DNA damage-induced phosphorylation of histone H2A at serine 15 is linked to DNA end resection

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ABSTRACT

The repair of DNA double-strand breaks (DSBs) occurs in chromatin and several histone post-translational modifications have been implicated in the process. Modifications of histone H2A N-terminal tail has also been linked to DNA damage response, through acetylation or ubiquitination of lysine residues that regulate repair pathway choice. Here, we characterize a new DNA damage-induced phosphorylation event on chromatin, at serine 15 of H2A in yeast. We show that this SQ motif functions independently of the classical S129 C-terminal site (γ H2A) and mutant mimicking constitutive phosphorylation increases cell sensitivity to DNA damage. H2AS129ph is induced by both Tel1^{ATM} and Mec1^{ATR}, and loss of Lcd1^{ATRIP} or Mec1 signaling decreases γ H2A spreading distal to DSB. In contrast, H2AS15ph is completely dependent on Lcd1^{ATRIP}, indicating that this modification only happens when end resection is engaged. This link is supported by an increase of RPA binding in the H2AS15E phosphomimic mutant, reflecting higher resection. This serine on H2A is replaced by a lysine in higher eukaryotes (H2AK15), which undergoes an acetylmonoubiquityl switch to regulate binding of 53BP1 and therefore resection. This regulation seems functionally conserved with budding yeast H2AS15 and the 53BP1-homolog Rad9, utilizing different post-translational modifications between organisms but achieving the same function.

INTRODUCTION

The genetic information of the cell is challenged by various endogenous and exogenous cues which leads to DNA damage. If unchecked this can lead to mutations, translocations, loss of genetic information and in higher eukaryotes be the underlying cause for diseases such as cancer (1, 2). One of the most harmful form of DNA damage is DNA double strand break (DSB). To endure and mitigate such damage to the DNA, cells orchestrate cell cycle arrest and DNA repair pathways, collectively termed DNA damage response (DDR). Two main pathways to repair DSBs are non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ involves direct ligation of the two broken DNA ends which may require processing, thus making it a more error prone pathway (3). In HR, single stranded DNA (ssDNA) is generated through a process called resection and involves use of homologous sequence or sister chromatid as a template to copy the genetic information, thus ensuring a lower error rate (4).

Phosphatidylinositol-3-OH kinase related kinases (PIKKs) family has been implicated to play an important role in DDR. This includes *S. cerevisiae* Tel1 and Mec1 and the mammalian ataxia telangiectasia-mutated (ATM), AT-related (ATR) and DNA-dependent protein kinase (DNA-PK) (5, 6). One of the first DNA damage-dependent chromatin modification to be identified was γ -H2A(X), which is the phosphorylation of histone H2A at S129 in yeast and H2A.X at S139 in mammals (7-9). This phosphorylation is carried out by both Tel1/ATM and Mec1/ATR. In yeast H2AS129ph spreads more than 50Kb on both sides flanking the DSB, whereas in mammals it spreads up to a megabase (10, 11). This histone mark has been implicated in DNA damage response and required for proper recruitment of chromatin remodelers/modifiers such as Ino80, Swr1 and NuA4 complexes (12-15). Mice model for H2AX^{-/-} is radiation sensitive, growth retarded,

immune deficient and show recruitment defect for variety of repair factors such as Nbs1, 53BP1 and BRCA1 (16).

