Rif1 S-acylation mediates DNA double-strand break repair at the inner nuclear membrane

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Rif1 is involved in telomere homeostasis, DNA replication timing, and DNA double-strand break (DSB) repair pathway choice from yeast to human. The molecular mechanisms that enable Rif1 to fulfill its diverse roles remain to be determined. Here, we demonstrate that Rif1 is S-acylated within its conserved N-terminal domain at cysteine residues C466 and C473 by the DHHC family palmitoyl acyltransferase Pfa4. Rif1 S-acylation facilitates the accumulation of Rif1 at DSBs, the attenuation of DNA end-resection, and DSB repair by non-homologous end-joining (NHEJ). These findings identify S-acylation as a posttranslational modification regulating DNA repair. S-acylated Rif1 mounts a localized DNA-damage response proximal to the inner nuclear membrane, revealing a mechanism of compartmentalized DSB repair pathway choice by sequestration of a fatty acylated repair factor at the inner nuclear membrane.

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Rif1 (Rap1-interacting factor 1) supports diverse biological functions. First identified in *Saccharomyces cerevisiae* as a telomere-binding protein, Rif1 regulates telomere length by counteracting telomerase recruitment and attenuates DNA end resection at dysfunctional telomeres. These activities depend on telomere recruitment by Rap1, mediated by two Rif1 C-terminal regions, the RBM (Rap1-binding motif) and CTD (C-terminal domain), and a conserved N-terminal domain with intrinsic DNA-binding activity known as the HOOK domain. Cooperative binding of Rif1 to DNA ends produces a protective protease sheath, which excludes DNA end-processing factors including telomerase and the DNA end-resection machinery.

Rif1 also serves as a regulator of DNA replication origins, a role that is conserved from yeast to human. Rif1 targets PP1 to replication origins, leading to removal of activating phosphorylations on components of the replication machinery, locally attenuating origin firing and modulating replication timing globally.

Recent years, Rif1 has emerged as a critical regulator of DSB repair pathway choices. In mammalian cells, the Rif1–53BP1 axis antagonizes BRCA1-CTD-mediated 5′-DNA end-resection, a process that exposes 3′-DNA overhangs for homologous recombination (HR)-dependent DSB repair. Thus, Rif1 helps stabilize DSB ends, promoting repair by re-ligation.

We have shown previously that cells deleted for *RIF1* exhibit a ~2-fold increase in Zeocin resistance compared to wild-type control cells. A similar increase in survival upon Zeocin exposure was observed for cells deleted for *PFA4*, but not cells deleted for any of the other six palmitoyl-transferases present in budding yeast. Interestingly, the NHEJ defect of *pfa4Δ* cells was not aggravated by the deletion of *RIF1*, suggesting that Rif1 and Pfa4 may act jointly to facilitate NHEJ.

Across organisms, Rif1 promotes DSB repair by NHEJ through the attenuation of DNA end-resection. To test whether Pfa4 impacts on DNA end-resection, we measured the accumulation of single-stranded DNA (ssDNA) at the induced DSB at the *MAT* locus using a qPCR-based method (see Fig. 1a for details). Strikingly, loss of Pfa4 was associated with a ~2-fold increase in ssDNA (2 h time point) after DSB induction, phenocopying the effect caused by loss of Rif1, while the combined loss of Rif1 and Pfa4 did not increase ssDNA accumulation further (Fig. 1d). Thus, like Rif1, Pfa4 is required to prevent hyper-resection at DSBs.

These findings suggest that protein S-palmitoylation is important for NHEJ efficiency in yeast, and implicate Pfa4 and Rif1 in a common pathway of DSB repair pathway choice.

**Rif1-mediated NHEJ is dependent on residues C466 and C473.** Having observed an epistatic defect in NHEJ efficiency after disruption of *RIF1* and/or *PFA4*, we sought evidence that Pfa4 acts through Rif1 to promote NHEJ. To identify sites of palmitoylation involved in NHEJ, we performed a mutational analysis on Rif1 and screened for compromised NHEJ efficiency. This analysis was based on two assumptions: first, relevant S-palmitoylated cysteine residues must be contained within the N-terminal domain of Rif1 (residues 1–1322, hereafter referred to as Rif1NTD, for Rif1 N-terminal domain). This region of Rif1 was shown to be required and sufficient for promoting NHEJ, with cells expressing Rif1NTD from the endogenous *RIF1* locus being as effective in promoting NHEJ as cells expressing full-length Rif1 (ref. 3) (see also Supplementary Fig. 2a). Secondly, S-palmitoylation must occur on cysteines likely to be surface-exposed, which we identified using the available crystal structure information for Rif1NTD. Of 19 cysteines present in Rif1, 14 are contained within the nuclear membrane and reveal a mechanism of compartmentalized DSB repair pathway choice.

**Results**

**The palmitoyl acyltransferase Pfa4 promotes NHEJ.** Rif1 fulfills an evolutionarily conserved function in DSB repair pathway choice, stabilizing DNA ends and promoting DSB repair by simple re-ligation along the NHEJ pathway. We hypothesized that if the reported Pfa4-dependent S-palmitoylation of *S. cerevisiae* Rif1 (ref. 33) is important for Rif1’s role in NHEJ, deletion of *PFA4*, like a deletion of *RIF1*, should decrease the efficiency of NHEJ. To test this, we used a reporter strain containing an inducible DSB at the *MAT* locus that can only be repaired by NHEJ (see Fig. 1a for details). As expected, cell viability upon DSB induction was fully dependent on core NHEJ factor Ku70 (Fig. 1b). Consistent with previous results, deleting *RIF1* led to a marked decrease in cell viability by ~40% after 2 h of transient DSB induction, reflecting compromised NHEJ in absence of Rif1. Under the same conditions, loss of palmitoyl transferase Pfa4 caused a comparable decrease in cell survival (Fig. 1b), implicating Pfa4 in NHEJ. In line with previous observations, loss of Pfa4 did not affect Rif1 protein levels (Supplementary Fig. 1a). Interestingly, the NHEJ defect of *pfa4Δ* cells was not aggravated by the deletion of *RIF1* (Fig. 1b). Disruption of NHEJ in yeast results in increased cell survival during chronic exposure to radiomimetic drugs such as Zeocin, consistent with HR being the more optimal pathway of DNA repair under these conditions.

We have shown previously that cells deleted for *RIF1* exhibit a ~2-fold increase in Zeocin resistance compared to wild-type control cells. A similar increase in survival upon Zeocin exposure was observed for cells deleted for *PFA4*, but not cells deleted for any of the other six palmitoyl-transferases present in budding yeast (Supplementary Fig. 1b). Furthermore, Zeocin resistance levels of *rif1Δ pfa4Δ* double-mutant cells were no greater than those of *rif1Δ* or *pfa4Δ* single mutant cells (Fig. 1c). These results suggest that Rif1 and Pfa4 may act jointly to facilitate NHEJ.

