Interplay of Histone Marks with Serine ADP-Ribosylation

Highlights
- Histone serine ADP-ribosylation and proximal acetylation are mutually exclusive
- PARP inhibitors block the DNA damage-induced histone H3 deacetylation
- Tyrosine residues are ADP-ribosylation targets
- Inverted polarity electrophoresis simplifies the study of histone marks

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In Brief
Bartlett et al. demonstrate that serine ADP-ribosylation and proximal phosphorylation and acetylation are mutually exclusive at the N-terminal tails of core histones. They also expand the repertoire of ADP-ribosylation target residues by providing evidence for tyrosine ADP-ribosylation on HPF1 and several other proteins.
Interplay of Histone Marks with Serine ADP-Ribosylation

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SUMMARY

Serine ADP-ribosylation (Ser-ADPr) is a recently discovered protein modification that is catalyzed by PARP1 and PARP2 when in complex with the eponymous histone PARylation factor 1 (HPF1). In addition to numerous other targets, core histone tails are primary acceptors of Ser-ADPr in the DNA damage response. Here, we show that specific canonical histone marks interfere with Ser-ADPr of neighboring residues and vice versa. Most notably, acetylation, but not methylation of H3K9, is mutually exclusive with ADPr of H3S10 in vitro and in vivo. We also broaden the O-linked ADPr spectrum by providing evidence for tyrosine ADPr on HPF1 and other proteins. Finally, we facilitate wider investigations into the interplay of histone marks with Ser-ADPr by introducing a simple approach for profiling posttranslationally modified peptides. Our findings implicate Ser-ADPr as a dynamic addition to the complex interplay of modifications that shape the histone code.

INTRODUCTION

ADP-ribosylation (ADPr) is a clinically important posttranslational modification (PTM) that controls many cellular processes, including DNA repair, transcription, translation, and chromatin remodeling (Gupte et al., 2017; Posavec Marjanović et al., 2017; Palazzo et al., 2017; Cohen and Chang, 2018). The ADPr reaction consists of the enzymatic transfer of ADPr from positively charged nicotinamide adenine dinucleotide (NAD⁺) onto an acceptor molecule with the simultaneous release of nicotinamide (Gupte et al., 2017; Pascal and Ellenberger, 2015). Poly(ADPr) polymerases (PARPs) are the major family of enzymes that perform ADPr, and 17 PARP family members are encoded in the human genome (Barkauskaite et al., 2015). Many studies have already been conducted on various histone modifications, yet all of them have overlooked Ser-ADPr because this PTM remained elusive until recently (Leidecker et al., 2016). Conversely, despite their focus on histones, studies centered on Ser-ADPr have so far investigated this PTM independent of other histone marks (Leidecker et al., 2016; Bonfiglio et al., 2017b; Fontana et al., 2017; Bilan et al., 2017). In this paper, we provide insights into the interplay between Ser-ADPr and canonical histone marks. Furthermore, by characterizing the PARP/HPF1-catalyzed ADPr consensus motif, we determine the relative significance of the preceding basic residue and discover tyrosine as an acceptor for ADPr. The resulting interplay analysis examines the effect of surrounding histone PTMs and shows that certain specific acetylation and phosphorylation marks can inhibit Ser-ADPr and vice versa. To broaden and improve studies of histone marks interplay, we introduce a method for visualization of modified as well as unmodified counterpart peptides.
RESULTS

Factors Influencing Ser-ADPr of Histone Peptides

To analyze the substrate properties that influence Ser-ADPr, we investigated sequence features that may affect the efficiency of in vitro histone peptide ADPr reactions. Our previous proteomics data provided a short consensus motif for in vivo Ser-ADPr with either Lys or Arg N-terminal to the target Ser (Leidecker et al., 2016; Bonfiglio et al., 2017b). Based on these observations, we incubated PARP1 and HPF1 with a variety of histone peptides, each containing an Lys-Ser (KS) motif known to be the modification site in vivo (Leidecker et al., 2016). Similar to what we reported before (Bonfiglio et al., 2017b), we observed that two different histone H3 peptides as well as H2A and H4 peptides were modified by the HPF1/PARP1 complex in vitro (Figure 1A). The Ser-ADPr glycosylhydrolase ARH3 (Fontana et al., 2017) was able to efficiently remove the ADP-ribose from all of the analyzed peptides (Figure 1A). We also compared the efficiency of H3 peptide 1–20 modification to that of the H3/H4 tetramer and the whole nucleosome. As shown in Figure S1A, peptide modification is not dramatically lower, especially considering the additional ADPr sites on the histone proteins and that this H3 peptide is mostly mono-ADPr in vitro (Bonfiglio et al., 2017b). These experiments establish that KS motifs in a variety of histone peptides can be modified efficiently and reversibly, demonstrating the utility of the histone peptide as a tractable in vitro assay for histone Ser-ADPr.

Next, we opted to focus on H3 Ser10 (H3S10) ADPr, because this site was previously shown to be the primary ADPr site on H3 in vivo (Palazzo et al., 2018). We investigated how alterations of the key KS residues affect the modification profile of the H3 histone peptide in vitro. Based on our previous finding that both PARP1 and PARP2 can modify this H3 peptide in the presence of HPF1 (Bonfiglio et al., 2017b), we examined both PARPs with variations on the KS motif. Substitution of Ser10 with alanine (Ala) led to a complete loss of the modification (Figure 1B), as we have previously shown (Bonfiglio et al., 2017b). Changing the neighboring Lys residue into Arg or Ala had varying effects on histone Ser-ADPr. The H3 peptide containing the K9R mutation was still modified, albeit to a lesser extent than wild-type (WT) peptide. In contrast, the H3K9A mutation strongly (but not completely) inhibited histone H3 Ser-ADPr (Figure 1B), highlighting the importance of a basic residue preceding the Ser. Both PARP1 and PARP2 modified the peptide panel with similar profiles, although PARP1 catalyzed the reactions more efficiently under the conditions used.

We further confirmed the importance of the consensus KS motif for Ser-ADPr in vivo. We transfected 293T cells with FLAG-tagged histone H3 WT, K9A, K9R, K9Q, or S10A mutant H3 and assessed ADPr efficiency, as described previously (Palazzo et al., 2018). DNA damage was induced by the treatment with 2 mM hydrogen peroxide (H2O2), followed by FLAG-immunoprecipitation (FLAG-IP). Western blotting was performed using a pan-ADPr reagent that recognizes all forms of cellular ADPr (Figures 1C and S1B). The ADPr patterns obtained were similar to those observed in our in vitro reactions. To note, by using a specific anti-H3K9ac antibody, we show that the KS motif is also important for K9 acetylation in vivo (Figure 1C, FLAG-IP).

These data extend our previous findings that the KS and RS motifs are preferred targets for Ser-ADPr and exclude the possibility that Lys rather than Ser is the modification target.