Interestingly, budding yeast has another Tel1/Mec1-dependent SQ phosphorylation site at H2B C-terminus, H2BT129ph, which largely correlates with H2AS129ph but is modulated by it (10). Other DNA damage-induced phosphorylation marks have been mapped on chromatin but are not deposited by PIKKs. These include H4S1ph, H2AS122ph and H2AT126ph in yeast which have been linked to processes like chromatin restoration after repair, meiosis, HR and repair of fragile sites with CAG/CTG repeats (17-21). Apart from phosphorylation, other histone modifications like acetylation, methylation, ubiquitylation and SUMOylation have been implicated in DDR (22). In mammals H2AK15 is ubiquitinated by RNF168 and this mark is responsible for recruitment and repair foci formation of 53BP1 in combination with H4K20me₂, inhibiting resection and favoring NHEJ (23). At DSBs, the TIP60 complex can acetylate H2AK15 and binds H4K20me, thus antagonizing 53BP1 and favoring repair by HR (24). In budding yeast, Rad9 is the functional homolog of 53BP1, contains a Tudor domain which binds H3K79me (equivalent to mammalian H4K20) and a BRCT domain which recognizes γ -H2A (25-27). Both of these domains are required for the recruitment and retention of Rad9 at DSBs (27, 28). In contrast to 53BP1, Rad9 does not have a ubiquitin-binding domain neither the yeast H2A has lysine corresponding to human H2AK15 (Fig. 1A). Instead, yeast H2A has a SQ site at this position which has been found phosphorylated in a Mec1-dependent manner upon DNA damage, in a large phosphoproteomic study (29).

This led us to investigate whether H2AS15 had a role in the repair of DNA breaks. Our results indicate that H2AS15ph is induced by Mec1^{ATR} over a large domain of chromatin around

the DSB. We also present data arguing that H2AS15ph regulates Rad9 binding and DNA end resection. Altogether these results suggest an evolutionary conserved mechanism, similar to the mammalian H2AK15 ubiquitin/acetylation switch, in modulating yeast Rad9^{53BP1} binding in response to DSB repair.

RESULTS

H2AS15ph function is distinct from H2AS129ph in response to DNA damage

In order to investigate if H2A-S15 has a role in DNA damage response, we mutated H2A-S15 to alanine (non-phosphorylation mimicking) and glutamic acid (phosphorylation mimicking). In presence of DNA damaging agent methyl methanesulfonate (MMS), *H2A-S15E* mutant cells show a slight growth defect which is not seen for *H2A-S15A* (Fig. 1B). However, in combination with the *H2A-S129A* mutant, both *H2A-S15A/E* show stronger growth defect compared to single *H2A-S129A* mutant cells (Fig. 1B). Next, we tested if the *H2A-S15* mutants had any genetic interaction with the other PIKK-dependent histone phosphorylation induced in response to DSBs in yeast, H2BT129ph (10). *H2A-S15A/E H2B-T129A* double mutant cells do not show any additional growth defect compared to *H2A-S15* single mutants in presence of MMS (Fig. 1C). These results indicate that H2AS129 and H2AS15 phosphorylations play distinct roles in response to DNA damage induced by MMS.

To decipher if *H2A-S15* mutants affects H2AS129 phosphorylation *in vivo*, we performed ChIP-qPCR at an inducible DSB. We used the yeast background in which a single persistent DNA double strand break is created at the *MAT* locus by homothallic endonuclease (HO) inducible by galactose. The induced H2AS129ph signal detected on each side of the DSB in wild type (WT)

background corresponds to what was previously reported (10, 12). Both *H2A-S15A/E* mutants show similar pattern of H2AS129ph compared to WT cells, not affecting the induction or spreading of H2A-S129ph beside a very small increase in the *H2A-S15E* mutant (Fig. 1D-E). These results again support a role of H2AS15ph in DNA damage response/repair that is distinct of H2AS129ph.

The lysine residues of H2A and H4 N-terminal tails are known targets of the NuA4 acetyltransferase complex (30). Studies have described histone modifications modulating the occurrence of other modifications in chromatin, thus forming a network of histone crosstalk (22). H4S1ph carried out by CK2 has been shown to inhibit acetylation of various lysine residues on the N-terminal tail of H4 and play an active role in DSB repair as well as transcription (17, 18). This raised the question whether H2AS15ph could influence acetylation of lysine residues on the H2A and H4 N-terminal tails. Western blot analysis of whole cell extracts from yeast cells harboring the *H2A-S15* point mutants reveals a small decrease in both H2A and H4 acetylation more noticeable for the *H2A-S15A* mutant compared to wild type (Fig. 1F). We further investigated *in vivo* if the *H2A-S15* mutants affected the acetylation of histones around DSB. Both *H2A-S15A/E* mutants show a decrease in H4 acetylation signal but only close to the DNA break site (Fig. 1G). These results suggest that H2AS15ph has a small but significant effect on other histone modifications in regions surrounding the DSB.