**Posttranslational modifications have been implicated in the regulation of Rif1 functions.** For example, phosphorylation of Rif1 close to the RXV/SILK PP1-binding sites disrupts Rif1-PP1 interactions, leading to the activation of Rif1-repressed replication origins. Rif1 ubiquitination and SUMOylation are required for the timely dissociation of 53BP1–Rif1 complexes from DNA damage, access of BRCA1–CTIp, and DSB repair by HR.

Rif1 S-palmitoylation has been detected in budding yeast. Rif1 is a member of the evolutionary conserved DHHC (aspartate–histidine–histidine–cysteine) family of protein acetyltransferases that promote S-palmitoylation of proteins involved in NHEJ. To test this, we used a reporter strain containing an inducible DSB at the *MAT* locus using a qPCR-based method (see Fig. 1a for details). Interestingly, the NHEJ defect of *pfa4Δ* cells was not aggravated by the deletion of *RIF1* (Fig. 1b). Disruption of NHEJ in yeast results in increased cell survival during chronic exposure to radiomimetic drugs such as Zeocin, consistent with HR being the more optimal pathway of DNA repair under these conditions.

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Rif1NTD, with C466, C473, C906, C1022, and C1089 being surface-exposed (Fig. 2a). In addition, we included in the analysis C71 and C1292, for which structural information is not available. Notably, in silico analysis using Swisspalm/CSS-Palm predicted one of the cysteines that we selected, C466, as a residue of potential Rif1 S-palmitoylation (Supplementary Fig. 2b).

We grouped Rif1NTD residues C906, C1022, C1089, and C1292 (referred to as cluster 1), and C71, C466, and C473 (cluster 2) to facilitate the mutational analyses (Fig. 2a), targeting endogenous Rif1 for alanine substitutions of all cysteines in cluster 1 and 2, alone and in combination, in the NHEJ reporter strain (see Fig. 1a). Single or combined alanine substitutions within cluster 1 had no effect on NHEJ efficiency as assessed by cell survival after DSB induction (Fig. 2b). In contrast, cluster 2 mutants Rif1 C71A/C466A/C473A and Rif1 C466A/C473A were associated with reduced survival after DSB induction, phenocopying Rif1 (Fig. 2b) and/or PFA4 (Fig. 1b) deletions. Single-site mutants Rif1 C464A or C473A had no effect on cell survival after DSB formation. Consistent results were obtained upon chronic exposure of cells to Zeocin, where the rif1 C466A/C473A allele led to increased Zeocin resistance, similar to what is observed for rif1Δ, pfa4Δ, or rif1Δ pfa4Δ cells, while the rif1 C464A and rif1 C473A single-mutation alleles had no effect (Fig. 2c, see also Fig. 1c). All cluster 2 mutations had little or no impact on protein stability (Supplementary Fig. 2c). These results indicate an impairment of NHEJ by combined, but not individual loss of potential Rif1 S-acylation sites C464 and C473.

Rif1 DSB end-protection and targeting requires C466 and C473. To assess the functional consequences of mutating potential Rif1 S-acylation sites C464 and C473, we measured DNA end-resection using the inducible DSB at the MAT locus. Compared to cells expressing wild-type Rif1NTD, cells expressing Rif1NTD C464A/C473A, but not those expressing Rif1NTD with a single C464A mutation, showed a ~2-fold increase of ssDNA formation by DNA end-resection as determined by qPCR (Fig. 1b). Time-lapse analysis indicated an impaired efficiency of NHEJ by combined, but not individual loss of potential Rif1 S-acylation sites C464 and C473.

Fig. 1. The palmitoyl acyltransferase Pfa4 promotes NHEJ. a) Schematic representation of S. cerevisiae chromosome III harboring a MATα HO-endonuclease cut site. Upon DSB formation by induction of HO endonuclease, cell survival is possible by NHEJ while DNA end-resection leads to cell death due to lack of an HR repair template (hmlΔ/hmrΔ). ssDNA formation progressively inactivates the annotated Alu restriction sites, leading to increased qPCR product yield (primers indicated in red), providing a qualitative read-out for DNA end-resection. b) NHEJ efficiency for the indicated strains measured by cell viability following 2 or 4 h of HO-endonuclease induction. Data are presented as mean values ± s.e.m. (n = 6 independent experiments). c) Cell viability in the presence of Zeocin (70 μg/mL). Data are presented as mean values ± s.e.m. (n = 6 independent experiments). d) ssDNA formed by DNA end-resection as determined by qPCR. Data are presented as mean values ± s.e.m. (n = 6 independent experiments). PE, plating efficiency; PEWT, plating efficiency without HO-endonuclease induction; PEHΔ, plating efficiency of Rif1 wild-type reference strain; WT, wild-type. For statistical analysis, one-way analysis of variance (ANOVA) and a post-hoc Tukey-Kramer multiple comparison test was performed, comparing wild-type to the indicated mutants. See also Supplementary Fig. 1. Source data are provided as a Source Data file.
Fig. 2 C466 and C473 are required for Rif1-mediated NHEJ. a Top, Budding yeast Rif1 (1916 amino acid residues) contains four identifiable functional domains: RVxF/SILK Ppi1-interacting domain; RBM and CTD Rap1-binding motifs; HOOK DNA-binding domain. The Rif1 N-terminal domain (Rif1NTD, residues 1–1322) supports the protein’s function in NHEJ. The positions of cysteine residues, representing potential Rif1 S-acylation sites, are indicated. Bottom, surface representation of Rif1 (residues 177–1283) structure bound with DNA3, identifying surface-exposed cysteines. For mutational analyses, cysteines were grouped by proximity into clusters 1 and 2. C1292 and C71, for which structural data is not available, were included in cluster 1 and 2, respectively. b NHEJ efficiency for Rif1NTD cysteine clusters 1 and 2 mutants, determined as in Fig. 1b after 2 h of HO-endonuclease induction. Data are presented as mean values ± s.e.m. (n = 3 independent experiments). c Viability of the indicated strains in the presence of Zeocin (70 µg/ml). Data are presented as mean values ± s.e.m. (n = 6 independent experiments). Statistical analysis was performed by one-way Anova and a post-hoc Tukey multiple comparison test, comparing wild-type to the indicated mutants. PE, plating efficiency; PET0, plating efficiency for Rif1NTD cysteine clusters 1 and 2 mutants, determined as in Fig. 1b after 2 h of HO-endonuclease induction. Data are presented as mean values ± s.e.m. (n = 3 independent experiments). Statistical analysis was performed by one-way Anova and a post-hoc Tukey comparison test, comparing wild-type to the indicated mutants. PE, plating efficiency; PET0, plating efficiency for Rif1NTD cysteine clusters 1 and 2 mutants, determined as in Fig. 1b after 2 h of HO-endonuclease induction. Data are presented as mean values ± s.e.m. (n = 3 independent experiments). Statistical analysis was performed by one-way Anova and a post-hoc Tukey multiple comparison test, comparing wild-type to the indicated mutants. PE, plating efficiency; PET0, plating efficiency for Rif1NTD cysteine clusters 1 and 2 mutants, determined as in Fig. 1b after 2 h of HO-endonuclease induction. Data are presented as mean values ± s.e.m. (n = 3 independent experiments). Statistical analysis was performed by one-way Anova and a post-hoc Tukey comparison test, comparing wild-type to the indicated mutants.