Discovery of Tyrosine as a Target Residue for ADPr

ADPr of Ser led us to question whether a hydroxyl group is sufficient and necessary to target an amino acid for ADPr when adjacent to Lys. We therefore decided to substitute H3S10 with threonine (Thr) and tyrosine (Tyr), the two other residues that contain hydroxyl groups, and additionally Glu and Asp as further controls. Not only were we unable to detect ADPr on Glu and Asp but also on Thr residues (Figure 2A). This suggests that although chemically similar to Ser, the additional methyl group on Thr interferes with the ADPr reaction mediated by PARP1/HPF1. In fact, in none of our previous proteomic analyses (Leidecker et al., 2016; Bonfiglio et al., 2017b) were we able to detect Thr-ADPr. Conversely, we identified a reproducible modification of Tyr when we introduced this amino acid instead of Ser10 (Figure 2A). Because Tyr has not previously been described as a substrate for ADPr, we sought mass spectrometric evidence for Tyr-ADPr. Although we could not detect Tyr-ADPr in our histone proteomics data (Leidecker et al., 2016), we confidently identified Tyr-ADPr of HPF1 in an in vitro reaction containing PARP1 (Figures 2B and S2B). We could also identify Ser97 in HPF1 as another site modified in this reaction (Figure 2C). These data suggested that PARP1 was the enzyme responsible for HPF1 Tyr-ADPr modification. To follow up on this point, we modified recombinant HPF1 using a panel of different PARPs and radioactively labeled NAD. We could observe a low but reproducible modification by PARP1 and possibly by PARP2 (Figures 2D, S2A, and S2E). This modification is at least partly dependent on the assembly of the PARP1/HPF1 complex, because the modification of the HPF1 R239A mutant protein (previously shown to be deficient in interacting with PARP; see Gibbs-Seymour et al., 2016) was significantly reduced (Figure 2E).

To confirm the ADPr of HPF1 in vivo, we overexpressed and immunoprecipitated FLAG-tagged HPF1 WT, S97A, and Y238A mutant proteins from 293T cells, as was described above for histone H3. We observed that HPF1 is significantly modified in cells even in undamaged conditions (Figure 2F). We did not detect a major effect of the S97A mutation on the modification.

Figure 1. Modifiers of Serine-ADP-Ribosylation of Histone Peptides

(A) Autoradiogram showing ADPr, and subsequent ARH3-mediated glycohydrolysis of H3 1–20aa, H3 27–45aa, H2A 1–17aa, and H4 1–23aa peptides. Coomassie staining of the SDS-PAGE is included and represents the loading control.

(B) Autoradiogram showing PARP1/2 + HPF1-mediated ADPr of H3 peptide with Lys9 substituted by Ala and Arg, and Ser10 substituted by Ala. Coomassie staining of the SDS-PAGE is included.

(C) 293T cells were transfected with the same amount of empty vector (EV) or plasmid expressing WT, K9A, K9R, or S10A FLAG-tagged histone H3 protein and treated for 10 min with H2O2. Inputs (A) and FLAG-IPs (B) were analyzed by western blotting.
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of HPF1. However, mutation of the Tyr238 site to Ala had a profound effect on the HPF1 ADPribosylation signal (Figure 2F). This defect may be at least partly due to a reduced ability of the Y238A mutant to interact with PARP1 and to stimulate ADPribosylation (Gibbs-Seymour et al., 2016). To further prove that HPF1 ADPribosylates depend on PARP1, we performed FLAG-IP in PARP1 knockout (KO) 293T cells. As can be seen in Figure 2G, HPF1 ADPribosylation is largely missing in PARP1 KO cells. It is likely that the remaining HPF1 modification is due to PARP2 activity. ADPribosylation of Tyr238 is not essential for the global HPF1-dependent ADPribosylation of histones because the non-modifiable Y238F HPF1 mutant supports this activity both in cells and in vitro (Figures S3A and S3B).

While it appears that there may be multiple ADPribosylation sites on HPF1, we were able to confirm the ADPribosylation of Y238 on HPF1 in cell extracts by ADPribosylation mapping through reprocessing (Matic et al., 2012) of a published dataset (Bilan et al., 2017) (Figure S2D). Reanalysis of a large-scale ADPribosylation dataset (Martello et al., 2016) revealed four additional high-certainty Tyr-ADPribosyl target proteins (Figures S2C and S2E–S2G). Although the type of mass spectrometric analysis used to generate the latter dataset is suboptimal (for additional information about the inadequacies of the high-energy collisional dissociation [HCD] technology for ADPribosylation site mapping, please refer to Bonfiglio et al., 2017a), our discovery of a Tyr-ADPribosylation peak (Figure S2C) enhances the confidence of Tyr-ADPribosylation site mapping.

**Canonical H3 Histone Marks Reduce the Efficiency of H3S10ADPribosylation on H3 Peptide**

We observed that removal of the positively charged Lys through synthesis of an H3 peptide containing an Ala in position 9 instead of an Lys almost completely abolished Ser-ADPribosylation (Figures 1B and 1C). It is known that acetylation neutralizes the positive charge of Lys residues, whereas methylation maintains the charge. Thus, the presence of this frequently modified residue in our consensus motif led us to hypothesize that modifications of the Lys preceding the Ser may have different effects on Ser-ADPribosylation, a potential mechanism of interplay between the known histone modifications in the H3S10 environment and H3S10ADPribosylation. Additionally, the recent discovery of PARPs conjugating ADPribosylation to phosphorylated DNA (Talhaoui et al., 2016; Munnur and Ahel, 2017) raised the intriguing possibility that this dynamic interplay.

Because these endogenous histone PTMs (histone marks) are highly dynamic in cells and organisms, an interplay is likely to have important biological consequences. By examining “marked” histone peptides in vitro, we can generate “snapshots” of this dynamic interplay.

We therefore set out to investigate the effect on H3S10ADPribosylation of the histone mark environment around H3S10, which is a particular PTM-rich and biologically important histone region (Huang et al., 2015). H3K9ac severely inhibits histone H3S10ADPribosylation (Figure 3A), as shown in a recent report (Liszczyk et al., 2018), and reversal of Lys9 acetylation by using deacetylase enzymes (HDAC2, SIRT2) re-established this peptide as a substrate for Ser-ADPribosylation by PARP1/HPF1 (Figure S4A). In comparison, H3K9me1 causes only a very mild reduction of H3S10ADPribosylation levels compared to the unmodified peptide. Phosphorylation of the target residue, Ser10, completely blocked ADPribosylation of the peptide, confirming that ADPribosylation and phosphorylation of the Ser10 site are mutually exclusive (Figure 3A). In agreement with this, we did not find any mass spectrometric evidence for ADPribosylation of a phosphorylated Ser. This indicates that PARP1-mediated ADPribosylation of DNA on a phosphate group (Talhaoui et al., 2016; Munnur and Ahel, 2017) is mechanistically different from HPF1-dependent ADPribosylation by PARP1 on protein substrates.

Given that H3K9me1 did not notably compromise S10ADPribosylation levels, we analyzed whether dimethylations or trimethylations, both commonly observed in the histone code, would have a greater impact on the reaction. We noted a stepwise decrease in H3S10ADPribosylation on H3K9me1, H3K9me2, and H3K9me3 substrates, with H3K9me3 permitting only a very modest degree of PARP1/HPF1-dependent H3S10ADPribosylation (Figure 3B). Because H3K9me and H3S10ADPribosylation modifications could coexist on the H3 peptide, we investigated whether the recently identified enzyme that removes Ser-ADPribosylation, ARH3, could still access and remove H3S10ADPribosylation in the presence of H3K9me. Our analysis showed that ARH3 was still active against H3S10ADPribosylation, irrespective of the H3K9me marks, and could efficiently erase H3S10ADPribosylation signals from modified H3 peptides (Figure S4B).