H2AS15ph is induced over a large chromatin domain upon DNA double strand break formation

To characterize the dynamics of H2AS15ph in response to DNA damage, we produced and purified an antibody against the *S. cerevisiae* H2A N-terminal portion with Ser15 bearing the

phosphorylation mark. To demonstrate that H2AS15ph is indeed regulated by DNA damage, we performed immunoblotting with purified yeast native chromatin. As shown in (Fig. 2A), H2AS15ph signal increases with MMS treatment, similarly to H2AS129ph. A control using phosphatase treatment of the chromatin confirms that the signal detected by the antibody is indeed phosphorylation-specific (Fig. 2B).

To determine whether H2AS15ph occurs at the DSB, we performed ChIP-qPCR after induction at the *MAT* locus. The signal detected for H2AS15ph shows a clear increase around the DSB site in WT background spanning over 10 kilobases on each side of the break (Fig. 2C). As a control to check the specificity of the signal detected, the *H2A-S15A* mutant cells do not show such signal over a large domain after the induction of DSB (Fig. 2C). However, close to the break site, *H2A-S15A* mutant cells do show a slight increase which likely reflects some non-specific cross-reactivity of the antibody with another mark. To characterize the dynamics of H2AS15ph appearance around the DSB, the ChIP-qPCRs were performed at different time-points after induction of the break. Interestingly, appearance of H2AS15ph seems delayed in comparison to H2AS129ph, reaching maximum levels throughout the region after 3hrs (Fig. 2D-E). Altogether, these data clearly demonstrate that H2AS15ph is actively deposited over a large region of chromatin upon formation of DNA double strand breaks.

Mec1^{ATR} and not Tel1^{ATM} phosphorylates H2AS15 at DSBs

Tel1^{ATM} and Mec1^{ATR} signaling kinases phosphorylate histone H2AS129 to form γ -H2A (7, 11, 31). They also target various proteins to modulate DSB repair and checkpoint activation. Recruitment of Tel1 to DSB requires Xrs2 (mammalian NBS1), subunit of the Mre11-Rad50-Xrs2 (MRX)

complex (32). Deletion of *XRS2* affects Tel1 dependent checkpoint signaling and telomere maintenance functions (33). Yeast Lcd1/Ddc2 (mammalian ATRIP) is responsible for the recruitment of Mec1 to RPA-coated single stranded DNA (ssDNA) produced during end resection (34). Mec1 has been shown to be involved in spreading of γ -H2A in *trans* onto adjacent undamaged chromosomes in close physical proximity (10, 35). Deletion of *LCD1* is known to incur loss of Mec1 recruitment to RPA-coated ssDNA and therefore Mec1-dependent cell signaling in response to damage (36, 37).

lcd1Δ and *lcd1Δxrs2Δ* mutant cells lead respectively to loss of Mec1 and both Mec1 and Tel1 at the DSB. In *lcd1Δ*, where Tel1 recruitment and signaling is unaffected, ChIP-qPCR experiments show that the signal for γ -H2A is similar to WT close to the break (Fig. 3A). However, farther from the break, at 7Kb and 10Kb, the signal is significantly reduced, showing its relation to Mec1. In *lcd1Δxrs2Δ*, both Mec1 and Tel1 recruitments being abrogated, the γ -H2A signal is completely abolished (Fig. 3A). This is similar to what is observed for *mec1Δ* and *mec1Δtel1Δ* mutant cells in previous studies (10). Loss of *MEC1* affects γ -H2A spreading away from the break site, whereas loss of *MEC1* and *TEL1* results in complete loss of γ -H2A.

