Targeting of SUMO substrates to a Cdc48-Ufd1-Npl4 segregase and STUbL pathway in fission yeast

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In eukaryotes, the conjugation of proteins to the small ubiquitin-like modifier (SUMO) regulates numerous cellular functions. A proportion of SUMO conjugates are targeted for degradation by SUMO-targeted ubiquitin ligases (STUbLs) and it has been proposed that the ubiquitin-selective chaperone Cdc48/p97-Ufd1-Npl4 facilitates this process. However, the extent to which the two pathways overlap, and how substrates are selected, remains unknown. Here we address these questions in fission yeast through proteome-wide analyses of SUMO modification sites. We identify over a thousand sumoylated lysines in a total of 468 proteins and quantify changes occurring in the SUMO modification status when the STUbL or Ufd1 pathways are compromised by mutations. The data suggest the coordinated processing of several classes of SUMO conjugates, many dynamically associated with centromeres or telomeres. They provide new insights into subnuclear organization and chromosome biology, and, altogether, constitute an extensive resource for the molecular characterization of SUMO function and dynamics.
The small ubiquitin-like modifier (SUMO) can be conjugated to hundreds of protein species, following an evolutionarily conserved enzymatic cascade akin to ubiquitylation1. SUMO is thereby believed to participate in the regulation of multiple biological processes by changing the interaction properties of the proteins to which it is conjugated. Prior to conjugation, SUMO is processed to expose a conserved diglycine motif (diGly) at its carboxy terminus. Conjugation is then catalysed by the sequential action of an E1-activating, an E2-conjugating and an E3-ligating enzyme, which ultimately leads to the formation of an isopeptide bond between the carboxy terminus of SUMO and the ε amino-group of a lysine in the substrate. Self-modification of SUMO at internal lysine residues can furthermore result in the formation of polymeric SUMO chains2. The fission yeast sumoylating enzymes comprise the E1 heterodimer Rap31/Fub2 (Aos1/Uba2), the E2 enzyme Ubc9 (also known as Hus5) and two identified E3s of the SP-RING family; that is, the PIAS homolog Pll1 and the Mms21 homolog Nse2 (ref. 3). A fundamental aspect of sumoylation is its reversible nature. Desumoylation is carried out by SUMO-specific proteases (Ulp/SNP proteins) that by hydrolyzing SUMO isopeptide bonds allow proteins to return to their non-sumoylated form.

An alternative to desumoylation is the elimination of sumoylated proteins through the STUbL pathway, STUbLs, as represented by the Rfp1/Slx8 and Rfp2/Slx8 dimers in Schizosaccharomyces pombe, the Sls5/Slx8 dimer in Saccharomyces cerevisiae, and homo-dimeric RNF4 in mammals, are conserved ubiquitin E3 enzymes that target sumoylated proteins for ubiquitylation and subsequent degradation or recycling4–8. The activity of STUbLs is determined by a RING domain, commonly found in ubiquitin E3 ligases, and by several SUMO-interaction motifs (SIMs) that bind poly-sumoylated specie5,9. High-molecular weight SUMO conjugates accumulate in STUbL-deficient cells and this phenotype is associated with genomic instability and general cellular dysfunction5,7,10. The best described STUbL substrate to date is the mammalian PML protein11,12. Other reported STUbL substrates include the transcriptional regulator Mot1 (ref. 12), the inner kinetochore protein CENP1 (ref. 13), the DNA damage protein MDC1 (refs 10,14), the cohesin motif 1,000 sumoylated lysines and to compare sumoylation levels for a subset of these sites between fission yeast wild-type and mutant strains deficient in STUbL (shx8-1) or Ufd1 (ufd1ΔCt213–342) function. These data suggest that certain substrates associated with centromeres and telomeres are coordinately processed by the combined action of the STUbL and Ufd1 pathways.

**Results**

**Engineering of a His6SUMO109K variant for proteomic studies.** We replaced the endogenous fission yeast SUMO gene (pmt3) with a His6-tagged version in which the leucine at position 109 was changed to a lysine (His6SUMO109K variant; Fig. 1b). A lysine at position 109 will, on digestion with the endoproteinase Lys-C, leave a diGly remnant on SUMO acceptor sites suitable for the purification of modified peptides with a diGly–Lys-specific antibody (anti-K-ε-GG)29. This antibody was originally prepared against ubiquitylated peptides for which a diGly remnant is left on trypsin digestion30. As ubiquitin contains an arginine at the position upstream its diGly sequence, Lys-C will not cleave at this site. Similarly, Lys-C digestion of proteins conjugated to other ubiquitin-like proteins generates long peptide-branches not recognized by the diGly–Lys antibody (Fig. 1b). Thus, Lys-C digestion of Ni2+-purified His6SUMO109K conjugates allows specific enrichment of SUMO diGly-modified peptides, facilitating MS analysis of the diGly-modified lysines based on their unique mass-to-charge ratio.

