and heated 90 °C for 5 min. The whole mixes were separated on a 12% denaturing polyacrylamide gel and reaction products visualized by phosphorimaging. Images product and excised product ratio was determined with ImageQuant 5.2 (Molecular Dynamics). For evaluation of 14-3-3 isoforms impact on excision activity reactions were supplemented with excess recombinant GST-14-3-3 or His-14-3-3 ζ WT or K49E.

Statistical comparisons were determined by GraphPad Prism 5.04 software (La Jolla, CA, USA) by use of a Paired Student's *t* test, two tail, confidence interval 99%). All values shown (graphs 2B) are the mean $\pm$ S.D., significant are indicated with a *p* value based on six separate biological experiments.

2.6. In vitro DNA replication assay

In vitro DNA replication assays were performed essentially as described in [38]. Briefly, HeLa cell cytosolic and nuclear extracts were incubated for 1 h at 30 °C with XL-Topo or XL-Topo containing the p186 minimal region of the *ors8* origin of DNA replication previously propagated in *dam* positive bacteria, in the presence of α -³²P-dCTP and α -³²P-TTP. Half of the reaction was left untreated while the other half was digested with *DpnI*. The samples were separated by agarose gel electrophoresis, the gels were dried and the reaction products were visualized by autoradiography. Recombinant hEXO1, 14-3-3 ϵ , or both were incubated with the cellular extracts on ice for 30 min before addition to the *in vitro* replication assay.

2.7. Confocal laser scanning microscopy

Transient transfections in NIH-3T3 and HeLa cells were performed with ExGen500 (Fermentas) and Fugene 6 (Roche), respectively, according to manufacturer's instructions. Briefly, around 2×10^5 cells/dish were seeded on glassbottom dishes (WillCo Dish, HBSt 5040) 24 h prior to transfection. Cells were transfected with 3 μ g relevant DNA vectors in total and incubated for 24–48 h before expression and sub-cellular localization of transiently expressed CFP and YFP fusion proteins were examined with a confocal Zeiss LSM510 microscope, using a 40 $\times$ oil objective. The microscope was coupled to a computer controlling image acquisition and data processing using integrated software.

2.8. Micropoint laser irradiation

HeLa cells were transfected with lipofectAMINE2000 (Life Technologies Inc., USA) according to manufacturer's instructions using 4 μ g of the relevant vectors. Targeted DNA damage was introduced, using the MicroPoint® Ablation Laser System from Photonic Instruments as described in [1]. Fluorescent protein recruitment was monitored in the appropriate channel for at least 2 min and the cells were then fixed and stained with antibodies against γ H2AX (Ser 139, 05–636, dilution 1:250, Upstate) and XRCC1 (H-300, Sc-11429, dilution 1:250, Santa Cruz), as described in [1]. Antibody signals were detected with a Nikon eclipse 2000E confocal microscope containing five imaging modules.

2.9. Yeast two-hybrid assay

A modified version of the yeast two-hybrid system, the interaction trap, was used essentially as previously described in [39,40]. Briefly, *Saccharomyces cerevisiae* EGY48 (*MATa trp1 ura3 his3 LEUL2::pLexAop6-LEU2*) harboring pSH18-34, the LacZ reporter plasmid, was used as a host for all interaction experiments. For protein–protein interaction studies, EGY48/pSH18-34 was cotransformed with pEG202 derivatives and pJG4-5 derivatives. Transformants were selected by complementation of auxotrophies using *TRP1*, *URA3*, and *HIS3* markers on plasmids. Positive protein–protein interactions were visualized by streaking colonies on selective plates with galactose (2%) and raffinose (1%) containing chlorophenyl-red- β -D-galactopyranoside (Roche) as a chromogenic substrate for β -galactosidase reporter.

2.20. Immunoprecipitation

HEK-293 cells were transiently transfected with Omni-tagged hEXO1 deletion mutant constructs and immunoprecipitation was performed using a pan-14-3-3 antibody (rabbit polyclonal SA-483 BIOMOL) as described in [33]. Proteins were detected with the same pan-14-3-3 antibody or with an Omni antibody (goat polyclonal M21 sc- 499 Santa Cruz).

3. Results

3.1. hEXO1 interacts specifically with checkpoint regulatory 14-3-3 proteins

Three isoforms of 14-3-3 were recently found to interact with N-hEXO1 in a YTH screen employed to identify new interaction partners for hEXO1 [33]. In this study, the reverse approach, intended to map the 14-3-3 protein interactome, identified hEXO1 as a novel

binding partner for 14-3-3 ζ , when a human HeLa cell cDNA library was used to screen for gene products that interact with 14-3-3 proteins [39,41]. To examine whether the interaction of hEXO1 with 14-3-3 is specific for the ζ isoform, each of the human 14-3-3 isoforms (ζ , β , ϵ , γ , η , σ , and τ) were cloned into yeast two-hybrid vector pJG and tested for interaction with hEXO1. As indicated by the appearance of blue color (Fig. 1A), hEXO1 showed positive interaction with all 14-3-3 isoforms, including ζ , in this assay. Co-immunoprecipitation data from HEK-293 cells transiently transfected with Omni-tagged hEXO1 using a pan-14-3-3 antibody confirmed *in vivo* complex formation between hEXO1 and 14-3-3 (Supplementary data, Fig. S1).

Coordination to all isoforms suggests that hEXO1 utilizes the well defined ligand-binding site, the amphipathic groove in 14-3-3 proteins [42] for binding. To evaluate the specificity of interaction, we utilized well-characterized mutants of 14-3-3 ζ deficient in client protein binding, including mutants of K49 and V176, two key residues in the basic cluster and hydrophobic patch in the 14-3-3 amphipathic groove. Similar to Raf-1, a well characterized 14-3-3 ligand [39–43], hEXO1 showed decreased interaction with 14-3-3 ζ K49E and 14-3-3 ζ V176D (Fig. 1A; lower panel), suggesting that 14-3-3 specifically interacts with hEXO1 through key residues in its amphipathic groove that are utilized by other known binding partners.