To investigate the effect of known histone marks in a wider context, we broadened the scope of our analysis of residues surrounding H3S10ADPribosylation by testing H3K4ac, H3K4me3, H3K14ac, H3K18ac, and H3K18me3 peptides. Of these additional histone marks, only H3K14ac notably affected the subsequent addition of ADPribosylation to H3S10 (Figure 3C).
Because our earlier experiments had determined that H4 1–23 and H3 27–45 peptides were suitable for PARP1/HPF1-dependent Ser-ADPr modification, we tested both for crosstalk between nearby acetylation and methylation modifications with H4S1ADPr and H3S28ADPr. We found that the modification of the KS motif at S28 has effects similar to those seen for Ser10 (Figure 3D). Alternatively, H3K36me1, H3K36me2, or H3K36me3 did not reduce the H3S28ADPr modification signals, while the H3K36ac had only a modest effect (Figure S4C). We also found that none of the H4R3me2, H4K5ac, or H4K8ac marks had a discernible effect on H4S1ADPr levels compared to the unmodified peptide (Figure S4C).

Ser-ADPr on H3S10 Prevents the Efficient Incorporation of H3K9 Acetylation and H3S10 Phosphorylation

We conducted reciprocal experiments based on our above findings, this time modifying histone H3 peptide first with PARP1/HPF1 complex (Figure S5), then subsequently incubating the reaction products in acetylation, phosphorylation, and methylation reaction mixtures using the purified catalytic domain of p300, the activated fragment of Aurora B kinase (Baronase; to phosphorylate H3S10) and Dim5 methyltransferase (Nunes Bastos et al., 2013; Moonat et al., 2013; Zhang et al., 2003). We detected the acetylated products of Ser-ADPr H3 peptides using a specific H3K9ac antibody and observed that K9ac is effectively prevented if the peptide is previously ADPr (Figure 4A, lane 5). To control for any possible interference of ADPr with western blot detection, we incubated the Ser-ADPr H3 peptide with p300, stopped the reaction, and removed Ser-ADPr from the peptide using ARH3. This assay showed only a negligible amount of H3K9ac (Figure 4A, lane 6). In a similar experiment, we saw that Ser-ADPr of H3 peptide prevented subsequent H3S10 phosphorylation (Figure 4B, lane 5). Finally, we incubated an Ser-ADPr H3 peptide in an Lys methylation reaction and found that H3S10ADPr did not preclude the incorporation of H3K9me3, although it did substantially reduce the efficiency of the reaction compared to the unmodified H3 peptide (Figure 4C, lane 3 versus lane 5).

An Approach for Rapid and Easy Analysis of ADPr Peptides

Our approaches above use [32P]NAD as a detection method, but this radioactive technique is expensive and requires strict safety procedures. Furthermore, using [32P]NAD and standard gel electrophoresis only allows the detection of modified product, rather than an analysis of unmodified and modified peptides together (i.e., substrates and products). These limitations, together with the clear importance of studying the interplay of Ser-ADPr and other known histone marks, motivated us to look for a simpler approach that could be implemented in virtually any biological laboratory. Given that ADP-ribose is a nucleotide, we reasoned that an electrophoresis system capable of resolving a one-nucleotide difference in the length of oligonucleotides would allow a clear separation of ADPr and unmodified substrate peptides. However, the negatively charged nucleic acids are separated by migrating toward the positively charged anode. In contrast, the histone tail peptides have a net positive charge, even when modified by ADP-ribose and would therefore migrate in the wrong direction. By changing the polarity of the electrodes, the positively charged substrate peptides can be driven into gels intended for electrophoresis of short nucleic acids and be separated according to their charge. Following ADPr, peptides become less positively charged and therefore migrate more slowly, which allows a clear spatial separation between the bands of the modified and unmodified peptides (Figure S5A). After the run, both species (unmodified and modified) can be clearly visualized and quantified by Coomassie-based staining, which reveals by band shift how much of the starting peptide has been ADPr (Figure 5A). Figure 5B shows an exemplar of this technique, comparing unmodified H3 peptide during a time course with PARP1, HPF1, and H3 peptide, with the modified peptide shifted upward at later time points. Incubating modified H3 peptide with ARH3 reverses the band shift to the unmodified state (Figure 5C). We then expanded this method to investigate ADPr efficiency on H3 peptides with a variety of histone marks. We observed that H3K4me mildly reduced ADPr levels compared to WT, whereas H3R8me peptides were modified efficiently (Figure 5D), H3K9ac, H3K9me, and K14ac modification profiles were comparable to the [32P]NAD experiments, reinforcing the value of this Coomassie-based approach for estimating the efficiency of a reaction. Additionally, we examined an H3T11ph peptide, which showed only a very slight ADPr band, suggesting a strong inhibition of HPF1/PARP1-catalyzed Ser-ADPr by the adjacent phosphorylation (Figure 5D). These combined experiments produced a map of the histone marks within a local region around H3S10 that affect the efficiency of H3S10ADPr (Figure 5E). Notably, histone marks other than ADPr also generated a band shift compared to the unmodified counterpart peptide (Figure 5D, left). This implies that the utility of our approach is not limited to ADPr and that this technique can be used to study the dynamics of other histone marks at the peptide level, such as the interplay between phosphorylation and acetylation (Latham and Dent, 2007).

H3K9ac and S10ADPr Are Mutually Exclusive Histone Marks in Human Cells

We sought to assess whether the results generated using histone H3 peptides could be replicated with intact human nucleosomes in vitro. WT and H3K9ac recombinant human mononucleosomes were incubated with PARP1 in the presence and...
We observed a clear contrast between the WT and H3K9ac nucleosomes when incubated with PARP1/HPF1, with WT displaying a higher level of Ser-ADPr (Figure 6A). The H3K9ac nucleosomes were still significantly modified, albeit to a lower degree, presumably due to modifications of other previously observed histone tail sites, such as H3S28 and H2BS6 (Leidecker et al., 2016). Similarly, we performed an assay using a nucleosome substrate to test the reciprocal reactions, namely Ser-ADPr, and then acetylation of the nucleosome (Figure 6B). We saw that prior Ser-ADPr reduced subsequent H3K9 acetylation, as detected by the specific anti-H3K9ac antibody (Figure 6B). These results suggest that the interplay that we observe between Ser-ADPr and acetylation of neighboring Lys residues on the peptide level also occurs in the context of whole nucleosomes and in vivo. We also observed that prior H3S10 phosphorylation of the nucleosome also significantly reduced subsequent p300-mediated acetylation of H3K9 (Figure 6B).