Functionality of the His6SUMO109K variant was verified by growth assays, microscopic examination, western blotting and MS. Cells expressing His6SUMO109K as their sole copy of SUMO, from the endogenous pmt3 locus, displayed a wild-type morphology and growth rate (Fig. 1c,d). This is in sharp contrast to the growth defects of pmt3A cells. The proteome of strains expressing His6SUMO109K appeared unaltered compared with cells expressing wild-type SUMO (Fig. 1e and Supplementary Data 1). Also, His6SUMO109K cells responded to heat shock as wild-type, by a massive increase in high-molecular weight conjugates (Supplementary Fig. 1a). We combined His6SUMO109K with the shx8-1 STUbL mutation5 and with the ufd1ΔCt213–342 mutation truncating the C-terminal half of Ufd1 (ref. 19). There again, growth and morphological analyses found that shx8-1 and ufd1ΔCt213–342 cells expressing His6SUMO109K were phenotypically similar to their wild-type SUMO counterparts at 30°C (Fig. 1c,d). By western blotting, overall conjugation appeared slightly reduced for His6SUMO109K as previously observed for an analogous S. cerevisiae SUMO109K mutant32 (Fig. 1f). Reduced sumoylation has been shown to alleviate the growth defect of the shx8-1 mutant at 36°C (refs 5,7).
Figure 1 | Strains engineered to test the effects of STUbLs and Cdc48–Ufd1–Npl4 on the sumoylated proteome of fission yeast. (a) Model for the processing of sumoylated species by STUbLs and Cdc48–Ufd1–Npl4. Sumoylated proteins can be targeted for degradation by STUbL-catalysed ubiquitylation. Cdc48–Ufd1–Npl4 may act directly on sumoylated proteins, or on proteins co-modified by SUMO and ubiquitin, to facilitate their recycling or proteasomal degradation. The strains shown in b–f were designed to identify substrates in these pathways. (b–f) Design and phenotypes of the His6SUMOL109K variant. (b) Sequences of S. pombe ubiquitin-like proteins upstream of their carboxy-terminal diGly motifs. Residues highlighted in blue indicate the branch left on conjugates after endoproteinase Lys-C digestion. (c) Growth analysis of wild-type, ufd1ΔCt213–342 and slx8-1 strains expressing SUMO or His6SUMOL109K. (c, left) Tenfold dilution series of the indicated strains were spotted onto rich medium (YES) and incubated at 33 °C for 3 days. From top to bottom: JK424, JK408, JK425, JK414, JK426 and JK418. (c, right) Growth curves. Cells were propagated at 30 °C in EMM2 minimal medium with required supplements and culture absorbance was recorded at 600 nm at the indicated time intervals. S.e. were computed from three independent cultures for each strain. (d) Micrographs of strains expressing SUMO or His6SUMOL109K. (e, left) 2D scatter plot showing the log2 relative abundance of 2,266 proteins between the samples His6SUMO/SUMO (x-axis) or His6SUMOL109K/SUMO (y-axis). Each point corresponds to a single protein and is coloured from green to red according to the density estimation value, reflecting the density of data points from less to more compressed. Proteins that are regulated by more than twofold are coloured in purple, however, none of these changes are statistically significant according to the multiple-sample analysis of variance test (Supplementary Data 1). (f) Western blot of wild-type SUMO and His6SUMOL109K conjugates. Whole-cell extracts of the indicated strains were probed with a SUMO antibody. Tubulin was used as a loading control. Cultures were propagated at 30 °C unless indicated otherwise. Asterisks indicate cross-reactivity of the SUMO anti-serum.
and indeed this was also the case with slx8-1 His6SUMO\textsuperscript{L109K} cells (Supplementary Fig. 1b). However, the sumoylation patterns detected in strains expressing His6SUMO\textsuperscript{L109K} or SUMO were globally very similar (Fig. 1f), in particular the changes in sumoylation occurring in the slx8-1 and ufd1\textsuperscript{AC}\textsuperscript{213–342} mutants compared with wild type were conserved, pointing to the suitability of His6SUMO\textsuperscript{L109K} to globally map sumoylation sites of fission yeast wild-type and mutant cells.

**Proteome-wide identification of sumoylation sites.** To identify the sumoylated species that accumulate in Ufd1 mutants\textsuperscript{19,20}, we isolated His6SUMO\textsuperscript{L109K}-modified proteins from ufd1\textsuperscript{AC}\textsuperscript{213–342} cells. Cells were lysed under denaturing conditions and His6SUMO\textsuperscript{L109K} conjugates were isolated by Ni\textsuperscript{2+} chromatography. The purified material was digested with Lys-C, or Lys-C and Glu-C to generate shorter peptides, and diGly–Lys peptides were immunoprecipitated with anti-K-\varepsilon-GG antibody prior to MS analysis (Fig. 2a). This strategy identified a total of 841 unique diGly-modified lysines mapped with a localization probability of $>0.75$. Together these sumoylated sites defined a total of 414 proteins (Supplementary Data 2).

The His6SUMO\textsuperscript{L109K} variant was then employed in conjunction with SILAC-based proteomics\textsuperscript{33} to quantitatively compare the sumoylated proteomes of wild type and mutants defective in Ufd1 (ufd1\textsuperscript{AC}\textsuperscript{213–342}) or STUbL (slx8-1). Two SILAC experiments were conducted in parallel, one experiment comparing a heavy lysine (Lys8) slx8-1 culture with a light lysine (Lys0) wild-type culture, and the other experiment comparing the Lys8 slx8-1 culture with a Lys0 ufd1\textsuperscript{AC}\textsuperscript{213–342} culture (Fig. 2b). The experiments were conducted in triplicate starting from isolated colonies, propagating cells at 30 °C and shifting them to 33 °C for the last 12 h to induce the slx8-1 temperature-sensitive phenotype at the semi-permissive temperature of slx8-1 (ref. 5). The slx8-1 cultures were split, mixed separately in 1:1 ratios with wild-type and ufd1\textsuperscript{AC}\textsuperscript{213–342} cultures, and lysed. Small samples of cell lysates were subjected to in-solution digestion with Lys-C and Glu-C to generate shorter peptides, and diGly-modified peptides were isolated from the remaining material. In total, the SILAC experiments detected 618 distinct sumoylation sites in 301 proteins. About 400 sites identified in the ufd1\textsuperscript{AC}\textsuperscript{213–342} strain had already been identified in the pilot experiment performed under non-SILAC conditions, indicating a high degree of experimental reproducibility (Supplementary Fig. 2). The higher complexity of SILAC peptide samples, where very intense SILAC pairs can hinder detection of less abundant species, might account for the fact that fewer sites were identified in the SILAC experiments than in the non-SILAC experiment even though each experiment used approximately the same amount of starting material. Together, the SILAC and non-SILAC experiments produced a comprehensive list of 1,028 sumoylation sites distributed between 468 proteins. This list is shown in Supplementary Data 2, the SILAC results are shown in Supplementary Data 3, and the total proteome analyses are shown in Supplementary Data 4. The lists comprise proteins ranging in abundance from very low to high according to the absolute quantifications in the study by Marguerat et al.\textsuperscript{35}, indicating no experimental bias towards abundant species (Supplementary Fig. 3 and Supplementary Data 5).

