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Proteome-wide analysis of SUMO2 targets in response to pathological DNA replication stress in human cells

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ABSTRACT

SUMOylation is a form of post-translational modification involving covalent attachment of SUMO (Small Ubiquitin-like Modifier) polypeptides to specific lysine residues in the target protein. In human cells, there are four SUMO proteins, SUMO1–4, with SUMO2 and SUMO3 forming a closely related subfamily. SUMO2/3, in contrast to SUMO1, are predominantly involved in the cellular response to certain stresses, including heat shock. Substantial evidence from studies in yeast has shown that SUMOylation plays an important role in the regulation of DNA replication and repair. Here, we report a proteomic analysis of proteins modified by SUMO2 in response to DNA replication stress in S phase in human cells. We have identified a panel of 22 SUMO2 targets with increased SUMOylation during DNA replication stress, many of which play key functions within the DNA replication machinery and/or in the cellular response to DNA damage. Interestingly, POLD3 was found modified most significantly in response to a low dose aphidicolin treatment protocol that promotes common fragile site (CFS) breakage. *POLD3* is the human ortholog of *POL32* in budding yeast, and has been shown to act during break-induced recombinational repair. We have also shown that deficiency of POLD3 leads to an increase in RPA-bound ssDNA when cells are under replication stress, suggesting that POLD3 plays a role in the cellular response to DNA replication stress. Considering that DNA replication stress is a source of genome instability, and that excessive replication stress is a hallmark of pre-neoplastic and tumor cells, our characterization of SUMO2 targets during a perturbed S-phase should provide a valuable resource for future functional studies in the fields of DNA metabolism and cancer biology.

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; FACS, fluorescence-activated cell sorting; HA, human influenza hemagglutinin; HCD, higher-energy collisional dissociation; HPLC, high performance liquid chromatography; PCNA, proliferating cellular nuclear antigen protein (*Homo sapiens*); PCR, polymerase chain reaction; RPA, replication protein A; SILAC, stable isotope labeling with amino acids in cell culture; Strep, streptavidin; STRING, search tool for the retrieval of interacting genes/proteins.

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1. Introduction

Small Ubiquitin-like Modifiers (SUMOs) are a conserved family of polypeptides that are covalently attached to and detached from proteins to modify their cellular function. SUMOs are conjugated to their substrates via an enzymatic cascade analogous to that involved in protein ubiquitylation (reviewed in [1]). In yeast (*Schizosaccharomyces pombe* or *Saccharomyces cerevisiae*), there is only one form of SUMO, while in human cells there are four distinct SUMO isoforms: SUMO1–4 [2,3]. SUMO1–3 are expressed in all cell types, and SUMO4 is mainly expressed in the kidney, lymph nodes and spleen. There is 95% amino acid sequence similarity between SUMO2 and SUMO3, whereas the level of similarity between SUMO1 and SUMO2/3 is approximately 50%. It is thought that SUMO2/3 form a subfamily with cellular roles distinct from those of SUMO1. Indeed, previous studies have indicated that the conjugation status of SUMO2/3 and SUMO1 are different in several ways. First, non-conjugated SUMO2/3 is present in molar excess over conjugated SUMO2/3 in normally growing cells, while SUMO1 is virtually all conjugated constitutively to target proteins [4]. Second, when cells are exposed to various toxic stresses, including heat shock and oxidizing agents, SUMO2/3 become conjugated to substrates, while the level of conjugated SUMO1 remains unchanged [4]. Third, previous proteomic studies have revealed that distinct groups of substrates are conjugated to SUMO1 or SUMO2/3 in unstressed growth conditions [5,6] or following heat shock [7]. Fourth, the sub-cellular localization of SUMO2/3 and SUMO1 is generally different. In interphase cells, SUMO2/3 are distributed throughout the nucleoplasm, while SUMO1 is specifically localized to the nuclear envelope and the nucleolus [8]. Finally, only SUMO2 and SUMO3 contain within their polypeptide sequence a SUMO consensus modification motif (ψKxE/D) and can, therefore, form polySUMO chains [9]. Genetic studies in yeast have indicated that polySUMO chains play a role in chromosome segregation, recovery from checkpoint arrest, the DNA damage response and in meiosis [10–12].