A large scale phosphoproteomic study has detected phosphorylation of H2AS15 and linked it to Mec1 (29). To investigate this, we carried out ChIP-qPCR using the H2AS15ph antibody to study its spreading around the DSB in *lcd1Δ* and *lcd1Δxrs2Δ* mutant cells. As shown above, wild type cells show enrichment for H2A-S15ph over a large region on each side of the DSB (Fig. 3B). However, *lcd1Δ* as well as *lcd1Δxrs2Δ* mutant cells do not show any H2AS15ph signal. Overall, the above data demonstrate that, unlike γ -H2A which is brought about by the kinase

activity of Mec1 and Tel1, H2AS15ph is carried out solely by Mec1 and is therefore linked to resection after DSB formation.

H2AS15 is required to maintain fidelity of resection

Previously studies have shown that the *H2A-S129A* non-phosphorylation mutant leads to faster resection from the HO-induced DSB compared to wild type cells (38, 39). This increased resection may be attributed to the inability of Rad9 to bind H2AS129ph. Yeast Rad9 like its mammalian homolog 53BP1 functions in slowing down or keeping a check on end resection rate (40-43). To investigate whether H2AS15ph may affect resection, we used an antibody against Rfa1 (RPA largest subunit) as a proxy for the ssDNA generated by resection in ChIP-qPCR at the HO-induced DSB. Interestingly, *H2A-S15E* (phosphorylation mimicking) mutant cells show increased resection, whereas *H2A-S15A* mutant cells behave like wild type (Fig. 4A). The double mutants *H2A-S15A/E H2AS129A* have similar increased resection patterns as *H2A-S129A* cells, maybe slightly less than *H2AS15E* cells (Fig. 4A). An interesting possibility is whether H2AS15ph is also recognized by the BRCT domain of Rad9, this domain being well characterized for its ability to bind H2AS129ph (26). Surprisingly, *in vitro* peptide binding assays with recombinant Rad9 indicate a clear binding preference for the histone H2A tail peptide, but in its non-phosphorylated form (Fig. 4B). Importantly, this binding is also confirmed using yeast whole cell extracts in peptide pull-downs and detecting endogenous Rad9 by immunoblotting (Fig. 4C). In this case, inducing DNA damage in the cells does not affect endogenous Rad9 ability to bind the H2A peptide *in vitro*. This ability of H2AS15ph to block Rad9 binding to the H2A histone tail may in part explain the increased end resection detected in *H2A-S15E* mutant cells but not in *H2A-S15A*

(Fig. 4A). Altogether, these results implicate the DNA damage-induced H2AS15ph histone mark in the end resection fidelity at DSBs.

DISCUSSION

Histone tails are sites of various post-translational modifications (PTMs) such as phosphorylation, acetylation, methylation, ubiquitylation and SUMOylation. These modifications are recognized by reader proteins containing specialized domains such as ubiquitin binding domains, BRCT and FHA domains which bind phosphorylation, Chromodomains, MBT domains, Bromodomains and PHD fingers which recognize methylation or acetylation. Proteins and protein complexes use these interactions for their proper recruitment, retention and orientation at the target sites to carry out their respective functions (44, 45).

In this study, we characterized yeast H2AS15ph as a novel histone modification in the context of DNA damage. This modification is induced in presence of DNA damaging agent MMS, which is a known alkylating agent and produces DSBs as cells replicate their DNA in S-phase. A large-scale proteomic study had identified H2AS15ph by mass spectrometry and implicated Mec1^{ATR} for its detection (29). Using *in vivo* analysis, we show that indeed Mec1, and not Tel1, is responsible for the induction of this mark around DNA breaks, unlike γ -H2A (H2AS129ph) which is brought about by either Tel1 or Mec1 (Fig. 3). While the spreading of γ -H2A at DSB is not affected in *H2A-S15A/E* mutant cells, a decrease of chromatin acetylation is detected in close proximity to the DSB (Fig. 1). We know from previous studies that acetylation of histone H4 and H2A by the NuA4 complex plays an important role for the recruitment of various chromatin

remodelers like INO80, RSC and SWI/ SNF required for repair by HR (12, 46, 47). Future work will be needed to determine if H2AS15 affects the recruitment of these remodelers.