**Comparing the effects of Ufd1 and STUbLs with SILAC.** Among the diGly-modified peptides identified in the SILAC experiments, only peptides identified in both heavy and light forms in at least two biological replicates of either slx8-1/wild type or slx8-1/ufd1\textsuperscript{AC}\textsuperscript{213–342} were used for quantification. This resulted in the calculation of heavy-to-light ratios for 327 distinct peptides (Supplementary Data 3). For the total proteome samples, peptides quantified in at least one biological replicate lead to the quantification of 3,115 crude proteins (Supplementary Data 4). SILAC ratios for crude proteins were normalized using the MaxQuant software\textsuperscript{36}, while the mean of their non-normalized ratios was used to normalize the ratios from the purified material\textsuperscript{25}. Normalized SILAC ratios were used to derive median values for peptides measured in 3 biological replicates, or mean values for peptides quantified in only 2 biological replicates, resulting in the quantification of 243 and 261 diGly-modified peptides in the slx8-1/wild type and slx8-1/ufd1\textsuperscript{AC}\textsuperscript{213–342} experiments, respectively (Supplementary Data 3). As 171 of these peptides were quantified for both slx8-1/wild type and slx8-1/ufd1\textsuperscript{AC}\textsuperscript{213–342} (Fig. 2c) their relative abundance in ufd1\textsuperscript{AC}\textsuperscript{213–342} and wild type could also be calculated. Figure 2d compares the slx8-1/wild type and ufd1\textsuperscript{AC}\textsuperscript{213–342}/wild-type ratios. Sumoylation was increased $\geq2$-fold for 51 sites (in 33 proteins) in the slx8-1 mutant and for 40 sites (in 31 proteins) in the ufd1\textsuperscript{AC}\textsuperscript{213–342} mutant. Conversely, sumoylation was reduced $\geq2$-fold for 14 and 21 sites in the 2 mutants, respectively. Regression analysis showed the two data sets are correlated with a Pearson coefficient of $r=0.49$. Even with a great degree of variation, this supports the existence of common tendencies in sumoylation dynamics in the two strains.

**Comparison of sumoylation and total protein levels.** Should proteins with increased sumoylation in slx8-1 and/or ufd1\textsuperscript{AC}\textsuperscript{213–342} be normally targeted for degradation by STUbL-mediated ubiquitylation, these proteins may be stabilized in the mutant backgrounds. We could quantify protein levels for a subset of proteins with quantified sumoylation sites (112 and 79 proteins were quantified in both crude and diGly–Lys immunoprecipitated samples for slx8-1/wild type and ufd1\textsuperscript{AC}\textsuperscript{213–342}/wild type, respectively). For these proteins, changes in sumoylation were not generally correlated with a change in protein abundance (Fig. 2e,f). Importantly, the abundance of SUMO itself was unchanged in the mutants excluding the possibility that protein sumoylation is increased in the slx8-1 or ufd1\textsuperscript{AC}\textsuperscript{213–342} mutant due to elevated SUMO concentration. Two exceptions were Sl1, an uncharacterized orphan protein, which displayed a nearly twofold increase in ufd1\textsuperscript{AC}\textsuperscript{213–342} cells compared with a threefold increase in sumoylation, and Smc5, which increased at the protein level by nearly threefold in both mutants.

**General features of the SUMO proteome at the residue level.** As our study is the first global analysis of sumoylation in *S. pombe*, and one of the very first to identify sumoylation sites globally in any organism, it also provided important insights into sumoylation per se. Nearly half the proteins identified in our combined experiments displayed multiple sumoylation sites (Fig. 3a). Some proteins contained many sites with, for example, 14 sites identified in Uba2, 17 sites in the HMG-box protein Hmo1 or 22 sites in the topoisomerase Top2. We identified seven SUMO–SUMO branching points (K14, K30, K39, K51, K60, K63 and K71, with co-sumoylation of K14 and K30), and multiple sumoylation sites in ubiquitin (K6, K11, K27, K48 and K63). Doubly or triply modified peptides were detected for 22 proteins even though our approach detects co-modification only for sequential lysines, pointing to widespread multi-sumoylation. Altogether these observations are consistent with features of sumoylation recently proposed for mammalian cells\textsuperscript{29–31}. Half of the sumoylation sites identified conformed to the forward ($PK\varepsilon K/D/E$)\textsuperscript{37} or reversed (D/ExKnp)\textsuperscript{27} consensus motif.
proposed in other organisms, while the remaining sites did not conform to any identifiable motif (Fig. 3b).

In addition to multiply sumoylated peptides, sumoylated peptides bearing other post-translational modifications were also detected (Supplementary Data 6), including branched SUMO peptides phosphorylated on T20 and T24. Co-modifications suggestive of cross-talks were observed for the histones H3, H2A and H2B. In particular, concomitant phosphorylation of S121 and sumoylation of K119 occurred on H2A-alpha and -beta. H2AS121 is phosphorylated by Bub1, mediator of the spindle assembly checkpoint. H2AS121 phosphorylation might facilitate sumoylation at K119 by introducing a negative charge.