In order to further investigate and corroborate these results, *in vitro* pull-down assays were performed with GST tagged 14-3-3 proteins and radio labeled IVTT hEXO1 (Fig. 1B). Recombinant 14-3-3 proteins form homodimers similar to their *in vivo* homo- and heterodimerization [44,45] and the NH₂-terminal GST tags do not influence protein dimerization or ligand binding ability. These *in vitro* results clearly demonstrated that hEXO1 binds specifically to all GST-14-3-3 isoforms, although weakly to 14-3-3 σ (Fig. 1B). To verify the validity of the assay and stress the direct binding between hEXO1 and 14-3-3, the pull-down experiments were performed with GST-tagged, His-tagged or non-tagged hEXO1 expressed from various coding vectors. In conjunction, a GST pull-down assay with recombinant purified hEXO1 and GST-14-3-3 η and GST-14-3-3 σ confirmed the direct binding between hEXO1 and 14-3-3 η , while binding to 14-3-3 σ was undetectable under these conditions (Fig. 1C). Relevant MMR proteins were also tested for binding to select 14-3-3 variants in *in vitro* pulldown assays (Fig. 1D). These experiments showed no detectable interaction between MSH2, MSH6, MLH1 or PMS2 and the various 14-3-3 isoforms (β , γ , η and σ), but confirmed hEXO1 binding to the selected isoforms.

3.2. The nuclease activity of recombinant hEXO1 is affected by 14-3-3 association

In light of the fact that recombinant hEXO1 degrades dsDNA *in vitro* by 5' $\rightarrow$ 3' nucleotide excision [16] and binds GST-14-3-3 in the pull-down assay (Fig. 1B), we tested whether the enzymatic activity of hEXO1 is affected by 14-3-3 association (Fig. 2A and B). The experiment showed that addition of excess GST-14-3-3 η and σ to the *in vitro* exonuclease assay resulted in stimulation of hEXO1 on the DNA substrate. These data support the results in Fig. 1 showing that hEXO1 directly interacts with η and σ (Fig. 1A and B).

Additional experiments confirmed that addition of excess (2–18-fold) His-tagged 14-3-3 ζ or its binding deficient variant 14-3-3 ζ K49E does not significantly affect the nuclease activity

of hEXO1 (Fig. 2B). Finally, pull-downs with GST-MSH2 or GST-MLH1 and IVTT bovine 14-3-3 ζ (amino acid sequence is equal to human 14-3-3 ζ) confirmed non-binding of 14-3-3 ζ to these MMR proteins (Fig. 2C) and also showed that hEXO1 is unable to simultaneously bind 14-3-3 ζ and MSH2 or MLH1. These pull-downs display positive binding for hEXO1 regardless of concurrent addition of hEXO1 and 14-3-3 ζ , including pre-incubated IVTT reactions (lanes 5–6, 9–10). This experiment supports our previous findings (Fig. 1) that hEXO1-14-3-3 association is not directly related to excision regulation during *in vitro* MMR.

3.3. In vitro DNA replication is not affected by addition of hEXO1

Previous studies have shown that 14-3-3 proteins perform a regulatory role during initiation of DNA replication by binding to DNA cruciform structures formed early in assembly of the replisome [reviewed in [50]]. Certain isoforms (β , ϵ , ζ , γ and σ) associate specifically with DNA replication origins, particularly at the G1/S boundary of the cell cycle, and addition of 14-3-3 specific antibodies interferes with complex formation and inhibits *in vitro* DNA replication [46–48]. Concurrently, hEXO1 is implicated in DNA replication events as it is directly associated with PCNA replication foci *via* a conserved PIP-box motif located in the carboxyl terminus [1]. To examine whether association between 14-3-3 and hEXO1 is relevant during DNA replication initiation, we studied *in vitro* DNA replication in the presence of recombinant hEXO1 (25 ng), 14-3-3 ϵ (100 ng) or a combination of both proteins. The *in vitro* replication assay, described in [38,46–48], allows the study of proteins involved in DNA replication. Typically, the *in vitro* replication products include relaxed circular (form II), linear (form III) and supercoiled (form I) plasmid DNA (Fig. 3). The *in vitro* replication assay exploits the property that newly replicated DNA becomes refractory to digestion by *Dpn*I due to its unmethylated status (Fig. 3A, compare lanes 10 and 14 to lanes 8 and 12, respectively). Quantification of the *Dpn*I-resistant forms II and III DNA (indicative of replicated DNA) showed that the amount of undigested DNA is comparable regardless of the addition of recombinant hEXO1, either separately or in combination with 14-3-3 ϵ (Fig. 3B: 20.6%, 21.6%, and 19.6% undigested DNA in cell extracts not supplemented with recombinant proteins (lanes 9–10), supplemented with hEXO1 (11–12) or supplemented with hEXO1 and 14-3-3 (15–16), respectively). The assay was performed with minimal amounts of hEXO1 to avoid degradation of replication substrates and the amounts of 14-3-3 ϵ were accordingly adjusted. The results clearly demonstrate that hEXO1, either alone or in complex with 14-3-3, does not influence DNA replication initiation *in vitro*. More likely, 14-3-3/hEXO1 association could be relevant following DNA damage induction and damage signaling. It was recently demonstrated that 14-3-3 deficient yeast cells fail to induce EXO1 hyperphosphorylation and accumulate EXO1 dependent ssDNA gaps at stalled replication forks, in response to HU administration [33]. However, in contrast to the human system, Exo1 has not been observed at PCNA replication foci, but is instead recruited to the replication fork in response to replication inhibition [49], indicating that the protein regulation pattern could differ between the two species.