To compare the interplay observed in our in vitro system with that in cells, we analyzed U2OS cell extracts by high-resolution electron-transfer dissociation (ETD) mass spectrometry (Leidecker et al., 2016). We identified H3S10ADPr in the presence of mono-, di-, and trimethylation of H3K9 and with H3K14ac, but never with H3K9ac (Figures 6C and S6A–S6C). Any detection of H3K9ac was in the absence of H3S10ADPr, although we were able to detect H3K9ac in co-existence with marks other than H3S10ADPr, such as H3K14ac (Figure 6D). To test whether our failure to detect H3K9ac and H3S10ADPr together was due to technical limitations, we purified small amounts of H3K9acS10ADPr peptide generated from a highly inefficient reaction and analyzed it by mass spectrometry. We found that we were able to detect both histone marks on the same peptide (Figure S6D), further indicating that the apparent non-coexistence of these marks is due to the mutual exclusivity in vivo rather than our technical inability of detecting doubly modified H3K9ac/H3S10ADPr peptides. Our findings define two groups of histone H3 PTMs that can either coexist with or are mutually exclusive to Ser-ADPr (Figure 6E).

To further characterize the interplay between histone Ser-ADPr and other PTMs in vivo, we assessed the levels of H3S10ph, H3K9ac, H3K9me3, and several other PTMs around the H3S10ADPr site in 293T cells following DNA damage (Figure 7A). Our results confirmed previously published data showing reduction of H3K9ac in response to DNA damage (Tjeertes et al., 2009), because we also observed striking specific deacetylation of the H3K9 site after 120 min of treatment (Figure 7A). We also observed significant deacetylation of H3K14 under the same conditions (Figure 7A). DNA damage-induced deacetylation of both H3K9 and H3K14 was completely blocked by pre-treatment with a PARP inhibitor, olaparib. We did not observe DNA damage-induced deacetylation of H3K27ac or K36ac, or demethylation of H3K9me3 or H3K27me3, among other PTMs.
**A**

I - Standard polarity

II - Inverted polarity

- Power source
- Cathode
- Anode
- TBE gel

- Oligonucleotides
- Histone tail peptides
  - (negatively-charged)
  - (positively-charged)

**B**

H3 (1-21) peptide

| PARP1/HPF1 | 0 min | 15 min | 30 min | 60 min | 120 min | 180 min | HPF1, 180 min |
|------------|-------|--------|--------|--------|---------|---------|--------------|
|            |       |        |        |        |         |         |              |

- H3 (1-21) S10ADPr
- Unmodified H3 (1-21)

**C**

H3 (1-21) peptide

- PARP1/HPF1
- AATK
- H3 (1-21) S10ADPr
- Unmodified H3 (1-21)

**D**

H3 (1-21) peptide

- WT
- WT, S10ADPr
- K68
- R128
- R128, S10ADPr
- S10ADPr
- N6
- N6, S10ADPr
- T12
- T12, S10ADPr

**E**

Canonical histone H3 marks that can coexist with S10ADPr in vitro

- Canonical histone H3 marks that severely interfere with S10ADPr in vitro

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directly involved in the regulation of chromatin structure and proteins, detected by pan-acetylation antibody, displayed a different profile of increasing acetylation after DNA damage treatment (Figure 7B). These results combined suggest that interplay between histone ADPr and K9 acetylation and some other forms of histone modifications takes place in living cells. This knowledge can offer a framework for the further investigation of crosstalk between Ser-ADPr and other histone marks, and onward toward a wider understanding of the physiological function of Ser-ADPr as a histone mark and as a PTM.

DISCUSSION

Ser-ADPr is a recent addition to the array of PTMs found on mammalian proteins (Leidecker et al., 2016), and several studies have started to delineate its physiological relevance (Fontana et al., 2017; Bonfiglio et al., 2017b; Palazzo et al., 2018). Ser-ADPr is found on a large number of proteins (Leidecker et al., 2016; Abplanalp et al., 2017; Bonfiglio et al., 2017b), and Ser is the primary amino acid acceptor residue of ADPr following DNA damage in human cells (Palazzo et al., 2018). Histone proteins are subject to a large variety of PTMs, many of which are directly involved in the regulation of chromatin structure and transcription and play important roles in DNA replication and repair (Lawrence et al., 2016). This raises the question as to how Ser-ADPr functions in this densely modified environment and how the other histone marks affect the DDR as a consequence of their crosstalk with Ser-ADPr.

Our findings demonstrate the interplay between Ser-ADPr and a number of canonical histone marks, also showing that the process of Ser-ADPr is dependent on the context of the local histone code and vice versa. Whereas most of the histone Ser-ADPr sites examined in this work have previously been reported to be phosphorylated under certain conditions (Huang et al., 2015; Leidecker et al., 2016), ADPr of H3S10 and H3S28 are particularly intriguing, because H3S10ph and H3S28ph were suggested to play key roles in mitosis (Sawicka and Seiser, 2012; Lawrence et al., 2016). Moreover, acetylation of H3K9 and H3K27, which are mutually exclusive of ADPr at H3S10 and H3S28, respectively, is critical in the regulation of gene expression (Lawrence et al., 2016). This suggests that the prior modification of H3K9 and H3K27 could prevent the expansion of the Ser-ADPr signal or that, following DNA damage, neither H3K9ac/H3K27ac nor H3S10ph/H3S28ph marks would be “permitted” to initiate their responses. Because H3K9ac is frequently associated with transcriptionally active areas (Lawrence et al., 2016), it is logical that in the event of DNA damage, it would be undesirable to collect a large body of repair factors while the transcription machinery is still local and active. Accordingly, in response to DNA damage, H3K9Ac becomes diminished at promoter regions of cell-cycle-responsive and active gene sites and at the sites of DNA lesions (Tjeertes et al., 2009; Bártová et al., 2011; Meyer et al., 2016). Here, we also showed that H3K9ac decreases following H2O2 treatment in human cells. The levels of H3K14ac changed in a manner similar to H3K9ac, and K14 acetylation on H3 peptide also blocks H3S10ADPr in vitro. Given that H3K9ac and H3K14ac marks have already been shown to have similar patterns and roles in the recruitment of the switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex and spreading of γH2AX (Lee et al., 2010), it is not surprising that transient K14ac deacetylation could also be important for efficient DDR.

Notably, we confirmed previous findings that H3S10ph decreases after DNA damage treatment (Monaco et al., 2005), even more sharply than does H3K9ac, suggesting the importance of H3S10 dephosphorylation in DDR. Both DNA damage-induced H3K9 deacetylation and H3S10 dephosphorylation were prevented by treatment with the clinically approved PARP inhibitor olaparib, suggesting that both marks have a strict interplay with PARP1/2 activity. The inhibitory effect of olaparib on H3S10 dephosphorylation was already observed (Monaco et al., 2005). Because H3S10ph is correlated with transcriptional activation, mitosis, and meiosis (Lawrence et al., 2016), it is plausible that DDR-related histone marks could be a detrimental addition during some steps of the cell cycle or in some DNA repair pathways.

Alternatively, the methylation of H3K9 and H3K27, both of which are associated with transcriptional repression (Lawrence et al., 2016; Zhu and Wani, 2010), do not prevent the ADPr of the neighboring Ser sites. In concordance, we showed that cellular H3K9me3 levels remain stable after DNA damage, which was also previously reported (Tjeertes et al., 2009).