Figure 2 | Quantitative identification of sumoylated residues in wild-type and slx8-1 and ufd1D C213–342 mutants. (a) Two-step enrichment for sumoylated peptides using His6SUMO109K strains. (b) Experimental design for SILAC experiments (see text for details). (c) Overview of peptides quantified in the triplicate SILAC experiments. Only diGly–Lys peptides for which SILAC ratios were obtained in at least two biological replicates were used in the analyses. In total, 171 distinct diGly–Lys peptides were quantified in both the slx8-1/wild-type and slx8-1/ufd1DeltaC213–342 IPs, which enabled us to calculate ufd1DeltaC213–342/wild-type ratios. See also Supplementary Fig. 2. (d) The normalized log2 ratios of quantified diGly–Lys-modified peptides for ufd1DeltaC213–342/wild type and for slx8-1/wild type were plotted on a tsMAP, giving an overview of the relative change in abundance of individual peptides in each mutant. The two data sets correlate with a Pearson coefficient of r = 0.49. Coloured data points represent peptides originating from the same proteins or from functionally related proteins, identified in Fig. 5. (e, f) Changes in protein sumoylation are not generally correlated with changes in protein concentration in either the slx8-1 or ufd1DeltaC213–342 mutant. Among the proteins with quantified sumoylation sites, 112 and 79 were also quantified in the total proteome analyses of crude lysates for slx8-1/wild type and ufd1DeltaC213–342/wild type, respectively. Log2 SILAC ratios for sumoylated peptides were plotted against quantified ratios obtained for these proteins, yielding the maps in e and f.
Figure 3 | General features of sumoylation at the residue level. (a) Distribution of sites between sumoylated proteins. (b) Amino-acid context of sumoylated lysines. Four pLogo graphs display the amino-acid sequence context of all sumoylated lysines; of KxE/KxD consensus-site lysines; of lysines within the ExK/DxK inverted consensus site and of sites not conforming to either consensus (residual sites). The numbers in parentheses above each graph indicate the total number of sites in the category (first number) and, among them, the number of sites that covered the full 13-amino acids window required for the pLogo analysis (second number). The log-odds of the binomial probability (π) are represented on the y-axis. Significance threshold values of 3.68 (P<0.05) are shown in red. (c) Sumoylated residues identified in this study are shown in yellow in a cartoon structure of *S. cerevisiae* topoisomerase 2 (PDB#4GFH; 94% identical to *S. pombe*). The portion of the protein whose structure was solved is coloured in the *S. cerevisiae* Top2 representation on the right. Enlarged cartoon views show the tendency of sumoylation sites to fall into coils or end of helices, the proximity of sumoylation sites in three-dimension and the proximity of sites to DNA. (d) Distribution of 82 sumoylated lysines identified in this study and mapped onto existing crystal structures using PyMOL. The structures are shown in Supplementary Figs 4 and 5 and the sites are listed in Supplementary Data 7. ‘End-of-helix’ and ‘end-of-sheet’ refer to lysines at three or less amino acids from the end of a helix or sheet. (e) Distribution of lysines in secondary structures predicted with PSIPRED70 for respective sumoylated lysines, and for all the lysines in proteins with a sumoylated lysine. *P<0.001. P values were calculated with a hypergeometric probability test. See also Supplementary Data 8.
at the +2 position and effectively creating a consensus modification site.

To investigate the structural features of SUMO modification sites in proteins, we mapped sumoylation sites onto known protein structures. Of the 22 sites in Top2 (Fig. 3c), we mapped 10 to the DNA gate, with 6 at the interface of the ATPase domain and DNA-binding and cleavage core (Fig. 3c). One sumoylated lysine (K388 in S. pombe or K333 in S. cerevisiae) is in the K loop, whose interaction with DNA has been proposed to trigger ATP hydrolysis and release of DNA from Top2 (ref. 39). In addition, 12 sumoylation sites are in the last 302 amino acids of S. pombe Top2, in a part of the protein not present in the crystal structure but whose sumoylation is also conserved29,30,40 and likely to interfere with the functioning of the C gate. Supplementary Figure 4 displays sites in Cdc48 and in the nucleosome, and Supplementary Fig. 5 displays a total of 73 additional sites in 9 S. pombe proteins and 34 highly conserved proteins from other organisms (82 sites in total; Supplementary Data 7). Several conclusions could be reached from this analysis. First, nearly all sites were accessible at the surface of proteins indicating sumoylation occurs on folded proteins. Second, sites located in different domains of a protein could be very close in three-dimensional structures as for K388 and K978 in Top2, or in the case of the nucleosome where modification sites on distinct histones (H3K79 and H4K79) are close together in the nucleoprotein complex. Finally, sites were predominantly in loops or coils or at the very end of alpha helices or beta sheets (Fig. 3d). Consistently, a PSIPRED secondary structure prediction for the proteins in our list (Supplementary Data 2) found sumoylated lysines preferentially in coils, with an under-representation in helices (Fig. 3e and Supplementary Data 8).

**Functional clustering of sumoylated proteins.** Gene ontology analyses of the ensemble of identified SUMO targets (Supplementary Data 2) found an over-representation of proteins participating in chromosome organization and segregation, transcription, as well as DNA recombination and repair (Fig. 4a). Also enriched were proteins associated with ribosome biogenesis and nucleo-cytoplasmic transport. These enrichments are not surprising given the literature on SUMO and the predominantly nuclear localization of SUMO in S. pombe24,26,41.

Corroborating another hallmark of sumoylation24,26,42, many of the S. pombe proteins targeted for sumoylation were part of heavily sumoylated macromolecular complexes (Supplementary Data 2 and Fig. 4b). For example, the SWI/SNF chromatin-remodelling complex, the histone acetylase SAGA complex and the TFIID transcription factor and RNA polymerase core complexes were sumoylated on multiple subunits. The nucleolar U3 sno-RNA-containing complex was sumoylated at 15 residues, on 9 subunits. Other complexes highly targeted by sumoylation were the core cohesion, SMC5/6, and condensin complexes, with the latter modified on all 5 components with a total of 17 sumoylation sites.

**Functional classes of conjugates targeted by STUbL or Ufd1.** The SILAC data sets (Supplementary Data 3) were analysed to identify cellular functions relying on the processing of SUMO conjugates by STUbLs or Cdc48–Npl4–Ufd1. The peptides whose sumoylation was most affected by the slx8–1 or ufd1ΔACE213–342 mutations are displayed in bar graphs in Fig. 5. Peptides were loosely grouped in the graphs according to whether their sumoylation was increased in both mutants (Fig. 5a; the largest and most strongly affected class), increased specifically in one mutant (Fig. 5b) or decreased in either mutant (Fig. 5c). Peptides originating from the same proteins were placed side-by-side, revealing strongly correlated patterns of sumoylation within proteins. Some proteins contained sites whose sumoylation was below the cutoff used in Fig. 5; in general sumoylation at these sites changed with the same trends as for the peptides on display, just with a reduced amplitude (Supplementary Data 3). Peptides whose abundance was measured only in the slx8–1/wild-type SILAC experiment are shown in Fig. 5d. Several of these peptides originated from proteins represented in the upper graphs, in all cases corroborating the observed changes.

The function of the proteins whose sumoylation status strongly depends on Slx8 or Ufd1 was determined using the Pombase43 and Pubmed databases, allowing us to annotate the graphs in Fig. 5 accordingly. Protein localization data43 were also used, although not systematically displayed. Functions could be assigned for most proteins. Colour was added in both Fig. 5 and Fig. 2d to label peptides according to their functional groups.