3.4. hEXO1 binding to 14-3-3 is mediated by a motif enclosing amino acid S746

To provide a mechanistic basis and determine the specificity for the hEXO1/14-3-3 interaction, we mapped the recognition site of hEXO1 for 14-3-3. Although the amino acid

sequence of hEXO1 does not contain a perfectly matched 14-3-3 binding motif of either reported form (mode 1: RSx(pS/T)xP; mode 2: Rxxx(pS/T)xP), two motifs showing considerable similarity to mode 1 were identified (Fig. 4A, turquoise). Juxtaposing these motifs with the mapped phosphorylation sites in hEXO1 [32] (Fig. 4A, red) reveals that one motif encloses a phosphorylable residue, S598, marking this sequence motif as a likely target for 14-3-3 binding. Other sequence motifs surrounding mapped phosphorylation sites, including both basal and HU induced sites (Fig. 4A, red and star) resemble to a lesser degree, a 14-3-3 binding motif. These comprise sequences surrounding S376, S422, S454*, S623, S639, and S746. Mutating the principle Ser or Thr residue to Ala blocks protein phosphorylation at the particular site and should interfere with 14-3-3 association if the amino acid in question is enclosed in the relevant binding motif. We first examined a hEXO1 variant containing Ala substitutions at seven phosphorylation sites (hEXO1 7mut), including the three sites specifically induced by HU treatment; namely S454, T581 and S714, combined with four basally phosphorylated Ser residues; namely S422A, S598A, S674A and S746A. GST pull-down experiments with this particular variant revealed that it binds less efficiently to all seven 14-3-3 isoforms compared to WT hEXO1. Binding to GST-14-3-3 σ appears weaker for WT hEXO1, and is not observed for the 7 mut variant (Fig. 4B). However, interaction with GST-14-3-3 σ is generally weaker than observed for the other isoforms, which might account for the seemingly absent binding of this isoform. Furthermore, hEXO1 shows strongest binding affinity towards 14-3-3 η in this assay as also indicated by the initial pull-downs (Figs. 1A and 4B). These results were confirmed in additional pull-downs with selected 14-3-3 isoforms (Fig. 4C and data not shown).

To unravel the molecular basis for this apparent loss in binding affinity, single amino acid variants were tested for binding to selected 14-3-3 isoforms; β , γ , η and σ (Fig. 4B). This strategy proved efficient in implicating one specific amino acid in 14-3-3 association (Fig. 4C). Like hEXO1 7mut, variant hEXO1 S746A showed reduced binding towards all tested 14-3-3 isoforms (Fig. 4C, lane 6). This reduced binding is not a consequence of protein instability, as binding to MMR proteins MSH2 and MLH1 was unaffected (Fig. 4C, bottom). Binding of hEXO1 S746A to 14-3-3 is reduced but not fully abolished, indicating that hEXO1 might contain a second binding site for coordination to 14-3-3 proteins. Interestingly, mutation of S746 to phosphor mimicking Asp (D) also reduced association with GST-14-3-3 β , but not with GST-MLH1 (Fig. 4D), suggesting that amino acid substitution at this position might interfere with local polypeptide configuration. Further *in vitro* binding experiments indicate that pre-incubation with excess 14-3-3 binding peptide R18 appears to induce a slight reduction in binding affinity of WT hEXO1 to GST-14-3-3 β , while binding affinity of the two variants S746A and S746D seems unaffected (Fig. 4D), confirming the supposition of a second 14-3-3 binding motif in hEXO1. Such motif could mediate association with 14-3-3 *via* a second patch not involving the general hydrophobic binding pocket usually coordinating binding to R18. Curiously, Co-IP data suggest that the interaction domain on human Exo1 falls within an area spanning amino acids 348 – 548, since interaction is not detected with hEXO1 deletion mutant 1-348aa, but detectable with deletion mutant 1-548 (Supplementary data, Fig. S1). This region contains 3 phosphorylable residues (Fig. 4A) that are each enclosed in motifs vaguely resembling the defined 14-3-3 binding motifs. Two of these residues (S422 and S454*) were tested for involvement in

14-3-3 coordination, but each showed no reduction in binding upon mutation to Ala (Fig. 4C).

3.5. hEXO1 phosphorylation mutants are effectively imported to the nucleus and co-localize with PCNA in replication foci

To further explore the role of hEXO1 protein phosphorylation and interaction with 14-3-3 proteins, selected phosphorylation mutants were ectopically expressed as fluorescent fusion proteins in eukaryotic cell lines for visualization of their subcellular distribution. The phosphorable residue S422 is found directly adjacent to the functional NLS sequence (⁴¹⁸KRPR⁴²¹) of hEXO1, which could interfere with import factor binding and influence nuclear import efficiency. We therefore investigated the potential effects of mutating this site on protein interaction and subcellular localization. We found that both YFP-hEXO1 S422A and YFP-hEXO1 S422D are efficiently imported into the nucleus and they co-localize with PCNA in replication foci like WT protein (Fig. 5A and B). In agreement, *in vitro* pull-down assays with IVTT hEXO1 and GST-tagged import factors Importin $\beta/\alpha 3$ demonstrate equal binding affinities of the two variants and hEXO1 WT and binding is not affected by addition of excess recombinant His-14-3-3 ζ (Fig. 5C). In addition, several hEXO1 variants were examined for nuclear import efficiency and co-localization with PCNA, including the 7 mut variant and variant 4S4D mutated to Asp at four phosphorylation sites; S422, S598, S714 and S746 involved in 14-3-3 coordination (Fig. 4). All of these variants show efficient nuclear import (Fig. 5A) and they interact normally with the import factors Importin $\beta/\alpha 3$ *in vitro* (data not shown). Furthermore, nuclear foci can be observed in co-localization with PCNA for all variants (Fig. 5B), confirming the integrity of the PIP-box [1] in these variants. These results infer that both specific protein phosphorylation and subsequent 14-3-3 binding is not directly involved in regulating recruitment of hEXO1 to PCNA associated replication foci.

We recently demonstrated that transiently expressed YFP-hEXO1 is rapidly recruited to laser induced DNA double strand breaks (DSBs) [1]. We therefore tested selected hEXO1 phosphorylation variants for recruitment to DNA DSBs induced by micropoint laser irradiation to assess recruitment to the DNA damage site. No clear distinction in recruitment efficiency was observed between WT YFP-hEXO1 and the selected phosphorylation variants YFP-hEXO1 7 mut, 4S4D and S746D (Supplementary data, Fig. S3), demonstrating that post-translation modification by phosphorylation is most likely not involved in the immediate response of hEXO1 to laser induced DNA damage sites. This result is in agreement with DNA DSB recruitment experiments of YFP-hEXO1 truncation and deletion variants [1] as most phosphorylation sites are enclosed in the distinct deleted regions.

In conclusion, a comprehensive survey of the multiple and single phosphorylation variants involving described functional assays demonstrate that all variants behave normally in the respective assays, including interaction with MMR factors MLH1 and MSH2, with nuclear import factors Importin $\beta/\alpha 3$, nuclear import efficiency and co-localization with PCNA in replication foci (Supplementary data, Fig. S4). The only exceptions are variants, 7mut, S746A and S746D, displaying reduced interaction with the 14-3-3, as outlined (Fig. 4B–D).