Although we did not see the effects of H3K36me2 in our systems, this PTM was previously identified as a DNA damage mark that facilitates the recruitment of DNA repair proteins and others (Figure 7A). As evident from the patterns for the cell-cycle proteins cyclin A, B1, E1, Cdc2 T15P, PRC1 T481P, and p21 (Figure 7A), the cell cycle was unaffected by olaparib treatment in our experimental settings.

To test our hypothesis that Ser-ADPr specifically affects these canonical histone marks, we performed similar experiments in ARH3 KO cells. We have previously demonstrated that these cells have chronically increased histone ADPr, including the Ser10 site (Fontana et al., 2017; Palazzo et al., 2018). DNA damage-induced deacetylation was more robust in these cells, which was especially obvious at 10 and 120 min post-DNA damage (Figure 7B). It is worth mentioning that some other acetylated ADPr was especially obvious at 10 and 120 min post-DNA damage (Figure 7B). These results combined suggest that interplay between histone ADPr and K9 acetylation and some other forms of histone modifications takes place in living cells. This knowledge can offer a framework for the further investigation of crosstalk between Ser-ADPr and other histone marks, and onward toward a wider understanding of the physiological function of Ser-ADPr as a histone mark and as a PTM.

Figure 5. A Technique to Rapidly Analyze ADP-Ribosylated Peptides

(A) Schematic representation of the approach to rapidly and easily analyze the modification status of positively charged histone tail peptides.

(B) Imperial stained gel showing ADPr of H3 (1–21aa) peptides after addition of PARP1/HPF1 during a 6-hr time course. The upward band shift denotes ADPr of the H3 peptide.

(C) Imperial stained gel showing ADPr of H3 (1–21aa) peptides after addition of PARP1/HPF1 and subsequent addition of ARH3. The upward band shift denotes ADPr of the H3 peptide.

(D) Imperial stained gel showing H3 (1–21aa) WT, K4me1, K4me2, K4me3, R8me1, R8me2a, K9ac, K9me1, K9me2, K9me3, S10ph, T11ph, and K14ac peptides and subsequent ADPr following addition of PARP1 and HPF1. The upward band shift denotes ADPr of the H3 peptide.

(E) A schematic showing a map of histone H3 1–20aa with histone marks that interfere with Ser-ADPr on H3 peptide in vitro.
Figure 6. H3K9ac and S10ADPr Are Mutually Exclusive Histone Marks in Human Cells

(A) Autoradiogram showing PARP1 mediated ADPr in the presence of absence of HPF1, with either WT or K9ac human recombinant nucleosomes. Coomassie staining of the SDS-PAGE is included.

(B) Western blot showing PARP1/HPF1 ADPr of recombinant human nucleosome and subsequent p300-mediated acetylation. One reaction includes Baronase incubation instead of ADPr reaction, before p300 acetylation reaction. Membrane probed with H3K9ac antibody, with commercially obtained recombinant human H3K9ac nucleosome included as a positive marker.

(C) High-resolution ETD fragmentation spectrum of a H3 peptide modified by methyl on lysine 9 and ADP-ribose on serine 10 obtained from Leidecker et al. (2016). The chemical structure of methyl and ADP-ribose are depicted.

(D) High-resolution ETD fragmentation spectrum of a H3 peptide modified by acetylation on lysine 9 and lysine 14 obtained from Leidecker et al. (2016). The chemical structure of acetylation is depicted. *1, Peak corresponding to an unfragmented co-eluting, co-isolated +2 precursor deconvoluted into the +1 state. *2, Peak corresponding to an unfragmented co-eluting, co-isolated +3 precursor deconvoluted into the +1 state.

(E) Schematic summary of canonical histone H3 marks and their interactions with Ser-ADPr based on the mass spectrometry (MS) analysis of U2OS cell extracts from Leidecker et al. (2016). The marks depicted on the top are H3 marks that can coexist with Ser10 or Ser28 ADPr in vivo, while the H3 marks depicted on the bottom are mutually exclusive with ADPr on Ser10 or Ser28.
is crucial for efficient repair (Fnu et al., 2011). Changes in many other histone modifications have also been described during the DDR. Of note, PARP1 can change PTM landscapes, not only by means of ADPr but also indirectly by modulating the activity of chromatin remodelers and histone-modifying enzymes (Gupte et al., 2017). According to our results, not all of the histone marks are involved in the interplay with Ser-ADPr following a certain DNA damage stimulus. However, these histone marks may be involved in response to other agents and/or in control of different DNA repair pathways. Nevertheless, it is tempting to speculate that it is the specific combination of many histone PTMs that defines the exact molecular pathway that the cell will follow to attempt DNA repair.

The inhibitory effect of histone acetylation on Ser-ADPr could explain why combining PARP inhibitors (PARPis) with histone deacetylase inhibitors (HDACis) results in increased DNA damage and cellular sensitivity (Min et al., 2015; Chao and Goodman, 2014; Rasmussen et al., 2016; Konstantinopoulos et al., 2014; Zhang et al., 2012; Kim et al., 2017). While both PARPis and HDACis are already individually in clinical trials, our data showing that DNA damage-induced K9 deacetylation is blocked by PARPis further strengthen the pre-clinical rationale for their simultaneous administration. At the same time, future investigation of the effects of including HDACis into the known combination therapies involving PARPis (Dréan et al., 2016) could highlight other promising therapeutic strategies.

The field of Ser-ADPr has developed rapidly due to advances in proteomics methods to overcome longstanding technical challenges associated with unbiassed ADPr mapping (Leidecker et al., 2016; Bonfiglio et al., 2017a, 2017b). Beyond Ser-ADPr site identification, future efforts will be directed toward understanding the functional consequences of Ser-ADPr and the detailed molecular mechanisms. ADPr differs among the well-studied PTMs for its lack of experimental tools and techniques needed for progress in the field. This is beginning to change (Crawford et al., 2018), and to facilitate the investigation of Ser-ADPr, we have also developed approaches that can be readily adopted by the majority of
biological laboratories (Fontana et al., 2017; Palazzo et al., 2018). Here, we have also introduced a method to dramatically simplify and improve the detection of Ser-ADPr peptides. This strategy overcomes the limitations of traditional radioactive techniques and allows an estimation of the extent of modification, which is impossible with other available techniques. Thus, our approach will clearly facilitate investigations of the dynamics of canonical histone marks, such as phosphorylation and acetylation.

We have demonstrated that Ser-ADPr is a histone mark that is mutually exclusive with neighboring acetylation and phosphorylation both in vitro and in vivo. Further characterizing the interplay between Ser-ADPr and the PTMs of histone residues (Liszczak et al., 2018) and of other proteins is of great interest and will provide valuable insights into the complex crosstalk regulating the architecture and accessibility of chromatin. Given the conservation of the KS motifs in Ser-ADPr for hundreds of proteins, acetylation and phosphorylation of the KS motifs could likely be a general strategy to regulate ADPr of proteins involved in genome stability beyond histones.