Remarkable functional clustering was observed. More than a third of the proteins whose sumoylation coordinately increased in both mutants (7/20; Fig. 5a) are proteins associated with centromeres or telomeres. Figure 6 displays all the SUMO conjugates identified in our study localizing to these chromosomal regions and labels conjugates quantified in the SILAC experiments. In total, the sumoylation of 17 centromere- or telomere-associated proteins was quantified in both mutants. The sumoylation status of 13 of them was significantly increased in Ufd1 or Slx8 (Fig. 6a).

It was also notable that condensins and cohesins constituted more than half of the proteins whose sumoylation was specifically increased in the slx8–1 mutant (Fig. 5b), representing nearly all condensins and cohesins measured in the SILAC experiments. The sumoylation state of numerous transcription factors and chromatin remodelers also changed in the mutants, but not in a unique pattern, consistent with the diversity of function and subnuclear compartimentalization of this class of proteins (Fig. 5). Finally, sumoylation of both SUMO and ubiquitin was affected in the mutants, albeit differently. SUMO branches increased greatly in both mutants (Fig. 5a). Sumoylation of ubiquitin increased specifically in the ufd1ΔACE213–342 mutant (Fig. 5b).

**Comparison with sumoylation in S. cerevisiae.** We compared our S. pombe data (Supplementary Data 2) with five S. cerevisiae large-scale proteomic studies of sumoylation24,25,44–46 in YeastMine47, filtering for proteins that have orthologues in both species (Supplementary Fig. 6 and Supplementary Data 9). As noted previously45, the five studies are in poor concordance with each other. Comparing them individually with our more comprehensive data sets indicates the poor concordance is partly due to limited coverage. Merging four of the S. cerevisiae lists24,25,44,45 finds that sumoylation is conserved between the two species for at least 42% of the 412 S. pombe proteins listed in Supplementary Data 2 for which orthologues were found in S. cerevisiae.

The study by Albuquerque et al.35 is of particular interest since it identifies potential STUbL substrates in S. cerevisiae, using the slx5Δ mutant. Comparing with the potential substrates identified here for Slx8 reveals strong evolutionary conservation for some classes of substrates in particular cohesins, condensins and for the Smc5/6 complex (Supplementary Fig. 7). The kinetochore and DASH complex components identified here in the case of Slx8 were not identified for Slx5, but another kinetochore protein, CBP2, and regulators of kinetochore-microtubule attachment (SI15 and BIR1) were identified, suggesting STUbLs and Cdc48–Ufd1–Npl4 might have conserved roles in chromosome segregation. Divergence was observed for septins, a major class of proposed substrates for S. cerevisiae STUbL35, for which sumoylation appears much less prominent in S. pombe,
possibly reflecting diverged modes of septation between fission and budding yeast.

**Discussion**

Using a recently developed purification strategy, we have analysed the SUMO proteome of fission yeast wild-type and mutant cells, identifying over a thousand unique sumoylation sites in 468 different proteins in conditions where SUMO was expressed at endogenous levels. Our data sets highlight the participation of SUMO in higher order protein complexes and the widespread links between SUMO and chromosome transactions. They provide a quantitative overview of the effects of STUbLs and Ufd1 on the sumoylated proteome, showing that both STUbLs and Cdc48–Ufd1–Npl4 target discrete classes of sumoylated proteins and identifying potential substrates sequentially processed by STUbLs and Cdc48–Ufd1–Npl4. The modification status of SUMO itself, and of ubiquitin, suggest a sequential action of Slx8 and Ufd1 that would result in the accumulation of SUMO–SUMO linkages in both mutants, and of mixed ubiquitin–SUMO chains specifically in the Ufd1 mutant. Some of the protein groups affected individually or in a correlated manner by STUbLs and Ufd1 are discussed below.

Many potential targets of a STUbL/Ufd1 pathway were at centromeres/kinetochores, telomeres and at the nuclear envelope (NE) (Figs 5a and 6a). They include Mis17, Ask1 and Dam1. Mis17 associates with the inner kinetochore plate together with the Mis6–Mal2–Sim4 complex (CENP-I/-O/-K homologues) throughout the cell cycle, while Ask1 and Dam1 are part of the DASH complex that binds the outer kinetochore only during mitosis, to facilitate the attachment of kinetochores to the mitotic spindle and subsequent chromosome segregation. In human cells, RNF4-mediated turnover of the inner kinetochore homologue CENP-I has been proposed to help form the inner kinetochore plate. Our finding of elevated Mis17 sumoylation in STUbL and Ufd1 mutant cells...
indicates a conserved role for STUbLs and Ufd1 in regulating inner kinetochore structures. STUbL/Ufd1-mediated turnover of outer kinetochore components may similarly contribute to normal spindle and chromosomes dynamics, possibly accounting for some of the mitotic defects observed in STUbL mutants.

Figure 5 | Sumoylated peptides with a >2-fold change in abundance in the slx8-1 or ufd1ΔCt213–342 mutant. Colours highlight peptides originating from the same proteins or from proteins with related function. The same peptides are coloured in the tsMAP in Fig. 2d. (a) Peptides of increased abundance in both mutants. (b) Peptides of increased abundance in single mutants. (c) Peptides of decreased abundance in either mutant. (d) Peptides quantified for only slx8-1/wild type.
At telomeres, sumoylation of the shelterin proteins Rap1 and Ccq1 was increased in the third to sixfold range in both mutants (Fig. 5a,d). The shelterin complex protects the ends of chromosomes and regulates their replication by telomerase. In *S. cerevisiae*, non-functional poly-sumoylated versions of Rap1 are cleaved by Uls1, a dual STUbL and DNA-dependent ATPase/translocase, to sustain Rap1-dependent inhibition of telomeric end-to-end fusions.13 A similar function could be carried out by the combination of Skx8/Rip STUbL and Cdc48–Ufd1–Npl4-dependent ATPase activity.