4. Discussion

We report here the direct association between DNA repair protein hEXO1 and regulatory checkpoint proteins 14-3-3. Interaction is effective for all isoforms, however, the more selective isoform 14-3-3 σ displays weak affinity towards hEXO1. 14-3-3 σ deviates from other 14-3-3 isoforms in a number of short regions and it displays a different migration pattern during SDS-PAGE (Supplementary data, Fig. S2). Moreover, it generally shows divergent ligand specificity, which may very likely be attributed to structural differences manifested by the presence of a second ligand binding site specific to 14-3-3 σ and possibly involved in ligand discrimination [50]. Consequently, our pull-down results suggest that hEXO1 contains a general 14-3-3 binding motif mediating a general regulation scheme of hEXO1. In contrast, attenuated binding by 14-3-3 σ is consistent with a more specialized regulation mode of specific proteins, excluding hEXO1.

Interaction with 14-3-3 is specific for hEXO1 and not observed for any other of the tested MMR proteins *in vitro* (Fig. 1). It cannot be excluded that binding of specific MMR proteins might occur *in vivo* or to certain isoforms not tested, as 14-3-3 interaction is usually depended on a phosphorylated binding motif, and such might not be invoked in the *in vitro* transcribed and translated MMR proteins. However, hEXO1 does associate with the relevant 14-3-3 proteins under the particular assay conditions, indicating that 14-3-3 interactions are most likely not invoked during MMR excision. Rather 14-3-3 binding is possibly related to other repair or damage signaling functions for hEXO1 and most likely invoked to regulate its availability or activity, either directly or indirectly, as discussed in the following.

Binding to 14-3-3 involves amino acid S746 in hEXO1. The sequence surrounding S746 (⁷⁴³KSS(pS)AD⁷⁴⁸) lacks the +2 *cis* configured Pro residue found in defined 14-3-3 binding motifs, while the common Arg upstream is replaced by Lys. Although this substitution is conserved, Lys is rarely seen at this position in frequently exemplified 14-3-3 binding motifs [45]. Atypical 14-3-3 binding motifs have been identified in several proteins, for example, p53 contains an unusual 14-3-3 binding motif involving an upstream Lys residue; ³⁷³KGQST(pS)RH³⁸⁰ [51]. Mutation of S746 reduced binding towards 14-3-3 but interaction is not fully abolished, indicating the existence of a second 14-3-3 binding motif in hEXO1 yet to be identified. Co-IP data suggest that such motif could be situated between residues 348 and 548. S746 is phosphorylated under regular cellular environments, *i.e.* in non-damaged cells. Generally, 14-3-3 proteins bind their ligands following specific phosphorylation or other post translational modification induced by particular cellular events. For instance, IR treatment induces dephosphorylation of p53 which facilitates its binding to 14-3-3 mediated by a second phosphorylated Ser residue enclosed in the described unusual binding motif [51]. Cellular treatment with HU induces phosphorylation of hEXO1, which leads to protein ubiquitination and degradation, but mutation of all three phosphorylation sites specifically induced by HU shows no effect on 14-3-3 binding (Fig. 4C, right lane). It is possible that phosphorylation of the HU inducible sites enables 14-3-3 binding to the S746 site by induction of conformational changes, but this remains to be verified.

The regulation schemes of 14-3-3 proteins are as numerous and versatile as their protein ligands and their cellular functions. The most common reported regulation modes exerted by 14-3-3 proteins include modification of enzymatic activity, modulation of protein affinity and protein interaction, and regulation of protein subcellular localization. Our results suggest that association between 14-3-3 and hEXO1 is in some cases related to regulation of hEXO1 nuclease activity but not to modification of DNA replication initiation controlled by certain 14-3-3 isoforms, at least *in vitro*. Moreover, we found no indication of functional defects in MMR protein association, nuclear import or replication foci accumulation for selected phosphorylation mutants of hEXO1, including variants mutated at S422 located adjacent to the NLS sequence, at S746 involved in 14-3-3 interaction or at amino acids specifically phosphorylated upon replication inhibition by HU administration.

However, there are a number of studies that combined provide ours with some likely scenarios to explain the functional significance of this novel association. Most pertinent is the direct involvement of hEXO1 in the DNA damage response (DDR) combined with the checkpoint functions of 14-3-3 proteins. Phosphorylation of hEXO1 at the 3 HU inducible sites appear to be involved in regulating protein levels *via* protein ubiquitination and degradation following the immediate response to arrested replication forks [20,32]. This scenario could serve to hinder excessive DNA resection at the stalled forks. In yeast cells defective in signaling replication fork arrest (*RAD53*⁻), EXO1 counteracts fork reversal by resecting newly synthesized DNA thus generating ssDNA [49]. ATR dependent phosphorylation and subsequent binding by 14-3-3 could provide a means for regulating hEXO1 protein levels following DNA damage (Fig. 6A). Unfortunately, attempts to visually quantify differences between hEXO1 WT and 7mut protein degradation by treating YFP-hEXO1 transfected cells with HU have not been feasible (data not shown). Notably, mutation of the 3 HU inducible sites leads to increased protein stability but protein degradation is not fully prevented [32], suggesting that regulation of protein levels is more complex. Moreover, studies demonstrate that specific phosphorylation of S714 is only induced by HU and not upon IR treatment [32] implying that divergent mechanism might regulate hEXO1 function during DNA replication and DSB repair.

