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The RAG1 V(D)J recombinase/ubiquitin ligase promotes ubiquitylation of acetylated, phosphorylated histone 3.3

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ABSTRACT

Histone variant H3.3 is associated with transcriptionally active chromatin and accumulates at loci undergoing preparation for V(D)J recombination, a DNA rearrangement required for the assembly of antigen receptors and development of B and T lymphocytes. Here we demonstrate that the RAG1 V(D)J recombinase protein promotes ubiquitylation of H3.3 that has been heavily acetylated and phosphorylated on serine 31 (acetyl-H3.3 S31p). A fragment of RAG1 promoted formation of a mono-ubiquitylated H3 product that was identified using mass spectrometry as ubiquitylated acetyl-H3.3 S31p. H3 was ubiquitylated at multiple lysine residues, and correspondingly, di-, tri- and higher-order ubiquitylated products were detected at low levels. Ubiquitylation was dependent on an intact RAG1 RING finger/ubiquitin ligase domain and required additional regions of the RAG1 amino terminus that are likely to interact with H3. Acetylated residues within the H3 amino terminal tail were also required. Purified, recombinant H3.1 and H3.3 were not good substrates, suggesting that post-translational modifications enhance recognition by RAG1. A complex including damage-DNA binding protein has also been shown to ubiquitylate H3 in response to UV treatment, suggesting the H3 ubiquitylation may be a common step in multiple DNA repair pathways.

1. Introduction

V(D)J recombination—the assembly of variable, diversity and joining gene segments into mature antigen receptor coding genes—is a requisite step in the development of B and T cells in humans and all other jawed vertebrates [1–3]. The RAG1 and RAG2 proteins comprise a recombinase that introduces double-stranded DNA breaks at recombination signal sequences (RSS) adjacent to the V, D, and J segments [1,2]. The broken DNA ends are repaired by the general non-homologous end joining (NHEJ) DNA repair machinery [4], with gene coding segments being joined to form coding joints and RSS containing DNA ends joined to form signal joints. In addition to exhibiting DNA cleavage activity, the RAG1 protein includes a RING finger domain that can act as a ubiquitin ligase (E3) [5,6], working in collaboration with ubiquitin conjugating (E2) enzymes to add ubiquityl moieties to several target proteins. RAG1 promotes its own ubiquitylation and targets both karyopherin alpha 1 (KPNA1) and histone 3 (H3) [5,7,8], although in the case of H3 reactions were carried out in crude extracts and appeared to be partially independent of RAG1's RING finger domain [7].

Just as they do with transcription, epigenetic signals exert spatiotemporal regulation over V(D)J recombination. There is a large catalog of data regarding chromatin modifications associated with recombination loci, including enrichment in the H3 variant H3.3 [9]. H3.3 is highly conserved in all eukaryotes and is associated with actively transcribed chromatin [10–12]. Nucleosomes including H3.3 bind less tightly to DNA [13], thus its presence in chromatin may facilitate a wide variety of protein–DNA transactions. Many of the H3 modifications associated with active chromatin may in fact reside primarily on H3.3 [14,15], as it is the predominant H3 variant present at active loci. Likewise, removal of markers of inactive chromatin such as lysine methylation occurs through H3.1 replacement by H3.3 [11]. During mitosis H3.3 undergoes phosphorylation on S31 (H3.3 S31p) [16], a modification that cannot occur in either H3.1 or H3.2, which both encode an alanine at this position. The role of this modification on H3.3 is unclear.

The different steps in V(D)J recombination—DNA cleavage and joining—require separate, sequentially occurring chromatin modifications. For example, recombination loci are characterized by markers for active chromatin prior to DNA cleavage [9,17,18], but phosphorylation of histone 2A variant H2AX occurs only after DNA cleavage and is required for the joining phase of the reaction [19].

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H2AX phosphorylation is a common step in all DNA double-strand break repair pathways, but it is not the only histone modification associated with DNA repair. The ubiquitylation of H3 by a complex including damage DNA binding protein (DDB) after UV irradiation suggests that it may also be a common step in repair of DNA damage [20], perhaps acting to weaken nucleosome–DNA interaction and/or recruiting additional repair factors. Here we show that H3.3—the variant known to be present at recombining loci—is a substrate for RAG1-dependent E3 activity and that RAG1 appears to specifically target heavily acetylated H3.3 S31p. While H3.3 S31p has previously been found in specific regions of chromatin only in mitotic cells [16], we show that it occurs in cultured lymphocytes undergoing recombination. This observation may indicate a unique function for H3.3 S31p that is required both in recombining lymphocytes and mitosis.