A most interesting finding reported here argues that yeast H2AS15ph corresponds to an evolutionary conserved regulatory function to regulate 53BP1/Rad9 binding to the H2A tail and DNA end resection, consequently DSB repair pathway choice. The yeast H2AS15 residue seems the functional homolog of mammalian H2AK15 in modulating resection which in turn affects DNA repair pathway selection, albeit through different PTMs. In mammals, when 53BP1 binds H2AK15ub in the vicinity of DSBs, it inhibits resection and favors the NHEJ pathway. This binding in turn inhibits TIP60-mediated acetylation of H2AK15 which otherwise would block ubiquitylation by RNF168 and 53BP1 binding, favoring resection and HR (24). *H2A-S15E* mutant cells show increased resection, as seen with *H2A-S129A* mutant cells where Rad9^{53BP1} is incapable to bind and inhibit or slow down resection (Fig. 4A). *In vitro* data suggest that Rad9 binds the H2A N-terminal tail in its unmodified state and this binding is abolished when H2AS15 is phosphorylated (Fig. 4B-C). We proposed a model depicted in (Fig. 4D) where resection of DNA ends produces ssDNA which gets coated by RPA and recruits Mec1^{ATR} through its binding partner Lcd1/Ddc2^{ATRIP} (34, 36). Mec1 then phosphorylates H2AS15 which inhibits Rad9 binding and therefore increases resection. H2AS15ph may be a dynamic mark, switching between phosphorylation and non-phosphorylation states to fine-tune the process of resection. This conserved function of the H2A tail to regulate Rad9/53BP1 binding and resection is even further supported by a recent report showing phosphorylation of the ubiquitin moiety of H2AK15ub (H2AK15pUbT12) to inhibit 53BP1 binding and favor resection (48).

Future work will address whether specific proteins may bind H2AS15ph. While our peptide pull-downs with yeast extracts followed by mass spectrometry did not yield clear results (data not shown), we speculate that other DNA repair proteins such as Rtt107 and Dpb11 which have BRCT domains could recognize H2AS15ph. Rtt107 was recently shown to bind H4T80ph and γ -H2A to counteract Rad9-modulated checkpoint activity (49). Dpb11 has been shown to act as a scaffold protein for various checkpoint proteins and is involved in Rad9 recruitment/retention at DSBs (50, 51). H2AS15 may also act redundantly with other DNA damage induced histone phosphorylation marks and function as a scaffold for proper orientation or retention of repair factors.

MATERIAL AND METHODS

Yeast strains

All yeast strains used in the current study are from FY-406 (52) and JKM-179 (53) (*hta1-htb1Δ::NatMX*, *hta2-htb2Δ::KANMX*) background carrying deletion of both copies of H2A and H2B genes. These strains carry an ectopic copy of *HTA1-HTB1* on a low copy vector pRS413 (in FY-406) and pRS414 (in JKM-179). Gene deletion and point mutations were obtained through standard PCR techniques. Plasmid shuffling using 5-Fluoroorotic acid (5-FOA) was employed to introduce the plasmid with point mutations in respective backgrounds. Yeast background JKM139 (53) containing deletion of *sml1Δ::NATMX*, *lcd1Δ::KANMX*, *xrs2Δ::TRP1* was used in this study. QY-362 (JKM139 *RAD9-13MYC::KANMX*) was also used in this study for peptide pull down experiment. General lithium acetate method was used to transform the yeast cells. Cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C till early log phase. For spot assay 1:10 dilution of the culture was spotted and grown at 30°C for 3-4 days.