Not only were kinetochore and shelterin components heavily sumoylated in the *slx8-1* and *ufd1LAC213–342* mutants, but their anchors at the nuclear periphery were also increasingly sumoylated in the Ufd1 mutant. In *S. pombe*, the association of telomeres with the NE relies on interactions between telomeric shelterin proteins (Taz1 and Rap1) and the inner nuclear membrane proteins Bqt3 and Bqt4 (refs 52,53). During mitosis these associations are released to allow faithful chromosome segregation. Other factors implicated in telomere–NE anchoring include the integral nuclear membrane protein of the animal LEM (Lap2/Emerin/Man1) subfamily of lamin-associated proteins, Lem2 and Man1 (ref. 54). We detected more than fourfold increase in the sumoylation of Lem2 at K121, a lysine situated in the nucleoplasm, in the *ufd1LAC213–342* background. Additional five sumoylation sites, clustered near the amino-terminal chromatin-binding Helix–Extension–Helix domain of Lem2, were detected exclusively in the *ufd1LAC213–342* strain (Fig. 6b and Supplementary Data 2). In addition, a total of nine sumoylation sites were identified in Bqt4, also solely in *ufd1LAC213–342*. Telomere anchoring to the nuclear periphery is known to depend on sumoylation in *S. cerevisiae* and *Caenorhabditis elegans* but the targets of sumoylation are not precisely defined. Our findings suggest undiscovered modes of telomere–NE regulation through sumoylation of inner membrane proteins. Such regulation is likely to also apply to other heterochromatic regions associating with the NE, such as centromeres. For instance, Lem2 is highly enriched at the centromere/spindle pole body region and we detected several sumoylation sites on the two-centromere NE-anchoring proteins Sad1 (SUN domain protein) and Csi1 (Fig. 6b). Again, Sad1 and Csi1 sumoylated peptides were almost exclusively detected in the *ufd1LAC213–342* background. Since the Cdc48–Ufd1–Npl4 ATPase-driven translocation of membrane-bound proteins have been well-established at the endoplasmic reticulum, one may speculate on similar roles for Cdc48–Ufd1–Npl4 in the turnover of NE-associated proteins, possibly via Ufd1 SIM – SUMO interactions.

In addition to proteins associated with centromeres or telomeres, multiple chromatin modifiers and transcription factors were targeted by both Skx8 and Ufd1 including Mot1, a Swi2/Snf2 family ATPase that displaces the TATA-box-binding protein Tbp1 from promoters. Mot1 is an established STUbL substrate in *S. cerevisiae*.12 We observed increased sumoylation of *S. pombe* Mot1 at K101 and K105 in *slx8-1* and *ufd1LAC213–342* mutants (Fig. 5a), in a domain of the protein corresponding to the region sumoylated in *S. cerevisiae*. This implicates Cdc48–Ufd1–Npl4 in the processing of sumoylated Mot1. We also detected sumoylation of Tbp1 at K209, within the DNA-binding domain (Supplementary Fig. 5). Other global controllers of transcription and chromatin structure co-regulated by Skx8 and Ufd1 included the SWI/SNF complex subunit Snf59—the histone H3K9 methyltransferase Clr4, the co-repressor Tup12 and the iron-responsive transcription factor Fep1 known to associate with Tup12.

The accumulation of other classes of SUMO conjugates was specific to the *slx8-1* mutant. This was the case for the DNA-directed RNA polymerase I and III complex subunits, for the Cnd2, Cut3 and Cut14 condensin proteins, for the Psm1 and Psm3 core cohesion proteins and for the nuclear protein Dnt1. Strikingly, Dnt1 showed 1.5- to 7-fold increased sumoylation on 7 different lysines in *slx8-1*. The *S. cerevisiae* Dnt1 homologue, Tof2, is an established SUMO target. Tof2 is part of a network of proteins that tether the ribosomal DNA (rDNA) to the inner nuclear membrane and sumoylation may modulate these interactions.17 Proper localization of the rDNA at the nuclear periphery is required for retaining rDNA structure and silencing. Consequently, loss of sumoylation leads to decreased rDNA silencing, and an inability to form SUMO chains has been linked to gross defects in rDNA compaction/organization.18 Moreover, rDNA silencing is also alleviated in STUbL-deficient budding yeast cells,59 suggesting that a pathway involving both polysumoylation and STUbL activity is required for normal rDNA function. The identification of Dnt1 as a highly upregulated SUMO target in *slx8-1* cells suggests that Dnt1 may constitute a substrate for such a pathway.

Increased sumolation of the Psm1 and Psm3 core cohesion proteins in *slx8-1* is particularly interesting in light of a recent study conducted in *S. cerevisiae* Here the other core cohesion subunit, Mcd1 (Rad21 homologue) was shown to be targeted for STUbL-mediated degradation in the absence of the cohesin-associated factor Pds5, leading to premature sister chromatid separation.15 By promoting cohesin proteolysis, STUbLs may allow efficient chromosome segregation during anaphase in wild-type cells and/or they might regulate cohesion functions outside mitosis such as during DNA repair where the sumoylation of cohesins plays important, yet incompletely defined, roles.60

It may be expected that sumoylated proteins normally targeted for degradation by STUbL-catalysed ubiquitylation would accumulate in the *slx8-1* and *ufd1LAC213–342* backgrounds. However, we found changes in protein sumoylation generally not to be correlated with a change in protein abundance. This lack of correlation, as determined for at least a subset of *slx8-1* and *ufd1LAC213–342*-regulated SUMO substrates, is in line with the notion that only a minor fraction of a protein is typically modified by SUMO at any given time.28,61 Consequently, the total pool of a protein likely to be subjected to STUbL/Ufd1-mediated regulation is proportionally low and any change in total concentration of the target protein may thus go undetected. It is also possible that ubiquitin is added by STUbLs not to degrade sumoylated proteins (that is, K63 rather than K48-linked chains), but rather to allow their extraction from large complexes.

In summary, our SILAC experiments identified a number of proteins whose sumoylation is increased in the *slx8-1* and/or *ufd1LAC213–342* backgrounds, of which only some were discussed above. We cannot conclude that some conjugates might accumulate through indirect effects of the *slx8-1* or *ufd1LAC213–342* mutations, for instance, effects on the cell cycle might result in the enrichment of certain cell-cycle-regulated SUMO conjugates. Conversely, our data set might not include particular SUMO-modified peptides, whose chemical composition precludes identification using standard MS-based bottom-up proteomics approaches. This might be the case for a predicted sumoylated peptide that was not identified in this study. We trust that the proteins identified include *bona fide* STUbL substrates, based on the finding of already known STUbL targets in the list, and of proteins localizing to subcellular structures or protein complexes previously shown to be affected by STUbL activity. Comparatively few sites displayed reduced sumoylation in either or both mutants. Reductions were limited in both amplitude and number of sites affected, with the remarkable exception of Hmo1 and a predicted ZF-GATA-type transcription factor (SPCC1393.08), which together account for nearly all sites of
reduced sumoylation in both the \textit{slx8-1} and \textit{ufd1D}\textsuperscript{Ct213–342} mutants (Fig. 5). Common tendencies in sumoylation dynamics between the \textit{slx8-1} and \textit{ufd1D}\textsuperscript{Ct213–342} mutant strains lend support to recent genetics observations of a functional relationship between STUbLs and the Cdc48–Ufd1–Npl4 segregase, and should motivate the further characterization of the specific substrates through which STUbL and Cdc48–Ufd1–Npl4 act either in concert or individually.