Intriguingly, studies from budding yeast demonstrate specific phosphorylation of EXO1 following DNA damage induction, particularly DSB induction, DNA replication inhibition and telomere uncapping [52]. Protein phosphorylation is partially dependent on checkpoint protein Mec1 (ATR) and fully dependent on downstream effector kinase RAD53 [52]. In addition, the *RAD53* deletion mutant is sensitive to DNA damaging agents MMS, IR and UV, but the lethality of this mutant is significantly suppressed by simultaneously compromising EXO1 nuclease function [53]. Four specific phosphorylation sites (S372, S567, S587 and S692) have been mapped in yeast EXO1 using telomere uncapping mutants and it was shown that phosphorylation of these particular sites apparently reduces the enzymatic activity of the protein [52]. Amino acid sequence alignment reveals no convergence in reported phosphorylation sites between the yeast and human proteins (Supplementary data, Fig. S5), however, both BMH1 and BMH2 physically interact with RAD53 and directly facilitates its DNA damage checkpoint functions by positively modulating its activity [54]. In addition, it was recently proposed that the yeast 14-3-3

proteins control the phosphorylation status of EXO1 to promote fork progression following replication inhibition [33]. Chk1 (functional ortholog of RAD53 [55]) is specifically phosphorylated following UV induced DNA damage at a consensus 14-3-3 binding motif and 14-3-3 interacts with Chk1 in *in vitro* GST pull-down assays [56]. These findings could suggest that 14-3-3 scaffolding mediates hEXO1 phosphorylation by Chk1 and subsequent inhibition of protein activity (Fig. 6B). This scenario would require initial phosphorylation of hEXO1 or some other protein modification allowing 14-3-3 associations, but it is in accordance with S746 being important for 14-3-3 binding, while the HU inducible sites appear dispensable for association. In comparison, 14-3-3 deficient yeast cells fail to induce Mec1 dependent EXO1 hyperphosphorylation in response to HU administration [33], indirectly implicating them in the control of EXO1 phosphorylation following DNA stress.

hEXO1 appears to be implicated in apoptosis induction following DNA damage but the mechanism of this induction remains unresolved [9]. 14-3-3 isoforms are essential proteins for cell survival that generally display anti apoptotic functions effectuated by inhibition of pro-apoptotic signals [57]. Linking these observations it might be proposed that binding by 14-3-3 could serve to inhibit the apoptotic functions of hEXO1 (Fig. 6C). Assuming that the binding deficient variant S746A is more efficient in inducing apoptosis it could suggest that 14-3-3 binding regulates this ability of hEXO1. Moreover, directly blocking hEXO1/14-3-3 association should also have an effect on apoptosis induction in human cell lines.

Importantly, the potential roles for 14-3-3 mediated regulation of hEXO1 discussed herein are not mutually exclusive and could in principal function in parallel or might even be combined in one model. A possible scenario for a collective model would include initial binding by 14-3-3, leading to further phosphorylation of hEXO1 and inhibition of its enzymatic activity either directly or by promoting protein degradation. This would obscure any direct apoptotic signaling function of hEXO1.

hEXO1 is implicated in DNA replication, MMR, DSB processing, DNA damage signaling and apoptosis induction. The relevance of each of these pathways in molecular fitness is profound and exemplified by cancer predisposition of individuals displaying dysfunction in different DNA repair and signaling pathways. This study reveals new specific interaction partners for hEXO1 that might prove highly relevant for its cellular functions in these pathways and, consequently, its role in human disease. We mapped amino acids directly involved in coordination to 14-3-3, which will be useful in future investigations of the cellular activities of hEXO1 and 14-3-3 and their interplay during DNA damage, signaling and repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Liberti SE, Andersen SD, Wang J, May A, Miron S, Perderiset M, Keijzers G, Nielsen FC, Charbonnier JB, Bohr VA, Rasmussen LJ. Bi-directional routing of DNA mismatch repair protein human exonuclease 1 to replication foci and DNA double strand breaks. *DNA Repair (Amst.)*. 2011; 10:73–86. [PubMed: 20970388]
- Nielsen F, Jäger A, Lützen A, Bundgaard J, Rasmussen L. Characterization of human exonuclease 1 in complex with mismatch repair proteins, subcellular localization and association with PCNA. *Oncogene*. 2004; 23:1457–1468. [PubMed: 14676842]
- Qiu J, Cyan Y, Chen V, Guan MX, Shen B. Human exonuclease 1 functionally complements its yeast homologues in DNA recombination, RNA primer removal, and mutation avoidance. *J. Biol. Chem.* 1999; 274:17893–17900. [PubMed: 10364235]
- Genschel J, Bazemore L, Modrich P. Human exonuclease I is required for 5' and 3' mismatch repair. *J. Biol. Chem.* 2002; 277:13302–13311. [PubMed: 11809771]
- Gravel S, Chapman J, Magill C, Jackson S. DNA helicases Sgs1 and BLM promote DNA double-strand break resection. *Genes Dev.* 2008; 22:2767–2772. [PubMed: 18923075]
- Eid W, Steger M, El-Shemerly M, Ferretti LP, Pena-Diaz J, König C, Valtorta E, Sartori AA, Ferrari S. DNA end resection by CtIP and exonuclease 1 prevents genomic instability. *EMBO Rep.* 2010; 11:962–968. [PubMed: 21052091]
- Bolderson E, Tomimatsu N, Richard DJ, Boucher D, Kumar R, Pandita TK, Burma S, Khanna KK. Phosphorylation of Exo1 modulates homologous recombination repair of DNA double-strand breaks. *Nucl. Acids Res.* 2010
- Bardwell PD, Woo CJ, Wei K, Li Z, Martin A, Sack SZ, Parris T, Edelmann W, Scharff MD. Altered somatic hypermutation and reduced class-switch recombination in exonuclease 1-mutant mice. *Nat. Immunol.* 2004; 5:224–229. [PubMed: 14716311]
- Bolderson E, Richard D, Edelmann W, Khanna K. Involvement of Exo1b in DNA damage-induced apoptosis. *Nucl. Acids Res.* 2009; 37:3452–3463. [PubMed: 19339515]
- Dherin C, Gueneau E, Francin M, Nunez M, Miron S, Liberti S, Rasmussen L, Zinn-Justin S, Gilquin B, Charbonnier J, Boiteux S. Characterization of a highly conserved binding site of Mlh1 required for exonuclease I-dependent mismatch repair. *Mol. Cell Biol.* 2009; 29:907–918. [PubMed: 19015241]
- Maringele L, Lydall D. Exo1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70Delta mutants. *Genes Dev.* 2002; 16:1919–1933. [PubMed: 12154123]
- Schaetzlein S, Kodandaramireddy N, Ju Z, Lechel A, Stepczynska A, Lilli D, Clark A, Rudolph C, Kuhnel F, Wei K, Schlegelberger B, Schirmacher P, Kunkel T, Greenberg R, Edelmann W, Rudolph K. Exonuclease-1 deletion impairs DNA damage signaling and prolongs lifespan of telomere-dysfunctional mice. *Cell.* 2007; 130:863–877. [PubMed: 17803909]
- Tinline-Purvis H, Savory AP, Cullen JK, Dave A, Moss J, Bridge WL, Marguerat S, Bahler J, Ragoussis J, Mott R, Walker CA, Humphrey TC. Failed gene conversion leads to extensive end processing and chromosomal rearrangements in fission yeast. *EMBO J.* 2009; 28:3400–3412. [PubMed: 19798055]
- Tran PT, Fey JP, Erdeniz N, Gellon L, Boiteux S, Liskay RM. A mutation in EXO1 defines separable roles in DNA mismatch repair and post-replication repair. *DNA Repair (Amst.)*. 2007; 6:1572–1583. [PubMed: 17602897]
- Vallur AC, Maizels N. Distinct activities of exonuclease 1 and flap endonuclease 1 at telomeric g4 DNA. *PLoS ONE*. 2010; 5:e8908. [PubMed: 20126648]
- Lee B, Wilson Dr. The RAD2 domain of human exonuclease 1 exhibits 5' to 3' exonuclease and flap structure-specific endonuclease activities. *Biol. Chem.* 1999; 274:37763–37769.