This study and the tools we have developed have also led to the discovery of Tyr-ADPr (also noted recently by Leslie Pedrioli et al., 2018). As with the recent discovery of Ser-ADPr, this initial finding poses a tantalizing number of questions. While we show that PARP1/HPF1 is able to catalyze Tyr-ADPr, are there other writers of this modification? What are enzymes that remove Tyr-ADPr? How common is this modification and what is the physiological relevance? The HPF1 Tyr-ADPr site (Tyr238) constitutes the main PARP1-binding residue (Gibbs-Seymour et al., 2016), and substitution of Tyr238 (along with Arg239) to Ala prevents PARP1/HPF1 interaction. These data suggest that Tyr238 modification could be a secondary level of regulation for the stability and activity of the PARP1/HPF1 complex. In this context, our discovery of a Tyr-ADPr diagnostic peak will enhance future proteomics identifications of Tyr-ADPr sites and understanding of their physiological relevance. In summary, our study provides insights into the interplay of Ser-ADPr and other histone marks and provides evidence for an intriguing type of ADPr.

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**AUTHOR CONTRIBUTIONS**

I.M., J.J.B., E.B., and I.A. conceived the study. E.B. designed and performed the biochemical studies. J.J.B. and T.C. acquired and analyzed the mass spectrometric data. J.J.B., T.C., and I.M. designed and J.J.B. performed the inverted polarity native gel analysis. E.P. and F.Z. performed the cell biology experiments. E.B. wrote the manuscript, with contributions from all of the authors.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**SUPPLEMENTAL INFORMATION**

Supplemental information includes six figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.08.092;
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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, Peptides, and Recombinant Proteins | EpiCypher | Cat# 16-0006 |
| Recombinant human mononucleosomes | EpiCypher | Cat# 16-0006 |
| Recombinant human mononucleosomes H3 K9ac | EpiCypher | Cat# 16-0314 |
| Activated DNA | Trevigen | Cat# 4671-096-06 |
| NAD+ | Trevigen | Cat# 4684-096-02 |
| 32P-NAD+ | Hartmann Analytic | Cat# ARP 0141 |
| Histone H3 (1 - 21), Biotinylated | AnaSpec | Cat# AS-61702 |
| Histone H3K4me (1 - 21), Biotinylated | AnaSpec | Cat# AS-64355 |
| Histone H3K4me2 (1 - 21), Biotinylated | AnaSpec | Cat# AS-64356 |
| Histone H3K4me3 (1 - 21), Biotinylated | AnaSpec | Cat# AS-64357 |
| Histone H3R8me (1 - 21), Biotinylated | AnaSpec | Cat# AS-64607 |
| Histone H3R8me2 (1 - 21), Biotinylated | AnaSpec | Cat# AS-64972 |
| Histone H3R8me3 (1 - 21), Biotinylated | AnaSpec | Cat# AS-64972 |
| Histone H3R8me (1 - 21), Biotinylated | AnaSpec | Cat# AS-64607 |
| Histone H3K9ac (1 - 21), Biotinylated | AnaSpec | Cat# AS-64359 |
| Histone H3K9me (1 - 21), Biotinylated | AnaSpec | Cat# AS-64358 |
| Histone H3K9me2 (1 - 21), Biotinylated | AnaSpec | Cat# AS-64359 |
| Histone H3K9me3 (1 - 21), Biotinylated | AnaSpec | Cat# AS-64360 |
| Histone H3S10ph (1 - 21), Biotinylated | AnaSpec | Cat# AS-64611 |
| Histone H3T11ph (1 - 21), Biotinylated | AnaSpec | Cat# AS-64973 |
| Histone H3K14ac (1 - 21), Biotinylated | AnaSpec | Cat# AS-64362 |
| Histone H3 peptide 1-20aa, biotinylated | EpiCypher | Cat# 12-0001 |
| Recombinant human PARP1 protein | Langelier et al., 2011 | N/A |
| Recombinant human PARP2 protein | Langelier et al., 2014 | N/A |
| Histone H3 peptide K4ac 1-20aa, biotinylated | EpiCypher | Cat# 12-0002 |
| Histone H3 peptide K14ac 1-20aa, biotinylated | EpiCypher | Cat# 12-0004 |
| Histone H3 peptide K18ac 1-20aa, biotinylated | EpiCypher | Cat# 12-0005 |
| Histone H3 peptide K4me3 1-20aa, biotinylated | EpiCypher | Cat# 12-0009 |
| Histone H3 peptide K18me3 1-20aa, biotinylated | EpiCypher | Cat# 12-0015 |
| Histone H3 peptide K9ac 1-20aa, biotinylated | EpiCypher | Cat# 12-0003 |
| Histone H3 peptide K9me1 1-20aa, biotinylated | EpiCypher | Cat# 12-0010 |
| Histone H3 peptide K9me2 1-20aa, biotinylated | EpiCypher | Cat# 12-0011 |
| Histone H3 peptide K9me3 1-20aa, biotinylated | EpiCypher | Cat# 12-0012 |
| Histone H3 peptide S10ph 1-20aa, biotinylated | EpiCypher | Cat# 12-0041 |
| Histone H3 peptide 15-34aa, biotinylated | EpiCypher | Cat# 12-0016 |
| Histone H3 peptide 27-45aa, biotinylated | EpiCypher | Cat# 12-0020 |
| Histone H4 peptide 1-23aa, biotinylated | EpiCypher | Cat# 12-0029 |
| Histone H4 peptide K5ac 1-23aa, biotinylated | EpiCypher | Cat# 12-0030 |
| Histone H4 peptide K8ac 1-23aa, biotinylated | EpiCypher | Cat# 12-0031 |
| Histone H4 peptide R3me 1-23aa, biotinylated | EpiCypher | Cat# 12-0059 |
| Histone H2A peptide 1-17aa, biotinylated | EpiCypher | Cat# 12-0112 |
| Histone H3 peptide K36ac 27-45aa, biotinylated | EpiCypher | Cat# 12-0129 |
| Histone H3 peptide K36me1 27-45aa, biotinylated | EpiCypher | Cat# 12-0174 |
| Histone H3 peptide K36me2 27-45aa, biotinylated | EpiCypher | Cat# 12-0115 |
| Histone H3 peptide K36me3 27-45aa, biotinylated | EpiCypher | Cat# 12-0212 |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Recombinant human HPF1 protein | Gibbs-Seymour et al., 2016 | N/A |
| Recombinant human HPF1 Y238F protein | This paper | N/A |
| Recombinant human HPF1 Y238A protein | This paper | N/A |
| Recombinant human HPF1 Y238E protein | This paper | N/A |
| Recombinant human ARH3 protein | Fontana et al., 2017 | N/A |
| Imperial Protein Stain | Thermofisher Scientific | Cat# 24615 |
| Olaribib | Cayman Chemical | Cat# 10621 |
| Histone H3 (21-44), Biotinylated | AnaSpec | Cat# AS-64641 |
| Histone H3K27ac (21-44), Biotinylated | AnaSpec | Cat# AS-64637 |
| Histone H3 (21-44), Biotinylated | AnaSpec | Cat# AS-64440 |
| Histone H3K27me1 (21-44), Biotinylated | AnaSpec | Cat# AS-64365 |
| Histone H3K27me2 (21-44), Biotinylated | AnaSpec | Cat# AS-64366 |
| Histone H3K27me3 (21-44), Biotinylated | AnaSpec | Cat# AS-64367 |
| Histone H3/H4 tetramer | Mehrotra et al., 2011 | N/A |
| p300, human, recombinant, catalytic domain | Enzo Life Sciences | Cat# BML-SE451-0100 |
| Dim-5, recombinant, Neurospora crassa | Zhang et al., 2002 | N/A |
| Baronase | Gift from Francis Barr (University of Oxford) | N/A |
| PARP3, human, recombinant | Bonfiglio et al., 2017b | N/A |
| PARP10 catalytic domain, human, recombinant | Palazzo et al., 2016 | N/A |
| Recombinant human PARP1 E988Q protein | Langelier et al., 2011 | N/A |
| HDAC2, human recombinant | Active Motif | Cat# 31505 |
| SIRT2, human, recombinant | Rack et al., 2014 | N/A |