2. Materials and methods

2.1. Proteins and antibodies

RAG1[1–389], RAG1[1–389] P326G, and RAG1[218–389] were expressed as Xpress epitope-, histidine-tagged fusion proteins in *Escherichia coli* and purified as previously described [21]. Protein kinase tagged ubiquitin (PK-ubi) was expressed in *E. coli* and purified as previously described [5]. UbcH2 and E1 enzymes were purchased from Boston Biochem (Cambridge, MA, USA). Anti-H3 (ab1791-100) and anti-H3.3 s31p (ab9628) were purchased from Abcam (Cambridge, MA, USA). A panel of antibodies against acetylated histone H3 (#9921) was purchased from Cell Signaling Technology (Boston, MA, USA). Monoclonal antibody specific for the amino terminus of H3 (#39763) was purchased from Active Motif (Carlsbad, CA, USA). Anti-ubiquitin–protein conjugate was purchased from Biomol International (Plymouth Meeting, PA, USA). Bacterially expressed, purified histone H3.1 (M503S) and H3.3 (M507S) were purchased from New England Biolabs (Ipswich, MA, USA).

Mixed histones were purified from RAG1-negative pro-B cells (4×10^8) by acid extraction. Briefly, cells were washed twice in ice cold phosphate buffered saline, followed by incubation in 4 ml hypotonic buffer (10 mM Tris pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol) for 2 h, 4 °C, with gentle rotation. Nuclei were collected by centrifugation (10,000 × *g*, 10 min, 4 °C), resuspended in 0.4 ml of 0.4 N H_2SO_4, and incubated for 1 h, 4 °C, with gentle rotation. Debris was pelleted by centrifugation (16,000 × *g*, 10 min, 4 °C). Histones were precipitated from the supernatant by dropwise addition of trichloroacetic acid (132 μl) and incubation on ice (30 min), and collected by centrifugation (16,000 × *g*, 10 min, 4 °C). The histone pellet was washed with ice cold acetone and resuspended in sterile water (100 μl). For hydrolysis of the amino terminal tail, histone solution was incubated over night at 37 °C in reaction buffer (50 mM Tris pH 7.4, 2 mM ATP and 50 mM NaCl).

2.2. Ubiquitylation reaction

RAG1 (22 nM), UbcH2 (0.3 μM), E1 (45 nM) and PK-ubi (500 μM) were combined with mixed histones (0.2 μl), H3.1 or H3.3 (0.5 μg) in 10 μl reaction buffer (50 mM Tris pH 7.4, 2 mM ATP and 50 mM NaCl) for 4–5 h, 37 °C. The low concentration of RAG1 was due to poor solubility of the 1–389 fragment. Reaction products were separated on a 4–12% bis–Tris gel (Invitrogen, Carlsbad, CA, USA), blotted and probed with anti-H3 (1:5000), anti-H3.3 S31p (1:2000), or anti-ubiquitin–protein conjugate (1:2000), followed by secondary-HRP conjugate as appropriate (1:5000) and Super-Signal West Femto detection reagent (Rockford, IL, Pierce, USA). ECL was detected and quantified with a Kodak ImageStation 3000 (Kodak, Cambridge, MA, USA).

2.3. H3.3 S31p detection in cultured lymphocytes

Abelson murine leukemia virus transformed 103/Bcl-2/4 Pre-B cells (gift of Dr. Garnett Kelsoe, Duke University Medical Center, Durham, NC) carry a temperature-sensitive variant of the *abl* protein [22]. These cells were maintained at the permissive temperature of 34 °C in RPMI-1640 supplemented with 10% FBS (Invitrogen, Carslbad, CA, USA), penicillin/streptomycin (Mediatech, Manassas, VA, USA), L-glutamine (Mediatech, Manassas, VA, USA) and β-mercapto-ethanol. Signal joint and coding joint formation was induced and detected by PCR as previously described [24].

Cells were arrested by treatment with nocodazole (100 ng/ml, 24 h). To release arrest, cells were washed with phosphate buffered saline then resuspended in complete media. Samples taken at various time points and temperatures were collected by centrifugation, resuspended in buffer containing 6 M urea and sheared extensively. H3.3 S31p was detected by Western blot as described in Section 2.2.