Cells expressing Rif1NTD DNA-binding site mutant K437E/K563E/K570E (referred to as HOOK mutant)3 (Fig. 3a). Thus, like protective encapsulation of DNA ends by the HOOK domain, potential Rif1 S-acylation sites C466 and C473 are essential for Rif1-mediated DNA end-protection.

To analyze Rif1NTD occupancy at DSBs, we performed chromatin immunoprecipitation (ChIP) at the MAT DSB. As expected, Rif1NTD accumulated following cut induction, but occupancy compared to wild-type was reduced by ~40% (4 h time-point, 0.3 kb from DSB) when potential Rif1 S-acylation sites C466 and C473 were mutated, or when Pfa4 was deleted (Fig. 3b). Consistent with previous results3, a similar loss of Rif1NTD from the DSB was observed upon introduction of the DNA-binding site HOOK mutation (Fig. 3b), and combining the HOOK and C466A/C473A mutations reduced Rif1NTD occupancy at DSBs further (Supplementary Fig. 3d, e). As shown in Fig. 2a, C466 and C473 are pointing away from the DNA-binding site, mapping to the convex surface of the HOOK domain. To rule out the possibility that the C466A/C473A mutation might disrupt DSB occupancy and cause NHEJ deficiency by disturbing the DNA-binding site at the concave surface of the HOOK domain, we purified Rif1 constructs (spanning residues 100–1322) with and without the C466A/C473A and HOOK mutations (Fig. 3c) to compare their DNA-binding activity using electromobility shift assays (EMSAs). As shown previously3, the Rif1 DNA-binding site HOOK mutant K437E/K563E/K570E was impaired in its ability to retard the DNA substrate in EMSAs. In contrast, the Rif1 C466A/C473A mutant exhibited a wild-type pattern of retarded DNA species, binding DNA with similar apparent affinity to wild-type (Fig. 3d). These results show that the potential Rif1 acceptor site C466/C473 for Pfa4-dependent S-acylation and the HOOK domain’s DNA-binding site make separate contributions to Rif1’s ability to effectively engage an induced DSB, while the integrity of both sites is indispensable for Rif1-mediated DNA end-protection and NHEJ.

**Pfa4-dependent S-acylation of Rif1 C466 and C473 in vivo.** To address the possibility of Rif1 S-acylation at C466 and C473, we turned to selective chemical labeling of thioester-linked cysteines. First, we used acyl-biotin exchange (ABE) followed by biochemical enrichment of S-acylated proteins. In brief, free cysteines are blocked with N-ethylmaleimide (NEM) before cysteine-acyl thioester cleavage using hydroxylamine (HA), with subsequent biotinylation at reactivated cysteines, allowing the capture of S-acylated proteins using avidin42 (Fig. 4a). This procedure strongly enriched Rif1NTD from cell extracts in the avidin fraction compared to control reactions with HA omitted (Fig. 4b). Consistent with previous results33, deletion of PFA4 reduced the amount of Rif1NTD recovered by biotin-avidin affinity capture by ~5-fold. Importantly, capture of Rif1NTD C466A/C473A from extracts of Pfa4-proficient cells was more than 2-fold lower than wild-type (Fig. 4c).
Fig. 3 Rif1 C466 and C473 are required for DSB end-protection and targeting. a DNA end-resection upon DSB induction at the MAT locus in strains expressing wild-type (WT) Rif1NTD, the indicated Rif1NTD mutants, or deleted for RIF1. ssDNA formation was determined by qPCR as in Fig. 1d. Data are presented as mean values ± s.e.m. (n = 6 independent experiments). b Association of wild-type and mutant Rif1NTD-Myc with the DSB at MAT. Results obtained with the indicated primers are reported as fold enrichment relative to ACT1 ± s.e.m. (n = 6 independent experiments). Statistical analyses shown in panels a and b were performed by one-way Anova, followed by a post-hoc Tukey-Kramer multiple comparison test, comparing wild-type to the indicated mutants. c SDS-PAGE gel of purified Rif1 (residues 100–1322) variants used for DNA-binding assays, stained with Coomassie blue. Asterisk denotes an unspecific band, BSA added for enhanced Rif1 stability. d EMSA analysis assessing the DNA-binding activity of the indicated Rif1100–1322 variants (10–160 nM) using a 32P-labeled 30 bp dsDNA (1 nM). Left, representative EMSA with free and Rif1-bound DNA species indicated. Right, quantitation of bound DNA presented as mean values ± s.e.m. (n = 3 independent experiments). X-axis, log2 scale. See also Supplementary Fig. 3. Source data are provided as a Source Data file.

reduced compared to wild-type Rif1NTD (Fig. 4b). These findings support NHEJ-critical Rif1 residues C466 and C473 as in vivo Rif1 S-acylation sites.

We next devised an alternative method to more directly establish Rif1 C466 and C473 S-acylation, which we term acyl-carnabiodimethyl exchange (ACE). Different from ABE chemistry, ACE replaces fatty acylation with carbamidomethyl (CAM) rather than biotin and Rif1 is captured by immunoprecipitation, such that modified peptides can be recovered, and S-acylation sites are accessible for mapping using mass spectrometry (Fig. 4c). For ACE, unmodified cysteines and tris (2-carboxyethyl) phosphine (TCEP)-reduced cysteines (opening potential disulfide bridges) were first labeled with NEM. This procedure leaves cysteine S-acylation intact (Supplementary Fig. 4a). Cysteine-acyl thioesters were then cleaved by treatment with dithiothreitol (DTT), followed by cysteine carbamidomethylation, allowing the
identification of S-palmitoylation sites in peptides with high sensitivity (see Supplementary Fig. 4b, c). Immuno-precipitated Rif1NTD was subjected to parallel reaction monitoring (PRM), analyzing NEM and/or CAM-labeled C466 and C473 in tryptic peptide fragments spanning residues 463 to 479. Peptides containing NEM-labeled C466 and C473 were detected in wild-type and pfa4Δ cells, accounting for unmodified Rif1NTD. Tryptic fragments containing either CAM-labeled C466 or CAM-labeled C473 were detected in wild-type cells, providing site-specific evidence for Rif1 S-acylation in vivo (Fig. 4d). Under these conditions, CAM-modified Rif1 peptides from pfa4Δ cells were not detected (Fig. 4d, see also Supplementary Fig. 4 and Supplementary Table 1 for further peptide analyses), supporting the role of Pfa4 in Rif1 C466/C473 S-acylation. Alternative S-acylation at C466 and C473 might explain why only combined Rif1 C466A/C473A mutations result in defective NHEJ, suggesting site redundancy for NHEJ-relevant S-acylation by Pfa4 in vivo.
Fig. 4 S-acylation of Rif1 C466 and C473 in vivo. a Outline of the ABE protocol, including protein alkylation at free cysteines with NEM, removal of S-acyl groups including palmitoyl (Palm) using hydroxylamine (HA), and labeling with BMCC-biotin. Biotinylated proteins are captured on NeutrAvidin-coated beads and the presence of Rif1NTD is analyzed by western blotting (WB). b Representative western blots of ABE assays performed with cells expressing Myc-tagged Rif1NTD under control of a GAL1 promoter. Input: BMCC-biotin samples prior to biotin capture with and without HA. Input and AviF samples were probed with anti-Myc and anti-biotin antibodies as indicated. Fold enrichment of Rif1NTD in AviF relative to wild-type is presented as mean values ± s.e.m. (n = 3 independent experiments). c Outline of the ACE protocol, including treatment of proteins with TCEP (reducing potential disulfide bridges between cysteine residues), alkylation at free cysteines with NEM, removal of S-acyl groups using DTT, and alkylation of freed-up cysteines with chloroacetamide. Myc-tagged Rif1NTD is then immunoprecipitated for analysis by mass spectrometry (see Supplementary Fig. 4a for additional controls). d Mass-spectrometric analysis of tryptic Rif1 fragments spanning amino acids 463 to 479. Following ACE, Rif1NTD tryptic peptides were subjected to parallel reaction monitoring (PRM), measuring NEM (unmodified Rif1) and/or CAM-labeled (reflecting in vivo S-acylation) C466 and C473 in wild-type vs. pfa4Δ. Integrated PRM counts are presented as mean values ± s.e.m. and were normalized using measurements of the five non-modified Rif1 peptides shown on the right (see Supplementary Table 1 for additional information). Data are shown in logarithmic scale (n = 3 independent experiments). e Measurements of C466/C473 NEM and/or CAM-labeled peptides (left panel) and unmodified control peptides (right panel) of Rif1 in untreated vs. Zeocin-treated wild-type cells. PRM analysis of Rif1NTD peptides as in panel d. Mean values of integrated PRM counts ± s.e.m (n = 3 independent experiments) are shown in logarithmic scale. See Supplementary Fig. 4b, c for peptide transitions used in the experiment. Source data are provided as a Source Data file.