17. Orans J, McSweeney EA, Iyer RR, Hast MA, Hellinga HW, Modrich P, Beese LS. Structures of human exonuclease 1 DNA complexes suggest a unified mechanism for nuclease family. *Cell*. 2011; 145:212–223. [PubMed: 21496642]
18. Genschel J, Modrich P. Mechanism of 5'-directed excision in human mismatch repair. *Mol. Cell*. 2003; 12:1077–1086. [PubMed: 14636568]
19. Zhang Y, Yuan F, Presnell S, Tian K, Gao Y, Tomkinson A, Gu L, Li G. Reconstitution of 5'-directed human mismatch repair in a purified system. *Cell*. 2005; 122:693–705. [PubMed: 16143102]
20. El-Shemerly M, Janscak P, Hess D, Jiricny J, Ferrari S. Degradation of human exonuclease 1b upon DNA synthesis inhibition. *Cancer Res*. 2005; 65:3604–3609. [PubMed: 15867354]
21. Doherty KM, Sharma S, Uzdilla LA, Wilson TM, Cui S, Vindigni A, Brosh RM Jr. RECQ1 helicase interacts with human mismatch repair factors that regulate genetic recombination. *J. Biol. Chem*. 2005; 280:28085–28094. [PubMed: 15886194]
22. Sharma S, Sommers J, Driscoll H, Uzdilla L, Wilson T, Brosh RJ. The exonucleolytic and endonucleolytic cleavage activities of human exonuclease 1 are stimulated by an interaction with the carboxyl-terminal region of the Werner syndrome protein. *J. Biol. Chem*. 2003; 278:23487–23496. [PubMed: 12704184]
23. Zhu Z, Chung W, Shim E, Lee S, Ira G. Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell*. 2008; 134:981–994. [PubMed: 18805091]
24. Mimitou E, Symington L. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature*. 2008; 455:770–774. [PubMed: 18806779]
25. Nicolette ML, Lee K, Guo Z, Rani M, Chow JM, Lee SE, Paull TT. Mre11-Rad50-Xrs2 and Sae2 promote 5' strand resection of DNA double-strand breaks. *Nat. Struct. Mol. Biol*. 2010; 17:1478–1485. [PubMed: 21102445]
26. Nimonkar A, Ozsoy A, Genschel J, Modrich P, Kowalczykowski S. Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:16906–16911. [PubMed: 18971343]
27. Nimonkar AV, Genschel J, Kinoshita E, Polaczek P, Campbell JL, Wyman C, Modrich P, Kowalczykowski SC. BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. *Genes Dev*. 2011; 25:350–362. [PubMed: 21325134]
28. Muslin A, Tanner J, Allen P, Shaw A. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*. 1996; 84:889–897. [PubMed: 8601312]
29. Yaffe M, Rittinger K, Volinia S, Caron P, Aitken A, Leffers H, Gambin S, Smerdon S, Cantley L. The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*. 1997; 91:961–971. [PubMed: 9428519]
30. Rittinger K, Budman J, Xu J, Volinia S, Cantley LC, Smerdon SJ, Gambin SJ, Yaffe MB. Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for the nuclear export signal of 14-3-3 in ligand binding. *Mol. Cell*. 1999; 4:153–166. [PubMed: 10488331]
31. Gardino AK, Smerdon SJ, Yaffe MB. Structural determinants of 14-3-3 binding specificities regulation of subcellular localization of 14-3-3-ligand complexes: a comparison of the X-ray crystal structures of all human 14-3-3 isoforms. *Semin. Cancer Biol*. 2006; 16:173–182. [PubMed: 16678437]
32. El-Shemerly M, Hess D, Pyakurel A, Moselhy S, Ferrari S. ATR-dependent pathways control hEXO1 stability in response to stalled forks. *Nucl. Acids Res*. 2008; 36:511–519. [PubMed: 18048416]
33. Engels K, Giannattasio M, Muzi-Falconi M, Lopes M, Ferrari S. 14-3-3 Proteins regulate exonuclease 1-dependent processing of stalled replication forks. *PLoS Genet*. 2011; 7:e1001367. [PubMed: 21533173]
34. Guerrette S, Wilson T, Gradia S, Fishel R. Interactions of human hMSH2 with hMSH3 and hMSH2 with hMSH6: examination of mutations found in hereditary nonpolyposis colorectal cancer. *Mol. Cell Biol*. 1998; 18:6616–6623. [PubMed: 9774676]
35. Du Y, Masters SC, Khuri FR, Fu H. Monitoring 14-3-3 protein interactions with a homogeneous fluorescence polarization assay. *J. Biomol. Screen*. 2006; 11:269–276. [PubMed: 16699128]