Chromatin Immunoprecipitation (ChIP)

ChIP-qPCR was performed as described previously (54). In brief, cells were grown overnight in YP-Raffinose till OD600 was 0.5-1, at this point galactose (2%) was added to induced DSB at *MAT* locus for 3 hours. This was followed by cross-linking of cells with formaldehyde and lysing with bead-beater. Sonication was performed on Diagenode Bioruptor to obtain chromatin size of ~200-500bp. 100μg of chromatin was used to setup the immunoprecipitation reaction with anti-H2A-S129ph (Upstate 07-745), anti-H2A-S15Ph (antibody was produced in rabbit using the peptide sequence: Ac-GSAAKA(pS)QSRSAK-C. Affinity purification was performed to improve the specificity of the antibody. Antibody was produced by Thermo Fisher Scientific), anti-Rfa1

(Agrisera AS07214), anti-H4ac (Upstate 06-946) and anti-H4 (Abcam 7311) and reaction was allowed to mix on a wheel overnight at 4°C. Next morning Protein-A agarose beads were added followed by additional 3-4 hours on a wheel at 4°C. After multiple washing steps the DNA was eluted and incubated at 65°C overnight for de-cross linking followed by phenol-chloroform extraction of DNA. This DNA was resuspended in NTE and used for qPCR. LC480 LightCycler (Roche) was used to quantify DNA with the primer pairs as mentioned in the respective figures to calculate % of immunoprecipitation (IP)/ input. The data represents mean of two independent biological replicates. Error bars indicate the ranges between the two biological replicates. Fold enrichment represents ratio of %IP/input at indicated loci around DSB normalized on signal at negative-control locus IntergenicV. Primer sequences are available upon request.

Purification of native yeast chromatin

Native yeast chromatin was prepared as described previously (55), cells were treated with MMS (0.05%) or DMSO (control) for 2 hours to induce DNA damage. Lambda protein phosphatase (λ PP) (NEB) kit was used. The reaction mixture was incubated for 30 mins at 30°C followed by booster of λ PP and additional incubation for 30 mins at 30°C.

Recombinant protein purification

Recombinant Rad9 (C-terminal containing Tudor and BRCT domains) protein was purified as described previously (56). In short, bacterial cells were grown overnight at 16°C with IPTG induction. Next day cells were lysed with lysozyme followed by sonication. The soluble portion was incubated with glutathione sepharose (GE healthcare) beads for 3-4 hours at 4°C and eluted with glutathione. The eluted protein concentration was quantified by running on SDS-PAGE with known BSA standards followed by coomassie staining.

Peptide-pull down

Peptide-pull down was performed with synthetic peptide corresponding to H2A N-terminal (aa 1-23) obtained from AnaSpec. 1µg of peptide was incubated with 1µg of GST-tagged protein overnight on wheel at 4°C in binding buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1% NP-40, 1 mM PMSF and phosphatase inhibitors). Next morning streptavidin dynabeads was added to the reaction mixture and kept at 4°C for two additional hours followed by washing, running on SDS-PAGE and western blotting with anti-GST (Sigma G1160).

Peptide-pull down with yeast cell extract was performed by growing cells in YPD and treating with MMS (0.05%) or DMSO for 2 hours till OD₆₀₀ ~1.0-1.5. Cells were centrifuged and pellet was wash with cold mQ. Pellet was resuspended in sensitization buffer (100mM Tris-HCl pH 9.4, 10mM DTT) and incubated at 30°C on a wheel for 15 mins. Centrifuged at 500g for 5mins at 4°C and resuspended in 30ml of spheroplasting buffer (1.2M Sorbitol 20mM Hepes pH 7.5). Centrifuged and resuspended again in 40ml of spheroplasting buffer with lyticase, incubated on wheel at 30°C for one and half hour. Centrifuged at 500g for 5 mins and washed with wash buffer (1.2M Sorbitol, 20mM Pipes pH 6.4, 1mM MgCl₂) and resuspended in 25ml of nuclear isolation buffer; NIB (250mM Sucrose, 60mM KCl, 1mM PMSF, 14mM NaCl, 0.8% Triton X-100, 5mM MgCl₂, 1mM CaCl₂, 15mM MES) mixed well to remove clumps and kept on ice for 20 mins. Centrifuged and washed two times with NIB till white nuclear pellet was obtained. Resuspended in pull-down buffer (400mM NaCl, 1mM PMSF, 50mM Tris-HCl pH8, 0.5% NP-40, 5 mM β-glycerophosphate, 10 mM sodium butyrate, 5 mM NaF) to get 300mM NaCl in final volume and kept on rotating plate at 4°C for 20 mins. Centrifuged at 14,000rpm at 4°C for 30 mins and collected the supernatant (nuclear extract). Biotinylated peptides were bound to streptavidin