The PRM values for double NEM-modified Rif1 peptides spanning residues 463 to 479 are ~10 times higher than the values for peptides derived from Rif1 S-acylated at C466 or C473. This implies that a substantial fraction of 15–20% of Rif1 was S-acylated at either C466 or C473 in wild-type cells (Fig. 4d). DNA-damage treatment with Zeocin did not lead to gross changes in Rif1 S-acylation levels (Fig. 4e), consistent with constitutive Rif1 S-acylation.

Rif1 S-acylation allows a nuclear-peripheral damage response. To address the question how S-acylation may promote Rif1-mediated NHEJ, we first sought to determine whether the modifying enzyme, Pfa4, has access to the cell nucleus. Although membrane-associated, palmitoyl transferases have so far not been observed at the inner nuclear membrane. Pfa4-GFP has been localized to the endoplasmic reticulum (ER)⁴³, which is continuous with the nuclear envelope, but inner nuclear membrane access is selective, and whether Pfa4 can populate this subcompartment is unknown (Fig. 5a). Taking advantage of induced inner nuclear membrane proliferation following overexpression of nucleoporin Nup53, we asked whether Pfa4-GFP can access the resulting, distinctive membrane structures. These intranuclear lamellae have been shown to present in the form of so-called theta (θ) nuclei with transsecting membranes, providing the basis for a quantifiable fluorescence assay for testing inner nuclear membrane localization of GFP-tagged candidate proteins⁴⁴. As a positive control, we expressed ER membrane protein Sec61-GFP, which accessed the inner nuclear membrane, efficiently decorating θ structures⁴⁴ induced by Nup53 overexpression (Fig. 5b). As expected, cells expressing ER membrane protein Hrd1-GFP showed ~4-fold lower levels of fluorescent θ nuclei compared to Sec61-GFP (Fig. 5c), reflecting poorer inner nuclear membrane access. Pfa4-GFP showed an intermediate phenotype, populating θ nuclei at ~1.5-fold lower levels than Sec61-GFP (Fig. 5c). Upon DNA-damage treatment with Zeocin, the localization of Sec61-GFP or Hrd1-GFP to θ structures did not change, while Pfa4-GFP-associated θ structures increased ~2-fold (Fig. 5b, c). These data show that Pfa4 localizes to the inner nuclear membrane in unperturbed conditions, and this localization is enhanced after DNA damage. Using cell fractionation, we confirmed previous results indicating Pfa4-dependent membrane associations of Rif1, which proved partially dependent on Rif1 residues C466 and C473 (Supplementary Fig. 5). Together, these
results are consistent with Pfa4 having access to Rif1 in the nucleus, where NHEJ-relevant S-acylation at C466 and C473 may contribute to Rif1-membrane interactions.

To investigate whether S-acylation-mediated membrane anchorage may direct the actions of Rif1 in NHEJ to the inner nuclear membrane, we expressed fluorescently tagged Rif1NTD, which is unable to interact with Rap1 (ref. 2) and does not co-localize with telomere clusters (Supplementary Fig. 6a). In untreated conditions, we observed nuclear Rif1NTD-GFP foci in ~28% of wild-type cells. After DNA-damage treatment with Zeocin or ionizing radiation (IR), focus formation was strongly induced, reaching a peak ~30 min post-treatment (Supplementary Fig. 6b), when ~60% (Zeocin) and ~80% (IR) of cells exhibited Rif1NTD-GFP foci (Fig. 6a, b and Supplementary Fig. 6c, d). Moreover, while the majority of focus-positive cells in unperturbed conditions contained a single Rif1NTD-GFP focus, most
focus-positive cells exhibited multiple (up to four) foci after DNA damage treatment (Fig. 6c). DNA damage-induced Rif1NTD-GFP foci were observed in G1 and S/G2 cells with no overt cell-cycle dependence. DNA-damage treatment did not lead to increased Rif1NTD expression levels (Supplementary Fig. 6e), suggesting that focus formation reflected the redistribution of Rif1NTD-GFP into foci upon DNA damage.

Next, we analyzed Rif1NTD-GFP foci in pfa4Δ cells. Untreated cells were indistinguishable from wild-type with ~30% Rif1NTD-GFP focus-positive cells, the majority of which contained a single Rif1NTD-GFP focus (~36% and ~38% focus-positive cells after Zeocin and IR, respectively, see Fig. 6b and Supplementary Fig. 6d, f) and the formation of multiple Rif1 foci in response to DNA damage (Fig. 6c) was significantly abrogated in pfa4Δ cells. Protein levels of Rif1NTD remained unchanged upon loss of Pfa4 (Supplementary Fig. 6g). Importantly, in Pfa4-proficient cells, introducing the S-acylation mutation C466A/C473A also diminished the formation of DNA damage-induced Rif1NTD foci and the ability of cells to form multiple Rif1 foci (Fig. 6b, c). Like S-acylation mutant Rif1NTD C466A/C473A, the Rif1 HOOK DNA-binding mutant was strongly compromised in its ability to form foci in response to DNA-damage treatment (Fig. 6b, c). Combining the S-acylation and HOOK mutations led to a more severe phenotype compared to either the Rif1NTD C466A/C473A or the Rif1 HOOK mutant (Fig. 6b, c). Thus, Pfa4-dependent S-acylation of Rif1 at C466/C473 and the ability of Rif1 to bind DNA contribute to effective Rif1 accumulation upon DNA damage.