**Antibodies**

| Antibody | Source | Cat# or RRID |
|----------|--------|--------------|
| anti-pan-ADP-ribose (rabbit monoclonal) | Millipore | Cat# MABE1016; RRID:AB_2665466 |
| anti-PAR (rabbit polyclonal) | Trevigen | Cat# 4336-BPC-100; RRID:AB_2721257 |
| anti-Flag HRP-conjugated (mouse monoclonal) | Sigma-Aldrich | Cat# A8592; RRID:AB_439702 |
| anti-HPF1 (rabbit polyclonal) | Gibbs-Seymour et al., 2016 | N/A |
| anti-Flag M2 agarose-conjugated (mouse monoclonal) | Sigma-Aldrich | Cat#: A2220; RRID:AB_1006303 |
| anti-ARH3/ADPRH (rabbit polyclonal) | Atlas Antibodies | Cat#: HPAD27104; RRID:AB_1060133 |
| anti-histone H3, CT, pan (rabbit polyclonal) | Millipore | Cat#: 07-690; RRID:AB_417398 |
| anti-H3K9ac (rabbit monoclonal) | Cell Signaling | Cat#: 9649; RRID:AB_823528 |
| anti-H3K14ac (rabbit monoclonal) | Cell Signaling | Cat#: 7627S; RRID:AB_1083941 |
| anti-H3K27ac (rabbit monoclonal) | Cell Signaling | Cat#: 8173P; RRID:AB_1094988 |
| anti-H3K36ac (rabbit monoclonal) | Cell Signaling | Cat#: 27683 |
| anti-H3K9me3 (rabbit polyclonal) | Abcam | Cat#: ab8898; RRID:AB_306848 |
| anti-H3K27me3 (rabbit polyclonal) | Gift from Rob Klose (University of Oxford) | N/A |
| anti-pan-Kac (rabbit polyclonal) | Cell Signaling | Cat#: 9441; RRID:AB_331805 |
| anti-H3S10P (rabbit polyclonal) | Abcam | Cat#: ab5176; RRID:AB_304763 |
| anti-cyclin A (rabbit polyclonal) | Santa Cruz Biotechnology | Cat#: sc-751; RRID:AB_631329 |
| anti-cyclin B1 (rabbit polyclonal) | Millipore | Cat#: 05-373; RRID:AB_309701 |
| anti-cyclin E1 (mouse monoclonal) | Cell Signaling | Cat#: 4129; RRID:AB_2071200 |
| anti-PRC1 T481P (rabbit monoclonal) | Abcam | Cat#: ab62366 ; RRID:AB_944969 |
| anti-Cdc2 Y15P (rabbit monoclonal) | Cell Signaling | Cat#: 4539S; RRID:AB_560953 |
| anti-p21 (rabbit polyclonal) | Santa Cruz Biotechnology | Cat#: sc-397; RRID:AB_632126 |
| anti-pan-Kac (rabbit polyclonal) | Abcam | Cat#: ab21623; RRID:AB_446436 |
| anti-PARP1 (rabbit monoclonal) | Abcam | Cat#: ab32138; RRID:AB_777101 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ivan Ahel (ivan.ahel@path.ox.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Most of the experiments in these studies utilized recombinant protein and enzymes, as well as chemically synthesized peptides. For the cell biology experiments we used standard human model cell lines U2OS (ATCC HTB-96; osteosarcoma) and HEK293T (ATCC CRL-3216; embryonic kidney). The cells were grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS (GIBCO) and penicillin-streptomycin (100 U/ml, GIBCO) at 37°C with 5% CO₂. The generation of ARH3 KO U2OS cells was previously described (Fontana et al., 2017). Absence of mycoplasma contamination confirmed by MycoAlert Mycoplasma Detection Kit.

METHODS DETAILS

In vitro ADPr assays

A variety of in vitro ADPr assays were used to measure the ability of enzymes to modify or demodify different substrates.
Recombinant proteins and peptides
Recombinant proteins are purified as described previously (Langelier et al., 2011; Langelier et al., 2014; Gibbs-Seymour et al., 2016; Fontana et al., 2017; Dunstan et al., 2012). Peptides were purchased from EpiCypher or custom made. Nucleosomes were from EpiCypher.

Enzymatic preparation of the modified histone peptides
Recombinant Dim-5 (the homolog of human SUV39H1/2) and SIRT2 were purified as previously described (Rack et al., 2014; Zhang et al., 2002). Recombinant p300 was purchased from Enzo Life Sciences. HDAC2 was purchased from Active Motif. For histone phosphorylation reactions we used the activated Aurora B fragment called Baronase, which was a gift from the Barr lab (Nunes Bastos et al., 2013). H3 peptides were purchased from EpiCypher. H3 peptides (either WT or Ser-ADPr modified as described above) were incubated in either; HAT buffer (p300) - 50 mM Tris-HCl pH 8.0, 1mM DTT, 100 μM Acetyl-CoA, 10% glycerol for 30 min at 30°C; phosphorylation buffer (Baronase) - 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT for 60 min at 37°C; methytransferase buffer (Dim-5) – 50mM Glycine pH 9.8, 2 mM DTT, 10% glycerol for 20 min at room temperature. Reactions were then analyzed by SDS-PAGE and western blotting (detailed below). HDACi reactions (HDAC2, SIRT2) were performed in reaction buffer contained 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM MgCl₂, which were subsequently supplemented by PARP1/HF1, activated DNA and 50 μM NAD⁺ spiked with ³²PNDAT. The modification reaction proceeded at room temperature for 20 min before addition of the PARPi Olaparib at 1 μM. Reactions were then analyzed by SDS-PAGE and autoradiography.

Standard radioactivity-based ADPr assay
Recombinant proteins or peptides were ADPr by PARP1 in the presence or absence of HPF1 and histone peptides. PARP1 concentration in the assays was 1 μM unless stated otherwise, HPF1 was always equimolar to PARP1, histone peptides were used at 0.5 μg per reaction, and recombinant nucleosomes were at 1 μM. The PARP reaction buffer contained 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM MgCl₂, activated DNA and 50 μM NAD⁺ spiked with ³²PNDAT. The modification reaction proceeded at room temperature for 20 min before addition of the PARPi Olaparib at 1 μM. Reactions were then analyzed by SDS-PAGE and autoradiography.