36. Knudsen N, Nielsen F, Vinther L, Bertelsen R, Holten-Andersen S, Liberti S, Hofstra R, Kooi K, Rasmussen L. Nuclear localization of human DNA mismatch repair protein exonuclease 1 (hEXO1). *Nucl. Acids Res.* 2007; 35:2609–2619. [PubMed: 17426132]
37. Fu H, Coburn J, Collier RJ. The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family. *Proc. Natl. Acad. Sci. U.S.A.* 1993; 90:2320–2324. [PubMed: 8460141]
38. Pearson CE, Frappier L, Zannis-Hadjopoulos M. Plasmids bearing mammalian DNA-replication origin-enriched (ors) fragments initiate semiconservative replication in a cell-free system. *Biochim. Biophys. Acta.* 1991; 1090:156–166. [PubMed: 1657184]
39. Gyuris J, Golemis E, Chertkov H, Brent R. Cdi1 a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell.* 1993; 75:791–803. [PubMed: 8242750]
40. Zhang L, Wang H, Liu D, Liddington R, Fu H. Raf-1 kinase and exoenzyme S interact with 14-3-3zeta through a common site involving lysine 49. *J. Biol. Chem.* 1997; 272:13717–13724. [PubMed: 9153224]
41. Luhn P, Wang H, Marcus AI, Fu H. Identification of FAKTS as a novel 14-3-3-associated nuclear protein. *Proteins.* 2007; 67:479–489. [PubMed: 17256767]
42. Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure function and regulation. *Annu. Rev. Pharmacol. Toxicol.* 2000; 40:617–647. [PubMed: 10836149]
43. Wang H, Zhang L, Liddington R, Fu H. Mutations in the hydrophobic surface of an amphipathic groove of 14-3-3zeta disrupt its interaction with Raf-1 kinase. *J. Biol. Chem.* 1998; 273:16297–16304. [PubMed: 9632690]
44. Chaudhri M, Scarabel M, Aitken A. Mammalian and yeast 14-3-3 isoforms form distinct patterns of dimers in vivo. *Biochem. Biophys. Res. Commun.* 2003; 300:679–685. [PubMed: 12507503]
45. Aitken A, Baxter H, Dubois T, Clokie S, Mackie S, Mitchell K, Peden A, Zemlickova E. Specificity of 14-3-3 isoform dimer interactions and phosphorylation. *Biochem. Soc. Trans.* 2002; 30:351–360. [PubMed: 12196094]
46. Alvarez D, Novae O, Callejo M, Ruiz MT, Price GB, Zannis-Hadjopoulos M. 14-3-3sigma is a cruciform DNA binding protein and associates in vivo with origins of DNA replication. *J. Cell. Biochem.* 2002; 87:194–207. [PubMed: 12244572]
47. Novae O, Alvarez D, Pearson CE, Price GB, Zannis-Hadjopoulos M. The human cruciform-binding protein, CBP, is involved in DNA replication and associates in vivo with mammalian replication origins. *J. Biol. Chem.* 2002; 277:11174–11183. [PubMed: 11805087]
48. Zannis-Hadjopoulos M, Yahyaoui W, Callejo M. 14-3-3 cruciform-binding proteins as regulators of eukaryotic DNA replication. *Trends Biochem. Sci.* 2008; 33:44–50. [PubMed: 18054234]
49. Cotta-Ramusino C, Fachinetti D, Lucca C, Doksani Y, Lopes M, Sogo J, Foiani M. Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. *Mol. Cell.* 2005; 17:153–159. [PubMed: 15629726]
50. Wilker E, Grant R, Artim S, Yaffe M. A structural basis for 14-3-3sigma functional specificity. *J. Biol. Chem.* 2005; 280:18891–18898. [PubMed: 15731107]
51. Waterman M, Stavridi E, Waterman J, Halazonetis T. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nat. Genet.* 1998; 19:175–178. [PubMed: 9620776]
52. Morin I, Ngo H, Greenall A, Zubko M, Morrice N, Lydall D. Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response. *EMBO J.* 2008; 27:2400–2410. [PubMed: 18756267]
53. Diffley M, Segurado J. Separate roles for the DNA damage checkpoint protein kinases in stabilizing DNA replication forks. *Genes Dev.* 2008; 22:1816–1827. [PubMed: 18593882]
54. Usui T, Petrini J. The *Saccharomyces cerevisiae* 14-3-3 proteins Bmh1 and Bmh2 directly influence the DNA damage-dependent functions of Rad53. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:2797–2802. [PubMed: 17299042]
55. Branzei D, Foiani M. The checkpoint response to replication stress. *DNA Repair (Amst.)*. 2009; 8:1038–1046. [PubMed: 19482564]

56. Jiang K, Pereira E, Maxfield M, Russell B, Godelock D, Sanchez Y. Regulation of Chk1 includes chromatin association and 14-3-3 binding following phosphorylation on Ser-345. *J. Biol. Chem.* 2003; 278:25207–25217. [PubMed: 12676962]
57. Porter G, Khuri F, Fu H. Dynamic 14-3-3/client protein interactions integrate survival and apoptotic pathways. *Semin. Cancer Biol.* 2006; 16:193–202. [PubMed: 16697216]

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Fig. 1.
hEXO1 interacts with 14-3-3 proteins. (A) Yeast two hybrid assay demonstrating specific interaction between 14-3-3 isoforms and hEXO1. *S. cerevisiae* EGY48/pSH18-34 was transformed with pEG-hEXO1 (upper panel) or pEG-Raf-1 (lower panel) along with pJG vectors that express WT 14-3-3ζ, its mutant derivatives, or other 14-3-3 isoforms as indicated. The production of blue color reflects positive interactions. (B) GST pulldown assay between recombinant GST-14-3-3 proteins and radioactive labeled IVTT hEXO1, either S-tagged, His-tagged or non tagged, showing distinct interaction between hEXO1 and six of the seven 14-3-3 isoforms and non interaction in control reactions with GST or beads (right lanes). (C) GST pulldown between recombinant GST-14-3-3 proteins and purified hEXO1 detected by WB against hEXO1. Positive interaction is demonstrated for 14-3-3η. (D) GST pulldown assay between recombinant GST-14-3-3 proteins β, γ, η and σ and radioactive labeled IVTT proteins hEXO1, MSH2, MSH6, MLH1 and PMS2, showing specific interaction only for hEXO1.