To determine the sub-nuclear localization of Rif1NTD-GFP foci, we scored their position relative to the nuclear envelope marked by fluorescently tagged nuclear-pore component Nup49 (ref. 45). Dividing the nucleus into three concentric zones of equal area, we found a strong bias of Rif1 accumulation in outermost zone 1, at the nuclear periphery, in wild-type cells. Upon DNA damage, ~60% of Rif1NTD-GFP foci localized in zone 1 (Fig. 6d). While cells expressing the Rif1 HOOK DNA-binding mutant maintained a strong localization bias to zone 1, cells with compromised Rif1 S-acylation expressing Rif1NTD C466A/C473A, Rif1NTD C466A/C473A HOOK, or wild-type Rif1NTD in a pfa4Δ background, exhibited an increase in zone 2-localized foci at the expense of zone 1-localized foci. Thus, Rif1 S-acylation mutants display an apparent reduction in Rif1-innuclear membrane interactions in conjunction with a significant impairment in the formation of DNA damage-induced Rif1-foci observed in all mutant backgrounds tested (Fig. 6b, c and Supplementary Fig. 6c, d, f).

Taken together, these data are consistent with a model where enrichment of Rif1 at the inner nuclear membrane mediated by Pfa4-dependent S-acylation of C466/C473 and its intrinsic DNA-binding activity enable effective Rif1 accumulation at nuclear-peripheral DNA damage, promoting preferential repair of membrane-proximal DSBs along the NHEJ pathway (Fig. 7).

Discussion

Here, we show that the palmitoyl transferase Pfa4 is essential for Rif1-dependent DSB repair by NHEJ. Loss of Pfa4 caused a drop in NHEJ efficiency but did not exacerbate the NHEJ defect of rif1Δ cells, genetically implicating Pfa4 and Rif1 in a common DSB repair pathway (Fig. 1). Starting from a structure-guided approach, we identified Rif1 C466 and C473 as two cysteine residues required for efficient NHEJ in vivo (Fig. 2), accumulation of Rif1 at DSBs, and attenuation of DNA end-resection (Fig. 3). We demonstrate that C466 or C473 are modified by S-acylation in vivo in a strictly Pfa4-dependent manner, strongly suggesting they are direct Pfa4 S-palmitoylation targets (Fig. 4).

Residual avidin-enrichment after subjecting Rif1NTD C466A/C473A to ABE chemistry points to Rif1 S-acylation at additional, as-yet unmapped sites (Fig. 4b). While these potential additional sites are neither required nor sufficient for Rif1 to promote NHEJ, at least one Pfa4-dependent S-acylation event at C466 or C473 is essential for Rif1-mediated NHEJ (Fig. 2). Localizing to the inner nuclear membrane (Fig. 5), Pfa4 has access to nuclear Rif1 and promotes Rif1-membrane associations (Supplementary Fig. 5). Furthermore, we discovered the nuclear-peripheral, focal accumulation of Rif1NTD in response to treatment of cells with radiomimetic drug Zeocin or IR. This localized DNA-damage response of Rif1 occurred in cells in G1 and S phase of the cell cycle, and thus reflects a cell-cycle phase-independent process. Importantly, DNA damage-induced Rif1 focus formation was dependent on Pfa4, S-acylated Rif1 residues C466 and C473, and required Rif1’s DNA-binding activity (Fig. 6). As IR and Zeocin give rise to DSBs and ssDNA breaks, we cannot exclude the possibility that Rif1 responds to multiple types of DNA damage. However, Rif1 accumulates at endonuclease-induced DSBs in a Pfa4 and C466/C473-dependent manner to attenuate DNA end-resection and promote NHEJ (Fig. 3 and Supplementary Fig. 3a). We therefore propose that the focal accumulation of Rif1 at the nuclear periphery, induced by Zeocin or IR, is a direct reflection of DSB binding. This contrasts with HR-dependent DSB repair foci marked by Rad52, which are generally found within the nuclear lumen46, a compartment that was deprived of DNA damage-induced Rif1 foci. Based on our results, we suggest a model where posttranslational fatty acylation at C466 or C473 increases the affinity of Rif1 for the inner nuclear membrane, creating a nuclear-peripheral compartment of high local Rif1 concentration. Here, Rif1 is poised to mount an effective response...
to DNA damage, encapsulating DSBs with its HOOK domain, and dampening DNA end-resection to favor repair by NHEJ (Fig. 7).

It is tempting to speculate that membrane binding may provide Rif1 with additional means of promoting favorable DNA repair outcomes, for example by constraining the movement of DSB ends to suppress chromosome instability by ectopic recombination events. At the same time, membrane-attachment could allow Rif1 to harness the nuclear envelope as a scaffold to facilitate the coordination of DSB ends for re-ligation. For telomere maintenance, Rif1 S-acylation appears to be dispensable53, and this may be explained by the recruitment of Rif1 to telomeres through protein-protein interactions with Rap1 (ref. 2). However, S-acylation may strengthen telomere interactions at the nuclear periphery to reinforce the telomere position effect33, where Rif1 helps antagonize transcriptional silencing near telomeres47,48. Rif1 interactions with the nuclear envelope have also been implicated in chromatin architecture and DNA replication5,7,14,15,49,50. While the ability of Rif1 to suppress DNA replication origin firing does not depend on Pfa4 (ref. 8) or C466 and C473 (Supplementary Fig. 3c), it will be important to explore at high resolution whether Rif1 S-acylation mutants act at ectopic genomic sites to determine whether fatty acylation modulates replication timing programs in eukaryotes.

The link between Rif1 and nuclear-peripheral DNA repair described herein resonates with increasing evidence for DNA repair compartmentalization and the impact of DSB microenvironments on repair pathway choice across organisms51,52. In human cells, nuclear-peripheral DSBs bound at the proteinaceous nuclear lamina are preferentially repaired by NHEJ53. The compaction of silent heterochromatin and repetitive sequence elements in lamina-associated domains has been proposed to hamper the recruitment of HR proteins, leading to a local NHEJ bias53. Reported lamina interactions of Rif1 (refs. 57,49) raise the interesting possibility that peripheral sequestration of Rif1 might have a role in sub-nuclear compartments geared towards NHEJ in mammalian cells. Of note, mammalian Rif1 S-palmitoylation is predicted by Swisspalm/CSS-Palm11, but it remains to be determined whether these modifications occur in vivo and how they might relate to NHEJ. In yeast, S-acylated Rif1 may preferentially target DSBs for NHEJ in nuclear envelope-associated heterochromatin or near telomeres. Furthermore, persistent DSBs in yeast relocate to the nuclear periphery54–56, which may facilitate access by S-acylated Rif1.