dynabeads in peptide binding buffer (150mM NaCl, 50mM Tris-HCl pH8.0, 0.1% NP-40, 5 mM β -glycerophosphate, 10 mM sodium butyrate, 5 mM NaF) by incubating at room temperature on wheel for 30 mins. Peptide-dynabeads were washed with protein binding buffer (140mM NaCl, 50mM Tris-HCl pH 8.0, 0.1% NP-40, 0.5mM DTT, 10 μ M ZnCl₂, 5 mM β -glycerophosphate, 10 mM sodium butyrate, 5 mM NaF) followed by addition of 600ug of nuclear extract to each pull-down reaction and incubated at 4°C on wheel for 2 hours. Nuclear extract that was incubated with phospho peptide was initially incubated with non-phospho peptide, on the other hand, nuclear extract for non-phospho peptide was initially incubated with phospho peptide for 2 hours at 4°C on wheel. Washed 3-4 times with final wash buffer (350mM NaCl, 50mM Tris-HCl pH8.0, 0.1% NP-40, 0.5mM DTT, 10 μ M ZnCl₂, 5 mM β -glycerophosphate, 10 mM sodium butyrate, 5 mM NaF) followed by addition of laemmli buffer, boiled and resolved in SDS-PAGE followed by western blotting with anti-Myc (9E10 Babco MMS150R).

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494

FIGURE LEGENDS

Fig 1: *H2A-S15* mutants are sensitive to DNA damage and functions independently of γ -H2A.

(A) Sequence of H2A N-termini highlighting the SQ site at position 15 in *S. cerevisiae* and corresponding lysine at position 15 in *H. sapiens*.

(B, C) Phenotypic analysis of yeast cells expressing wild type or the indicated H2A mutants. Ten-fold serial dilutions of log phase cells were spotted on plates containing rich media alone or in presence of DNA damaging agent methylmethane sulfonate (MMS). *H2A-S15E* showed a slight growth defect in presence of the drug. Whereas, both *H2A-S15A/E* in background with *H2A-S129A* showed stronger sensitivity than their respective single mutants. *H2B-T129A* did not influence the growth of *H2A-S15A/E*.

(D, E) ChIP-qPCR showing γ -H2A at an inducible DSB is not affected by *H2A-S15A/E*. Cells were grown in YP-raffinose overnight till log phase followed by addition of galactose to induce DSB for 3 hours before cross-linking the chromatin. Primer pairs used flanked the DNA sequence on both left and right side at 3Kb, 7Kb and 10Kb of the DSB at *MAT* locus. Anti-H4 signal was used to normalize for histone occupancy. The data represents mean of two independent biological replicates. Error bars indicate the ranges between the two biological replicates.

(F) *H2A-S15A/E* affects the acetylation of histone H4 and H2A. Western blot analysis of yeast whole cell extracts (WCE) with anti-H4ac reveals a slight reduction in H4ac and H2Aac in *H2A-S15A* and to a lesser extent in *H2A-S15E* compared to wild type. Histone H3 was used as loading control.

(G) *H2A-S15A/E* show reduced H4ac signal in regions close to the DNA break site. Signal for H4ac was normalized for histone occupancy on total H4 signal. ChIP-qPCR was performed as described in (D), with primers flanking 150bp, 1kb, 3Kb left and 0.5Kb, 1kb, 3Kb right of DSB.