A major source of DNA damage occurs during the process of DNA replication. For example, replication forks can encounter lesions in the template or bound proteins that interfere with fork progression [13]. This perturbation of replication can cause deletions or gene rearrangements at several genomic loci; in particular, at common fragile sites (CFSs). These sites manifest as gaps or breaks visible in condensed metaphase chromosomes, and are considered part of normal chromosome structure and are present in nearly all individuals. They normally are not prone to breakage (called 'expression'), but can be induced to break by treating cells with replication perturbing agents such as a low dose of the DNA polymerase inhibitor aphidicolin (APH) [14,15] that still permits cells to traverse S phase. Previous studies have demonstrated that CFSs are a primary target for oncogene-induced DNA damage in pre-neoplastic lesions, suggesting that CFS instability could be a key player during tumorigenesis [16]. Mechanisms that regulate the expression of CFSs have been studied extensively and documented [17,18].

In *S. cerevisiae*, it has been shown that SUMO is essential for the viability [19], while in *S. pombe* SUMO is important for the cellular response to replication perturbation [20]. It has also been demonstrated in *S. cerevisiae* that DNA double strand breaks can trigger simultaneous multisite SUMOylation of several proteins involved in DNA damage response pathways [21,22]. In human cells, only a handful of proteins have been identified as being SUMOylated during the processes of DNA damage/repair and replication, including BLM [23,24], 53BP1 [25], BRCA1 [26], and TDG [27], most of which are targeted by SUMO1. The proteins that are modified by SUMO2 during DNA replication or repair events remain largely unknown. Considering the strong evidence that SUMO2/3 are involved in the cellular response to various stresses, we hypothesized that SUMO2/3 conjugation might play an important regulatory role in DNA replication and repair. Hence, we employed mass spectrometry (MS)-based, quantitative proteomics to identify proteins whose SUMOylation status is altered when cells are under DNA replication stress.

We performed two biological replicate experiments and identified 976 putative SUMO2 target proteins under both normal growth conditions and during replication stress. Moreover, we found that the SUMOylation status of 22 proteins increased more than 2-fold when cells are exposed to DNA replication stress. Most interestingly, POLD3 was shown to have the greatest increase in SUMO2 conjugation in cells exposed to conditions that activate CFS breakage. Because *POLD3* is the human ortholog of *POL32* in budding yeast, and plays a role in break-induced recombinational repair in both yeast and human cells, we subsequently analyzed the involvement of POLD3 in the DNA replication stress response. We found that human cells deficient for POLD3 had a more prolonged DNA damage stress response when the cells were treated with a low dose of APH that is known to activate CFS breakage. Our data suggest that POLD3 is SUMOylated in response to replication stress and could play an important role in the regulation of cellular response to DNA replication stress.

2. Materials and methods

2.1. Cloning and stable cell line generation

The complementary DNA (cDNA) of human SUMO-2 isoform a (NCBI reference sequence: NP_001005849.1) was amplified using the polymerase chain reaction (PCR) from an IMAGE clone. The PCR product was subcloned into a bacterial expression vector that contains Strep-HA tag (pcDNA4/TO-Strep-HA, made in-house). The Strep-HA-SUMO2 cDNA was then sub-cloned into the eukaryotic expression vector pcDNA3.1(+) (Life Technologies) at the KpnI and NotI sites. All constructs were verified by DNA sequencing. The pcDNA3.1-Strep-HA-SUMO2 vector was transfected into U2OS cells using FuGENE 6 transfection reagent (Promega). G418 resistant clones expressing Strep-HA-SUMO2 recombinant protein were selected.

2.2. Cells culture conditions and drug treatments

U2OS and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml). Where required, G418 (400 μg/ml) was added to the medium to maintain the selection of U2OS cells with pcDNA3-Strep-HA-SUMO2. To enrich for cells in a perturbed S phase, an asynchronously growing cell population was first synchronized at the G1/S boundary by incubation in medium containing 3 mM HU for 18 h, then rinsed twice with PBS and were released in medium without the drug for the length of time as indicated in Fig. 1A. To induce a perturbed S-phase, the HU-treated cells were released into medium containing 0.4 μM APH for 8 h.

2.3. Flow cytometry

Following the treatment under the various conditions listed above, the cells were trypsinized, washed twice in PBS, and suspended in 1 ml of ice cold 70% ethanol (added drop-wise) and incubated at −20 °C overnight. Fixed cells were rinsed twice in PBS, and were incubated 30 min at 37 °C in 1 ml of PBS containing 40 μg/ml propidium iodide (Sigma–Aldrich) and 200 μg/ml RNase A. DNA content was analyzed by measuring propidium iodide staining of the DNA using a FACS Calibur (Becton Dickinson). Percentages