Protein S-acylation has been detected on a wide range of cytosolic and nuclear proteins1,12,17, and dysfunctional fatty acylation has been implicated in human diseases including cancer18,39. However, the functional consequences for most targets of S-acylation remain to be determined. Interestingly, chemical inhibition of protein S-palmitoylation in mammalian cells led to a muted DNA-damage response60. Here, we uncover a role for protein S-acylation in DSB repair pathway choice. The reversible nature of S-palmitoylation is reminiscent of well-established posttranslational modifications with important roles in DSB repair, including protein phosphorylation and ubiquitination, potentially allowing the dynamic regulation of Rif1 at DNA damage. The essential requirement for S-acylation in Rif1-mediated NHEJ provides the first example of a direct involvement of fatty acylation in DNA repair.

**Methods**

**Yeast techniques.** The complete list of *S. cerevisiae* strains used in this study can be found in Supplementary Table 2. Deletions and epitope tagging of genes of interest were done by one-step PCR gene replacement61. Point mutations in the *RIF1* gene were introduced using delitto perfetto62 and/or CRISPR/Cas9-based methods63 (see Supplementary Table 3 for primer sequences). For overexpression of Myc-tagged Rif1NTD, Pfa4-GFP, and Nup53, a GAL1 galactose-inducible promoter was inserted genomically and expression was induced by addition of 2% (w/v) galactose (Formedium, GAL02) to cells cultured in YPLG. For drop assays, strains were grown exponentially, and serial 10-fold dilutions were spotted on YPAD agar plates. Control cells were removed prior to HO-endonuclease induction and plated on medium containing glucose. At the indicated time-points, cells were plated on glucose-containing YPAD, then diluted in YPLG and grown exponentially. For transient methods63 (see Supplementary Table 3 for primer sequences). For overexpression of Myc-tagged Rif1NTD, Pfa4-GFP, and Nup53, a GAL1 galactose-inducible promoter was inserted genomically and expression was induced by addition of 2% (w/v) galactose (Formedium, GAL02) to cells cultured in YPLG. For drop assays, strains were grown exponentially, and serial 10-fold dilutions were spotted on YPAD agar plates. Control cells were removed prior to HO-endonuclease induction and plated on medium containing glucose. HO-endonuclease cut-efficiency was routinely determined by qPCR (see Supplementary Table 3 for primer sequences) and data was normalized accordingly67. Colonies were counted 3 days after plating, and NHEJ efficiency was calculated as described64.

**NHEJ assays.** The efficiency of NHEJ as measured by cell survival was determined as described38,64. JKM179-derived strains (see Supplementary Table 2) were grown overnight in YPAD, then diluted in YPLG and grown exponentially. For transient HO-endonuclease expression, 2% (w/v) galactose was added to the culture medium. At the indicated time-points, cells were plated on glucose-containing YPAD agar plates. Control cells were removed prior to HO-endonuclease induction and plated on medium containing glucose. HO-endonuclease cut-efficiency was routinely determined by qPCR (see Supplementary Table 3 for primer sequences) and data was normalized accordingly67. Colonies were counted 3 days after plating, and NHEJ efficiency was calculated as described64.
incubation for 3 h. The reaction was stopped by adding 20 mM EDTA and EMSA1 was purified using an Illustra MicroSpin G-25 column (GE Healthcare, 27532501) into 10 mM NaCl and 0.1% Triton X-100, and resuspended, and beads buffer containing 20 mM 2-mercaptopethanol, and protease-inhibitor cocktail (Roche, 05 892 791 001), Triton X-100 was added to a final concentration of 1% before being loaded to AGE beads for 2 h at 4 °C on a rotating wheel. After chloroform-methanol precipitation, proteins were solubilized in resuspension buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2% SDS, 8 μl/mg). Following addition of 1-biotinamido-4-(4-[(maleimidomethyl) cyanoethylcarboxamido]butan-1-ol (MCBC-biotin, Thermofisher Scientific, 21900) buffer (50 mM Tris-HCl pH 7.5, 300 μM BCA, 1 μl PBS), samples were mixed and hydroxylamine (HA, Sigma-Aldrich, 438227) was added (HA) or not (-HA) to a final concentration of 1 M. Biotin extraction was performed for 2 h at 4 °C. After precipitation and solubilization, NeutrAvidin agarose beads (Thermofisher Scientific, 29201) in 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.1% Triton X-100 were added, and the mixture was incubated for 4 h at 4 °C on a rotating wheel. Chloroform-methanol precipitations were solubilized in resuspension buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2% SDS, 8 μl/mg). Removal of S-acetyl groups from cysteine residues was achieved by incubating the samples with beads buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.1% Triton X-100) containing 10 mM 1,4-dithiothreitol (DTT, Sigma-Aldrich, 438245) for 1 h at room temperature. Proteins were precipitated, resuspended, and beads buffer containing 20 mM 2-chloroacetamide (CAA, Sigma-Aldrich, 22790) was added, allowing disulfide bridges formed by closely-spaced cysteine residues, leaving S-acetylation intact (see also Supplementary Fig. 4). Final concentrations of 50 mM NEM and 0.5% Triton X-100 were added to the cleared lysates, and blotting of reactive cysteines was performed for 2 h at 4 °C on a rotating wheel. Chloroform-methanol precipitations were solubilized in resuspension buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl) were performed overnight at 4 °C on a rotating wheel. After extensive washing with high (50 mM Tris-HCl pH 7.5, 500 mM NaCl) and low salt (50 mM Tris-HCl pH 7.5, 150 mM NaCl) buffers, samples were subjected to tryptic digestion on beads and analyzed by mass-spectrometry.

**ACE and acyl-N-ε-lysine exchange on synthetic peptides.** Synthetic peptides designed on the tryptic Rif1 peptide spanning residues 463-479 were obtained from Thermofisher Scientific, and contained palmitoylated C466/CAM-labeled C473 (IYQ[C][PALMITOYL]LMSPVC[CAM][ETIPEK]), CAM-labeled C466/palmitoylated C473 (IYQ[C][CAM][ILSPVC][PALMITOYL][ETIPEK] or palmitoylated C466/palmitoylated C473 (IYQ[C][CAM][ILSPVC][PALMITOYL][ETIPEK]) or palmitoylated C466/palmitoylated C473 (IYQ[C][CAM][ILSPVC][PALMITOYL][ETIPEK]). Peptides were diluted in 20% CH3CN, aliquoted, and stored at −20 °C. To monitor acyl-CAM and acyl-NEM exchange following treatment with reducing agents, peptide aliquots were diluted in a solution containing 50 mM Tris-HCl pH 8, 40% CH3CN, and either 40 mM DTT or 40 mM TCEP, and reduction was carried out overnight at 4 °C. Aliquots with either 90 mM iodoacetamide (IAA, Sigma-Aldrich, R01003) or 90 mM NEM was performed for 1 h at room temperature. 1% trifluoroacetic acid (TFA, Pierce Biotech, 28904) was added, and samples were analyzed by mass spectrometry.