Methods

Strain constructions. The strains used in this study are listed in Supplementary Table 1. The endogenous \textit{S. pombe} \textit{SUMO} gene, \textit{pmt3}, was engineered to encode a \textit{SUMO} variant with an N-terminal His\textsubscript{6}-tag and a single amino-acid substitution changing the leucine at position 109, immediately upstream the C-terminal diGly sequence, into a lysine (His\textsubscript{6}SUMOL109K allele). This was performed using a ‘loop-in-loop-out’ strategy\textsuperscript{64}, where a plasmid containing His\textsubscript{6}SUMOL109K, \textit{pmt3}\textsubscript{+} and 5\textsuperscript{0} and 3\textsuperscript{0} flanking regions and a \textit{ura4}\textsuperscript{+} gene (pJBK142) was integrated into the \textit{pmt3}-flanking region by homologous recombination in a \textit{ura-D18} strain. ‘Loop-outs’ where His\textsubscript{6}SUMOL109K remained as the single \textit{pmt3} genomic copy were subsequently selected by 5\textsuperscript{0}-fluoroorotic acid resistance and they were verified by PCR amplification of the locus and sequencing. To produce the pJBK142 ‘loop-in’ plasmid, the \textit{S. pombe} \textit{SUMO} (\textit{pmt3}) open reading frame and its flanking regions were amplified in two separate PCR reactions: The open reading frame and its 5\textsuperscript{0} region were amplified with the forward primer GTO-492 (5\textsuperscript{0}-GCTCCAC\underline{AATTCTCACA}GACCC-3\textsuperscript{0}) and reverse primer GTO-548 (5\textsuperscript{0}-\underline{CGATGGATC}\underline{CCTAAAGGCATAGATGGGTGCAACCACC}TTTCTGTTCTAAG-3\textsuperscript{0}), changing the codon for leucin 109 (TTA) to a lysine codon (TTT), as underlined. The \textit{pmt3} downstream region was amplified with GTO-549 (5\textsuperscript{0}-\underline{GCATGGATCCAGTACG}\underline{Sad1}\underline{Tm}\underline{SUN} domain)
Filter-aided sample preparation and protein digestion. His,SUMO1-109K-conjugated proteins (~300 μg as determined by ultraviolet–visible spectrophotometry) were extracted under non-denaturing conditions using 30 μl of 30% glicerol mixed with 5 μl of SUMO109K. Allele replacements had been identified, a ura4+ gene was inserted at a tightly linked location 5’ of pmt3 to follow the allele through subsequent crosses. His,SUMO109K int:pJBK111(ura4-TAGTATTTTTAAGCTGTTTC-3’) was crossed into skb-1 and ufld1-1. Anti-SUMO rabbit antibodies (kindly provided by J. Seeler) were used for Western blotting, followed by horseradish peroxidase-conjugated swine anti-rabbit IgG secondary antibody (Dako PO217, diluted 1:50,000). Cysteine-rich SUMO proteins were detected with an ECL plus kit (GE Healthcare). Western blots detecting tubulin were performed with an anti-tubulin antibody (Sigma 00020911; diluted 1:25,000).

Cultures and lysis for label-free total proteome analysis. Strains JK424, JK354 and JK408 were grown in triplicates in Edinburgh minimal medium (EMM2) supplemented with 2 μg/ml fMet-Leu-Phe, harvested at an OD of 0.6, washed in ice-cold PBS before freezing in liquid nitrogen. Cells were lysed with NaOH/b-mercaptoethanol, as described below for SILAC experiments in section ‘Protein extraction and nickel affinity purification’, and resuspended in 6 M guanidinium-HCl; 100 mM NaHPO4, pH 7.5; 50 mM Tris-HCl pH 7.5; 150 mM NaCl and 20 mM N-ethylmaleimide using a FastPrep instrument (MP Biomedicals). Equal amounts of protein lysates were separated by SDS–polyacrylamide gel electrophoresis on 4–20% gradient gels (Lanza), transferred to a nitrocellulose/MCE membrane (Advantage) and immunoblotted using an anti-SUMO rabbit antibody (kindly provided by J. Seeler; antibody kindly provided by J. Seeler) and blotted using an anti-SUMO rabbit antibody (kindly provided by J. Seeler; antibody kindly provided by J. Seeler).

Cultures and conditions for SILAC experiments. The wild-type (JK408) and strain JK414 was grown in EMM2 supplemented with 5 mg/ml of ‘light’ (12C614N2, Lys0) or ‘heavy’ L-lysine (13C615N2, Lys8) (Cambridge Isotope Laboratories). The wild-type (JK408) and UFALC23-342 His-SUMO109K strain JK414 was grown in EMM2 supplemented with 30 mg/ml b-lysine at 30°C. Cell pellets were harvested by centrifugation, resuspended in 60 ml of binding buffer (6 M guanidinium-HCl; 100 mM NaHPO4, pH 8.0; 10 mM Tris-HCl, pH 8.0; 20 mM imidazole; 5 mM b-mercaptoethanol). Approximately 2.5% of each culture was harvested from exponentially growing cells (A600~0.9) and washed in ice-cold PBS before freezing in liquid nitrogen.