Fig 2: H2AS15ph is locally induced upon DNA double strand break formation and spread over several kilobases.

(A) H2A-S15ph signal increased when yeast cells are treated with DNA damaging agent, MMS. Western blot analysis was performed with 300ng of native chromatin purified from yeast cells treated with DMSO (untreated control) and MMS. Anti-H2AS129ph was used as positive control for the MMS treatment. Antibodies against histone H2B and H4 were used as loading control.

(B) Anti-H2AS15ph is specific for recognizing a phosphorylation mark on H2A. Western blot analysis was performed with 300ng of native chromatin purified from MMS treated yeast cells after incubation with or without lambda protein phosphatase (λ PP). Lane with λ PP treatment showed a significant decrease in signal for H2A-S15ph. Histone H4 was used as loading control.

(C) H2AS15ph spreads on both sides of the HO endonuclease induced single DSB at *MAT* locus. Signal for H2AS15ph showed enrichment after the induction of the DSB in wild type but not in *H2A-S15A*. ChIP-qPCR was performed as described in Figure 1D. Fold enrichment was calculated as ratio of %IP/ input normalized on total H4 for histone occupancy at indicated loci around DSB at *MAT* locus to the signal at negative-control locus intergenicV.

(D, E) Kinetics of H2S15ph and H2AS129ph accumulation after the induction of the HO-DSB. ChIP samples were analyzed after times 0, 30min, 1hr and 3hrs. Anti-H4 signal was used to normalize for histone occupancy. ChIP-qPCR was performed as described in Figure 1D.

Fig 3: Mec1^{ATR} mediates phosphorylation of H2AS15 *in vivo*.

(A) Phosphorylation of H2AS129 carried out by Mec1 and Tel1. Signal for H2AS129ph at DSB in *lcd1Δ* (loss of Mec1) is similar to wild type proximal to the break and much reduced distal to the break. H2AS129ph signal is abolished in *lcd1Δxrs2Δ* (loss of Mec1 and Tel1). The line representing the signal for GAL *lcd1Δxrs2Δ* is hidden behind and has similar values as uninduced/raffinose samples.

(B) Phosphorylation of H2AS15 is mediated by Mec1 and not Tel1. Loss of H2AS15ph signal in *lcd1Δ* and *lcd1Δxrs2Δ* compared to WT.

All yeast stains used in the above experiments have *sm1Δ* background. ChIP-qPCR was performed as described in Figure 1D, primer pairs flanking 1Kb left and right of DSB were also used.

Fig 4: H2AS15 regulates resection of DNA ends at DSB and Rad9^{53BP1} binding *in vitro*

(A) *H2A-S15E* mutant has increased DNA end resection compared to *H2A-S15A* and wild type backgrounds. *H2A-S129A* is used as a positive control. ChIP-qPCR was performed as described in Figure 1D with antibody against Rfa1 and primer pairs flanking 1Kb, 3Kb and 7Kb left and right of DSB.

(B) Peptide pull-down assay with GST-tagged recombinant Rad9 and indicated H2A N-terminal peptides. rRad9 binds to unmodified peptide over phosphorylated peptide. Empty GST is used as negative control.

558 (C) Peptide pull-down assay as in (B) but using extracts from yeast cells treated or not with MMS
559 and expressing myc-tagged Rad9.

560 (D) Model for the role H2AS15ph in DSB repair. Mec1 phosphorylates H2AS15 in the region
561 surrounding the DSB. This phosphorylation inhibits Rad9 binding and results in increased
562 resection. H2AS15ph may function as a dynamic switch to modulate resection, phosphorylation
563 enabling resection and non-phosphorylation preventing hyper-resection.

564

565

(A)

1 15
S.cerevisiae N-SGGKGGKAGSAAKASQSRSAK
H.sapiens N--SGRGKQGGKARAKAKSRSSR

(B)

(C)

(D)

(E)

(F)

(G)

FIGURE 2