**Mass spectrometric analysis of synthetic peptides.** Synthetic peptides were analyzed by capillary liquid chromatography tandem mass spectrometry (LC-MS). Peptides were loaded in 0.1% formic acid (Pierce Biotech, 28905), 10% acetonitrile (VWR, 11185), 4% isopropanol (VWR, 14263) and 96% water (VWR, 14261) at 50 µl/min flow rate with 15% acetonitrile (VWR, 11185) and 0.1% formic acid (Pierce Biotech, 28905) buffer B (buffer A: 0.1% formic acid. 10% acetonitrile in water; buffer B: 0.1% formic acid in acetonitrile). The column was mounted on a DVP ion source (New Objective)
Subcellular fractionation. Rate, as calculated in Scaffold. To 5 ppm and fragment ion mass tolerance to 0.01 Da. The results were validated by subtraction of the signal obtained in the Avf without HA and normalized to the input for data shown in Fig. 4b. Western blot signals for Rfi1-Enh in soluble or membrane fractions were quantified using Fiji software. Band intensities were background-corrected and normalized to tubulin for the soluble fraction and to the nuclear pore p90 band for the membrane-bound fraction. Results are shown in Supplementary Fig. 5. Scanned EMSA phosphorimages were analyzed with ImageJ. Total intensity of each individual lane was plotted and separated into the unbound DNA signal and the retarded DNA signal. The percentage of retarded DNA signal including all shifted bands (defined as “fraction bound”) was analyzed and plotted using GraphPad Prism v. 7 to produce the results shown in Fig. 3d. For quantification of the δ nucleus and Rif1NTD-GFP foci presented in Figs. 5 and 6, respectively, confocal microscopy images were analyzed using Fiji software. Violin plots shown in Fig. 6c were generated with Rstudio.

Priming report. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
A reporting summary for this article is available as a Supplementary Information file. The data underlies Figs. 1b–d, 2a, b, d, e, and 6b, c are provided as a Source Data file. All data supporting the findings of this study are available from the corresponding author upon reasonable request. Structural figures were prepared with PyMOL v. 1.8.4 (Schrodinger Inc.) and are based on published structures (PDB: SNVR and PDB: NWS5). The mass spectrometry proteomics data have been deposited at ProteomeXchange via PRIDE and can be accessed using access code PXD012137.

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Subcellular fractionation. Assumption of solubility of Myc-tagged Rif1NTD in wild-type or mutant strains was performed by differential centrifugation as reported.23 Exponentially growing cells were collected, spheroblasted, and resuspended in 100 mM KCl, 50 mM HEPES-KOH pH 7.5, 2.5 mM MgCl2, and 0.4 M sorbitol. After centrifugation (1500×g for 1 min at 4 °C), pellets were resuspended in an equal volume of extraction buffer (100 mM KCl, 50 mM HEPES-KOH pH 7.5, 2.5 mM MgCl2, 50 mM NaF, 5 mM Na2P04, 0.1 mM NaVO3, protease inhibitors). Spheroblasts were lysed on ice by addition of 0.25% Triton X-100, and the whole cell extract fraction was collected. Lyastes were underlayered with half a volume of 1 M sucrose and centrifuged at 150,000×g for 10 min at 4 °C. Pellets corresponding to the membrane-bound fraction, and soluble fraction were separated. Equal amounts of protein were loaded on 3–7% Tris-acetate gels. See "Quantification and statistical analysis" for a detailed description of the quantification of Rif1NTD enrichment in the soluble and membrane-bound fraction.

Spinning-disk confocal microscopy. Exponentially growing cells, cultured in sterile-filtered YPAD, were treated with 100 μg/ml Zeocin for 30 min or 100 Gy of irradiation by counteracting DDK activity. Cell Rep. 7, 53–61 (2016).

Hiraga, S.-I. et al. Rif1 controls DNA replication by directing protein recruitment and structural modification of the replication origins is limited by telomere sequestration. Genes Dev. 3677 (2012).

Hayano, M. et al. Rif1 is a global regulator of timing of replication origin firing in yeast. Genes Dev. 26, 137–150 (2012).

Yamazaki, S. et al. Rif1 regulates the replication timing domains on the human genome. EMBO J. 31, 3678–3690 (2012).

Hayano, M. et al. Rif1 is a general regulator of timing of replication origin firing in yeast. Genes Dev. 26, 137–150 (2012).

Davé, A., Cooley, C., Garg, M. & Bianchi, A. Protein phosphatase 1 recruitment by Rif1 regulates DNA replication origin firing by counteracting DDK activity. Cell Rep. 7, 53–61 (2016).

Hiraga, S.-I. et al. Rif1 controls DNA replication by directing protein kinase C to a novel Cdc7-mediated phosphorylation of the MCM complex. Genes Dev. 28, 372–383 (2014).

Hiraga, S.-I. et al. Rif1 controls DNA replication timing in yeast through the PPI phosphatase Pch1. Cell Rep. 7, 62–69 (2014).

Alver, R. C., Chadha, G. S., Gillespie, P. J. & Blow, J. J. Reversal of DDK-mediated MCM phosphorylation by Rif1-PPI regulates replication initiation and replisome stability independently of ATR/Chk1. Cell Rep. 18, 2508–2520 (2017).

Hafner, L. et al. Rif1 binding and control of chromosomal DNA replication origins is limited by telomere sequestration. Cell Rep. 23, 983–992 (2018).

Hafner, L., Shore, D. & Mattarocci, S. ChECing out Rif1 action in freely cycling cells. Curr. Genet. 65, 429–434 (2019).

Quantification and statistical analysis. Statistical analyses were performed using GraphPad Prism v. 7. The applied tests and number of independent observations are indicated in the corresponding figure legends. Western blot band intensities were quantified using Fiji software, and normalized using the indicated loading controls. Results shown in Supplementary Figs. 1a, 2c, 3b, 6c, and 6g were plotted with GraphPad Prism v. 7. Western blot signals for biotin-captured Rif1NTD (AviF) were quantified using Fiji software. Band intensities were background-corrected and normalized to tubulin for the soluble fraction and to the nuclear pore p90 band for the membrane-bound fraction. Results are shown in Supplementary Fig. 5. Scanned EMSA phosphorimages were analyzed with ImageJ. Total intensity of each individual lane was plotted and separated into the unbound DNA signal and the retarded DNA signal. The percentage of retarded DNA signal including all shifted bands (defined as “fraction bound”) was analyzed and plotted using GraphPad Prism v. 7 to produce the results shown in Fig. 3d. For quantification of the δ nucleus and Rif1NTD-GFP foci presented in Figs. 5 and 6, respectively, confocal microscopy images were analyzed using Fiji software. Violin plots shown in Fig. 6c were generated with Rstudio.
16. Chapman, J. R. et al. Rif1 is essential for S3BP1-dependent nonhomologous end joining and suppression of DNA double-strand break resection. Mol. Cell 63, 781–791 (2016).

17. Di Virgilio, M. et al. Rif1 prevents resection of DNA breaks and promotes immunoglobulin class switching. Science 339, 711–715 (2013).