Inverted polarity native gels for ADPr detection
This simple, non-radioactive method allows visualization of both substrates and products of ADPr reactions. ADPr reactions were performed as described above, except with 2 μg histone peptide per reaction, and in the presence of non-radioactive NAD⁺. Samples were mixed with TBE Sample Buffer and loaded on 20% TBE gels in TBE Running Buffer. Samples were run with inverted polarity at 200 V for 1 hr. Gels were then fixed for 30 min in 10% glutaraldehyde, washed in H₂O for 3 × 10 min, then stained with Imperial Protein Stain for 1 hr.

In vitro ADP-ribosyl glycohydrolase assays
The assays were as in Fontana et al., 2017. Briefly, H3 peptides were incubated with PARP1 and HPF1, under the conditions described above, and stopped by addition of Olaparib. ARH3 was then added to the reactions for incubation at room temperature for 30 min. Reactions were then analyzed by SDS-PAGE and autoradiography. ARH3 concentration was at 1 μM.

Reanalysis of published high-quality proteomics datasets
For the reanalysis of published high-quality proteomics datasets, public raw files were analyzed with MaxQuant proteomics suite of algorithms (version 1.5.3.17) (Cox and Mann, 2008), using the integrated search engine Andromeda (Cox et al., 2011).

Data from the published proteomics study of peptides enriched with an ADPr-binding macrodomain (Martello et al., 2016) were searched against the human proteome database (downloaded 09.10.2015 from UniProt) with the following parameters: the maximum allowed mass deviation was set to 4.5 ppm for precursor ions and 20 ppm for fragment ions; the minimum peptide length was set to 6 amino acids and the maximum number of missed cleavages was set to 5 with the maximum charge state 7. Variable modifications included acetylation (Protein N-term), Oxidation (M) and ADPr (DEKRSTCYNQHM). The variable modification ADPr allowed for neutral losses of adenine (m/z 136.0618); adenosine with loss of water (m/z 250.0935); AMP (m/z 348.0704); ADP (m/z 428.0367) and ADP-ribose (m/z 542.0684). FTMS top peaks per 100 Da were set to 20. We employed the annotated mass spectrometry (MS)/MS spectra generated by MaxQuant as the basis for our manual validation of spectra. To consider a peptide as modified on Tyr, we required the presence of fragment ions with either the intact ADP-ribose or phosphoribose (resulting from the loss of AMP) pointing to ADPr on Tyr. Unmodified “native” sequence ions were not considered as evidence for localization since it is impossible to distinguish between an original lack of modification and complete loss of ADPr during fragmentation. Two additional pieces of evidence supporting Tyr modification could also be observed in lower mass regions of these spectra. First, a peak matching the immonium ion of modified Tyr (+ ADPr – AMPloss) could be observed (albeit weakly) in these spectra at 330.0742 Da (+1). The native (unmodified) Tyr immonium ion (136.0762) was also generally very weak (~5%) in comparison to the immediately neighboring Adenine peak (136.0623) in these spectra, as opposed to those of peptides containing Tyr but with ADPr on serine. The significance of this ratio as support of Tyr modification can only be fully assessed with larger numbers of ETD-verified peptide spectra.

For the cellular ADPr characterization with HCD and EThCd study (Bilan et al., 2017), variable modifications included oxidation (M), acetylation (Protein N-term and K) and ADPr (DEKRSTCYNQHM). For confident identification of ADPr sites, we considered only ETD MS/MS spectra and required a minimum Andromeda score of 100, mass deviation smaller than 3 ppm after MaxQuant recalibration and a localization score above 0.9. In addition, we manually validated all the representative spectra by requiring extensive coverage of the peptide backbone fragment ions. For localization we required the clear presence of multiple high-intensity fragment ions pinpointing the modification site.
For the cellular MS analysis of endogenous histones study (Leidecker et al., 2016), variable modifications included oxidation (M), acetylation (Protein N-term and K), methylation (KR), dimethylation (K), trimethylation (K) and ADPr (DEKRSTYCNQHM). Similarly, we considered only ETD MS/MS spectra and required a minimum Andromeda score of 100, mass deviation smaller than 3 ppm after MaxQuant recalibration and a localization score above 0.9.

**Western blotting**

Human U2OS (ATCC HTB-96) and HEK293T (ATCC CRL-3216) cells were plated and grown overnight in DMEM (Sigma-Aldrich) supplemented with 10% FBS (GIBCO) and penicillin-streptomycin (100 U/ml, GIBCO). To induce DNA damage, cells were incubated with 2 mM H2O2 (Sigma-Aldrich) in DPBS with calcium and magnesium (GIBCO) for the indicated times. For PARPi, cells were pretreated with 10 μM Olaparib for 1 hr, and Olaparib was also added to the DPBS solution in case of the induction of DNA damage. Cells were lysed with Triton X-100 lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% Triton X-100) at 4°C. Right before use, the buffer was supplemented with 5 mM MgCl2, protease and phosphatase inhibitors (Roche), 1 μM ADP-HPD and 1 μM Olaparib. Benzonase (Sigma) was added to the cell lysates and incubated for 20 min at 4°C. After centrifugation at 14,000 rpm for 15 min, supernatants were collected. Protein concentrations were analyzed by Bradford Protein Assay (Bio-Rad). Proteins were boiled in NuPAGE LDS sample buffer (Invitrogen), resolved on NuPAGE Novex 4%-12% Bis-Tris gels (Invitrogen), and transferred onto nitrocellulose membranes (Bio-Rad) using Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in PBS buffer with 0.05% Tween 20 and 5% non-fat dried milk for 1 hr at room temperature, and incubated overnight with primary antibodies at 4°C, followed by 1-hour incubation with peroxidase-conjugated secondary antibodies at room temperature. Blots were developed using ECL (Invitrogen) and analyzed by exposing to films.

**Immunoprecipitation experiments**

Flag-immunoprecipitation followed by western blotting was used to analyze the modification status of the precipitated proteins and their mutant versions. Full-length human histone H3.1 and HPF1 cDNAs were cloned into the pDONR221 vector (Thermo Fisher Scientific). Point mutations were produced in pDONR-H3.1 and pDONR-HPF1 using QuikChange Lightning site-directed mutagenesis kit (Agilent). Mammalian expression constructs expressed H3.1 proteins with the C-terminal 3xFlag tag, and HPF1 proteins with N-terminal Flag tag. Wild-type proteins and their mutant versions were expressed in 293T cells. The cells were plated, cultured overnight, and transfected using Polyfect (QIAGEN) with an empty vector or a plasmid expressing the Flag-tagged protein of interest for 24 hr essentially as described (Palazzo et al., 2018). The cell lysates were obtained the same as for the western blotting. Protein concentrations were analyzed by Bradford Protein Assay (Bio-Rad), normalized, and then, the cell lysates were incubated with anti-Flag M2 agarose-conjugated mouse monoclonal antibody (Sigma-Aldrich) for 1 hr while rotating at 4°C. Beads were washed several times with Triton X-100 lysis buffer and eluted with NuPAGE LDS sample buffer (Invitrogen). The samples were then analyzed by Western Blotting as described above.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The qualitative gel-based assays were used to visualize the experimental results. Representative gels from at least three independent biological replicates were shown.