Fig. 2.

hEXO1 excision activity on blunt end DNA is affected by 14-3-3. hEXO1 nuclease activity assayed on 5' radioactive labeled 42-mer homoduplexes by incubation with recombinant purified hEXO1, non-tagged or His tagged. (A) hEXO1 nuclease activity assayed in the presence of excess 14-3-3. In absence of hEXO1 or in the presence of 14-3-3 no degradation of substrate occurs. The substrate was between 10% and 25% degraded and separated on 12% denaturing polyacrylamide gel. (B) Nuclease activity of hEXO1 on 5' labeled substrate after addition of 14-3-3 isoforms. Values are expressed in percentage excision of substrate by hEXO1 in presence of 14-3-3. The hEXO1 is set as control to normalize the addition the 14-3-3 isoforms. Excision activity of hEXO1 on the substrate is significantly stimulated by 14-3-3η and σ (mean ± SD.; * $p = 0.0158$; *** $p = 0.0009$). The control reaction with GST-14-3-3ε (lane 11) demonstrates the purity of the GST-14-3-3 preparations. (C) hEXO1 nuclease activity assayed in the presence of excess recombinant His-14-3-3ζ or the binding deficient form His-14-3-3ζ K49E (2-18 fold molar excess). Activities are defined as product band proportion of total band intensity and data given as mean ± SD from 4 independent experiments. (D) Double GST-pulldown assays in which IVTT hEXO1 and 14-3-3ζ (bovine) were added separately (lanes 4-5, 7-8) or in combination, simply added the reaction tubes simultaneously (lanes 5 and 9) or pre-incubated and then added (lanes 6 and 10).

Fig. 3. *In vitro* DNA replication is unaffected by addition of recombinant hEXO1. (A) *In vitro* DNA replication assay showing the ability of p186 to confer autonomous replication activity to non-origin plasmid XL-Topo. *DpnI* digests methylated (ii) but not unmethylated (i) pBluescript. HeLa cell extracts are incubated with XL-Topo or XL-Topo containing the p186 minimal region of the *Ors8* origin of DNA replication and either left untreated (–) or digested with indicated amounts of *DpnI* (+). Input methylated DNA is digested by *DpnI* whereas newly replicated DNA becomes resistant to digestion, as shown by the absence of digestion products (iii). (B) *In vitro* DNA replication assay showing no effect of recombinant hEXO1, 14-3-3ε, or both components on *in vitro* replication of XL-Topo containing p186. The arrows indicate the relaxed circular (form II) and linear (form III) plasmid DNA. Form I (supercoiled plasmid DNA) overlaps with the region of the *DpnI* digestion products, and is not detectable in the figure. Quantification of the *DpnI*-resistant forms Hand III DNA is shown at the bottom of panel B, as percentage over the total DNA.

Fig. 4.

Mapping of 14-3-3 binding motif in hEXO1 by GST pull-down assays. (A) Primary amino acid sequence of hEXO1 showing the N and I nuclease domains (grey), phosphorylated amino acids (red, *HU induced), known sequence motifs; NLS (blue), MIP-box (pink), PIP-box (yellow), and predicted 14-3-3 binding motifs (turquoise) highly resembling a mode 1 sequence peptide (RSxpS/TxP). Underlined residues (348-548) represent the 14-3-3 interaction domain on human Exo1 suggested by Co-IP data. (B) GST-pull-down assay between GST-14-3-3 isoforms and IVTT hEXO1 WT and the 7mut variant showing reduced interaction in comparisons. The 7mut is altered at S422A, S454A, S598A, T621A, S714A, S674A and S746A. (C) GST-pulldown assay between GST-14-3-3 isoforms and selected IVTT hEXO1 variants. The triple variant 3HU is mutated specifically at the HU induced sites: S454A, T621A and S714A. Control reactions with MMR proteins GST-MSH2 and GST-MLH1 demonstrates functional binding ability and protein stability of the separate variants. The table on the right indicates the relative binding affinities. Band intensities were estimated with ImageQuantTL software. Interaction affinities for the variants are estimated relative to WT interaction, which is set to be 100% for each interacting protein. (D) GST-pulldown assay between GST-14-3-3β and IVTT hEXO1 WT, S746A and S746D, in the

absence or presence of 14-3-3 inhibitor peptide R18. Binding to GST-MLH1 was included as a control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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Fig. 5.

hEXO1 phosphorylation variants co-localize with PCNA in the nucleus. (A) HeLa cells transiently expressing YFP-hEXO1 (WT or variant forms) visualized with confocal laser-scanning microscopy. The top row shows the YFP signal, while the lower panel represents the Nomarski view showing whole cells and nuclear YFP protein distribution. Variants include YFP-hEXO1 S422A, S422D, **4S4D**: S422D, S598D, S714D and S746D, **6mut**: S422A, S454A, S598A, T621A, S714A and S746A and **7mut**: S422A, S454A, S598A, T621A, S714A, S674A and S746A. (B) NIH-3T3 cells co-expressing CFP-PCNA (green) and YFP-hEXO1 (red, WT or variant forms) visualized with confocal laser-scanning microscopy. Yellow represents the overlay demonstrating protein co-localization. (C) GST-pulldown assay between GST-Importin $\beta/\alpha 3$ and IVTT hEXO1 WT, S422A and S422D in the presence of increasing amounts of recombinant His-14-3-3 ζ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 6.

Potential functional outcomes of hEXO1-14-3-3 association in the DDR. Model of three plausible outcomes hEXO1/14-3-3 association presumed to indirectly regulate hEXO1 excision activity upon DNA damage induction. (A) Extensive 5' → 3' DNA resection by hEXO1 at the DNA damage site is possibly hindered by protein phosphorylation and subsequent binding by 14-3-3 proteins, mediating its ubiquitination and degradation. (B) Active hEXO1 at the DNA damage site is initially phosphorylated or otherwise modified which enables 14-3-3 binding. Chk1 is also phosphorylated in response to DNA damage and associates specifically with 14-3-3 proteins. 14-3-3 scaffolding of Chk1 and hEXO1 could mediate further phosphorylation of hEXO1 by Chk1 inhibiting its activity and preventing excessive DNA resection at the damage site. (C) Binding of hEXO1 by 14-3-3 might be related to inhibition of its apoptotic signaling ability, either mediated by nuclear export or altered protein affinity towards other binding partners in the apoptotic pathway.