18. Escribano-Díaz, C. et al. A cell cycle–dependent regulatory circuit composed of S3BP1-Rif1 and BRCA1-CIP controls DNA repair pathway choice. Mol. Cell 49, 684–693 (2013).

19. Feng, L., Fong, K.-W., Wang, J., Wang, W. & Chen, J. Rif1 counteracts BRCA1-mediated end resection during DNA repair. J. Biol. Chem. 288, 11135–11143 (2013).

20. Zimmermann, M., Lottersberger, F., Buonorno, S. B., Steir, A. & de Lange, T. S3BP1 regulates DSB repair using Rif1 to control 5′ end resection. Science 339, 704–707 (2013).

21. Boersma, V. et al. MAD2L2 controls DNA repair at telomeres and DNA breaks by inhibiting 5′ end resection. Nature 521, 537–540 (2015).

22. Xu, G. et al. REV7 counteracts DNA double-strand break resection and affects PARP inhibition. Nature 521, 541–544 (2015).

23. Gupta, R. et al. DNA repair network analysis reveals shieldin as a key regulator of the HJF and PARP inhibitor sensitivity. Cell 173, 972–988.e23 (2018).

24. Tomida, J. et al. FAM35A associates with REV7 and modulates DNA damage responses of normal and BRCA1-defective cells. EMBO J. 37, e95943 (2018).

25. Noordermeer, S. M. et al. The shieldin complex mediates S3BP1-dependent DNA repair. Nat. Cell Biol. 16, 460–471 (2014).

26. Mirman, Z. et al. 53BP1 regulates DSB repair using Rif1 to control 5′ end resection. Nature 521, 541–544 (2015).

27. Dev, H. et al. Shieldin complex promotes DNA end-joining and counters homologous recombination in BRCA1-null cells. Nat. Cell Biol. 20, 954–965 (2018).

28. Ghezraoui, H. et al. S3BP1 cooperation with the REV7–shieldin complex underpins DNA structure-specific NHEJ. Nature 560, 122–127 (2018).

29. Zhang, H. et al. A cell cycle-dependent BRCA1-UHRF1 cascade regulates DNA double-strand break repair pathway choice. Nat. Commun. 7, 10201 (2016).

30. Kumazaki, R. & Cheek, C. F. Dynamics of Rif1 SUMoylation is regulated by PIA54 in the maintenance of genomic stability. Sci. Rep. 7, 17367 (2017).

31. Roth, A. F. et al. Global analysis of protein palmitoylation in yeast. Cell 125, 1003–1013 (2006).

32. Lam, K. Y. et al. Palmitoylation by the DHHC protein Pfa4 regulates the ER exit of Chs3. J. Cell Biol. 174, 19 (2006).

33. Park, S. et al. Palmitoylation controls the dynamics of budding-yeast heterochromatin via the telomere-binding protein Rif1. Proc. Natl Acad. Sci. USA 108, 14572–14577 (2011).

34. Chamberlain, L. H. & Shipston, M. J. The physiology of protein palmitoylation in cell. Physiol. Rev. 95, 341–376 (2015).

35. Lanyon-Hogg, T., Faronato, M., Serwa, R. A. & Tate, E. W. Dynamic protein acylation: new substrates, mechanisms, and drug targets. Trends Biochem. Sci. 42, 566–581 (2017).

36. Taddei, A., Schober, H. & Gasser, S. M. The budding yeast nucleus. Cold Spring Harb. Perspect. Biol. 2, a00612 (2010).

37. Fox, C. A. & Gartenberg, M. R. Palmitoylation in the nucleus: a little fat around the edges. Nucleus 3, 251–255 (2012).

38. Moore, J. K. & Haber, J. E. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. Mol. Cell. Biol. 16, 2164–2173 (1996).

39. Zhang, Y. et al. Role of Dnl4-Lif1 in nonhomologous end-joining repair complex assembly and suppression of homologous recombination. Nat. Struct. Mol. Biol. 14, 659–667 (2007).

40. Zierahr, C. & Difilipo, J. F. Break dosage, cell cycle stage and DNA replication influence DNA double strand break response. EMBO J. 27, 1875–1885 (2008).

41. Blanc, M. et al. SwissPalm: protein palmitoylation database. F1000Research 4, 261 (2015).

42. Wan, J., Roth, A. F., Bailey, A. O. & Davis, N. G. Large-scale profiling of protein palmitoylation in mammalian cells. Nat. Methods 6, 135–138 (2009).

43. Chen, B., Sun, Y., Niu, J., Jarugumilli, G. K. & Wu, X. Protein lipidation in cell signaling and diseases: function, regulation, and therapeutic opportunities. Cell Chem. Biol. 25, 817–831 (2018).

44. Resh, M. D. Palmitoylation of proteins in cancer. Biochem. Soc. Trans. 45, 409–416 (2017).

45. Oza, P., Jaspersen, S. L., Miele, A., Dekker, J. & Peterson, C. L. Mechanisms that regulate localization of a DNA double-strand break to the nuclear periphery. Genes Dev. 23, 912–927 (2009).

46. Martín, B. R. & Cravatt, B. F. Large-scale profiling of protein palmitoylation in mammalian cells. Nat. Methods 6, 135–138 (2009).

47. Foti, R. et al. Nuclear compartmentalization of DNA repair. Methods Mol. Biol. 17, 12 (2016).

48. Longtime, M. S. et al. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14, 953–961 (1998).

49. Stuckey, S., Mukherjee, K. & Storici, F. In vivo site-specific mutagenesis and gene collateral using the delitto perfetto system in yeast Saccharomyces cerevisiae. Meth. Mol. Biol. 745, 173–191 (2011).

50. D’Arico, J. E. et al. Genome engineering in Saccharomyces cerevisiae using CRISSPR-Cas systems. Nucleic Acids Res. 41, 4336–4343 (2013).

51. Lee, S. E., Pâques, F., Sylvan, J. & Haber, J. E. Role of yeast SIR genes and mating type in directing DNA double-strand breaks to homologous and non-homologous repair paths. Curr. Biol. 9, 767–770 (1999).

52. Rass, U. & West, S. Synthetic junctions as tools to identify and characterize Holliday junction resolvases. Methods Enzymol. 408, 485–501 (2006).

53. Iy, Y. et al. Direct detection of S-palmitoylation by mass spectrometry. Anal. Chem. 85, 11952–11959 (2013).

54. MacLean, B. et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 26, 966–968 (2010).

55. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).

56. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675 (2012).

57. Vizcaino, J. A. et al. 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 44, 11033–11033 (2016).

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Author contributions
G.A.F. conducted most experiments, with assistance by D.K.; G.A.F. established ACE with the help of D.H., who performed mass spectrometry. D.S. and S.M. provided ChIP and immunoprecipitation control data for N.H.T. and J.K.R., and D.K. purified Rif1 proteins and carried out DNA-binding experiments. B.F. helped with data analyses. G.A.F. and U.R. conceived the study and wrote the manuscript with input from all authors.
Additional information

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