Protein extraction and nickel affinity purification. Protein extraction and N2+ purification of His,SUMO1-109K conjugates from ufld1-1 was performed using similar protocols. The SILAC experiments, comparing skb-1 with wild type and skb-1 with ufld1-1 were performed using parallel Heavy and light labelled cells were mixed in a 1:1 ratio based on the weight of cell lysates prior to lysis. Mixed pellets were resuspended in 50 ml ice-cold H2O and lysed by adding an equal volume of NaOH and b-mercaptoethanol to a final concentration of 1.85 M and 1.85%, respectively (~12 mU per g of cell pellet). After incubation on ice for 30 min, proteins were precipitated with 25% trichloroacetic acid, washed twice with 50% trichloroacetic acid and resuspended in 60 ml of binding buffer (6 M guanidinium-HCl 100 mM NaHPO4, pH 8.0; 10 mM Tris-HCl, pH 8.0; 20 mM imidazole; 5 mM b-mercaptoethanol). Proteins were allowed to go into solution in binding buffer on a roller mixer for up to 1 h before lysates were finally clarified by high-speed ultracentrifugation (30,000 g, 4°C, 1 h). Proteins were eluted in three sequential steps with 5 ml of wash buffer pH 8.0 containing 200 mM imidazole and the three elution fractions of each biological sample were combined for further processing.
Data analysis. Raw mass spectrometric data files were processed with MaxQuant software (version 1.3.0.5)\(^9\) and searched against UniprotKB S. pombe reference proteome database (downloaded on 14 December 2013). UniprotKB gene names were used in Tables except for a few names that were updated with Pombase gene names. Protease specificity was set for Lys-C or Lys-C and Glu-C permitting up to three or five missed cleavages, respectively. Carbamidomethylation of Cys residues was added as a fixed modification and oxidation of Met, acetylation of protein N-termini and diGly adduction to internal Lys in the peptide sequence were set as variable modifications. In addition, phosphorylation of Ser, Thr and Tyr or acetylation of Lys were added as variable modifications and the minimum peptide length was set to seven amino acids. For SILAC samples, the minimum peptide length was set to 2, and Lys3 was selected as a heavy labelled counterpart. LFQ files were analysed using MaxQuant software version 1.5.2.8. Multiplicity was set to 1, trypsin was selected as an enzyme and up to two missed cleavages were permitted. LFQ\(^8\) and match between runs options were both enabled with a minimum LFQ ratio count of 2, match time window of 1 min and alignment time window of 20 min. All data sets were filtered by posterior error probability to achieve a false discovery rate of 1% at both, protein and peptide level.

Complete proteome results (Supplementary Data 4) report only proteins that were quantified in at least one biological replicate. Potential contaminants and proteins identified based on reverse decay database search were excluded. For each quantified protein, the normalized protein ratio of individual biological replicates was averaged and expressed as a log2. The number of ratio occurrences and the variance of these ratios between the individual biological replicates of each experimental condition are reported in Supplementary Data 4. Mean normalized ratio of JK414/JK408 was derived from the mean ratios of JK418/JK408 and JK418/ JK414, and was expressed as a log2. The list of proteins quantified in the LFQ experiment (Supplementary Data 1) includes proteins that were quantified in at least two biological replicates of one experimental condition, omitting potential contaminants and proteins identified based on reverse decay database search. LFQ values from individual biological replicates were averaged and the ratio of the median values of His6-SUMO/SUMO and His6-SUMO\(^{199}$SUMO were expressed as a log2. Each protein in the 2D scatter plot was coloured using a gradient from green to red according to the density estimation values calculated using Perseus software package (300 points per dimension; Perseus.xs') density function; version 1.5.2.6; downloaded from http://141.61.102.17/perseus_doku/doku.php?id=start). Perseus software was also used to calculate multiple-sample analyses of variance and a false discovery rate of 0.1% was accepted as significant for the comparison of the median values of His6-SUMO/SUMO and His6-SUMO\(^{199}$SUMO.

Analysis of His$_6$SUMO\(^{199}$SUMO-modified sites. Sequence motifs were visualized using pLogo\(^6\). Sequence windows of 13 amino acids centred on all SUMO-modified lysines identified were input. Protein N- or C-terminus sequences shorter than the 13-amino acid window were omitted from the output. UniprotKB S. pombe reference proteome database (downloaded on 14 December 2013) containing 145,132 lysines was used as a background data set and residues were scaled relative to their Bonferroni-corrected statistical significance.

Mapping of sumoylation sites onto existing protein structures. The ensemble of S. pombe proteins for which sumoylation sites were identified in this study (Supplementary Data 2) were used in a Blast search at NCBI against the Protein Data Bank. PDB files were retrieved for proteins with high identity with their S. pombe homologues. Lysines that are sumoylated in S. pombe were mapped onto structures with the PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC. The data are displayed in Fig. S3d, Supplementary Figs 4 and 5 and in Supplementary Data 7.

PSIPRED analysis. PSIPRED (version 3.5 for Linux; derived from the study by Jones\(^4\)) and the Uniref90 database (release 2014_10) were used to predict the secondary structure context (that is, helix, sheet, coil) of all identified sumoylated lysines and diGly residues in the same proteins (Supplementary Data 8 and Fig. 3e). The secondary structure context at positions +3 and –3 was also determined, using a custom script.

References

25. Hannich, J. T. et al. Analysis of His$_6$SUMO-SUMO1-modified sites. Sequence motifs were visualized using pLogo. Sequence windows of 13 amino acids centred on all SUMO-modified lysines identified were used as input. Protein N- or C-terminus sequences shorter than the 13-amino acid window were omitted from the output. UniprotKB S. pombe reference proteome database (downloaded on 14 December 2013) containing 145,132 lysines was used as a background data set and residues were scaled relative to their Bonferroni-corrected statistical significance.
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Author contributions
J.B.K. created and characterized the S. pombe strains used in the study, prepared samples for mass spectrometry and wrote the first draft of the manuscript. Mass spectrometry and downstream data analysis were performed by T.T. M.M.J. helped establish protocols for the purification and western blotting of SUMO conjugates, examined sumoylation following heat shock and mapped sumoylation sites on existing protein structures. N.N. was involved in the purification and western blotting of SUMO conjugates, performed downstream data analysis were performed by T.T. M.M.J. helped establish protocols for the purification and western blotting of SUMO conjugates, examined sumoylation following heat shock and mapped sumoylation sites on existing protein structures. N.N. established growth curves. All authors participated in experimental design, data analyses and writing of the manuscript.

Additional information
Accession codes. The raw mass spectrometry data and the associated tables have been deposited to the ProteomeXchange Consortium (http://protemecentral.proteomexchange.org/) via the PRIDE partner repository with the data set identifier PXD002972.

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