2.4. Mass spectrometry analysis

Ubiquitylated histone samples were lyophilized, re-suspended in LDS sample buffer, loaded on an Invitrogen 4–12% pre-casted bis–Tris NuPage gel with MOPS running buffer. The protein components separated by SDS-PAGE were stained with SimpleBlue® (Invitrogen, Carlsbad, CA, USA). Bands corresponding in size to mono-ubiquitylated H3 were excised from the gel. In-gel tryptic digestion was performed to extract the peptides from these gel bands [23]. Each peptide sample was desalted by C18 ZipTip (Millipore, Bedford, MA, USA), then, lyophilized and resuspended in 16 μl of 0.1% TFA for LC–MS analysis.

Each sample (6 μl) was loaded on an Agilent 1100 nano-capillary HPLC system (Agilent Technologies, Palo Alto, CA) with a 10 cm integrated μRPLC-electrospray ionization (ESI) emitter column (made in house), coupled online with a linear ion-trap (IT) mass spectrometer (LTQ XP, ThermoElectron, San Jose, CA) for mRPLC-MS/MS analysis. The integrated μRPLC-ESI emitter columns were made of 75 μm i.d. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ), which were slurry packed with 5 μm, 300 Å pore size C-18 silica-bonded stationary RP particles (Jupiter, Torrance, CA, USA) using a slurry packing pump (Model 1666, Alltech Associates, Deerfield, IL, USA). After sample injection, a 20 min wash with 98% of mobile phase A (0.1% formic acid) was applied and peptides were eluted using a linear gradient of 2% mobile phase B (acetonitrile with 0.1% formic acid) to 42% mobile phase B within 40 min at a constant flow rate of 200 ml/min. The seven most intense molecular ions in the MS scan were sequentially selected for subsequent collision-induced dissociation (CID) using a normalized collision energy of 35%. The mass spectra were acquired at the mass range of m/z 350–1800. The ion source capillary voltage and temperature were set at 1.5 kV and 200 °C, respectively. The MS/MS data were searched against a database with only RAG1 and ubiquitin protein sequences using BioWorks interfaced SEQUEST (ThermoElectron) operating on a 10 node Beowulf parallel virtual machine computer cluster (Dell, Inc., Round Rock, TX). Ubiquitylation, acetylation, methylation, phosphorylation and methionine oxidation were searched as differential modifications. Only tryptic peptides with up to two missed cleavage sites meeting a specific SEQUEST scoring criteria (delta correlation (ΔC_n) ≥ 0.10 and charge state dependent cross correlation (X_{corr}) ≥ 2.0 for $[M+H]^{1+}$, ≥2.5 for $[M+2H]^{2+}$ and ≥3.0 for $[M+3H]^{3+}$) were considered as legitimate identifications.

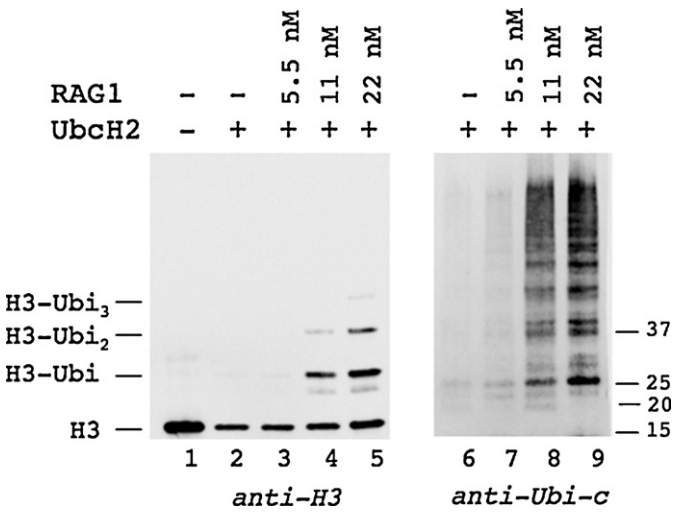

Fig. 1. Ubiquitylation of H3 promoted by RAG1. Mixed histones were subjected to ubiquitylation as described in Section 2.2, using various concentrations of UbcH2 and RAG1[1–389] as indicated. Products were separated on denaturing polyacrylamide gels, blotted and probed with polyclonal anti-H3 antibody or anti-ubiquitin–protein conjugate (ubi-c) antibody. The positions of unmodified H3 and ubiquitylated H3 species (H3-ubi) are indicated.

2.5. FACS analysis

103/Bcl2/4 cells were sampled at various time points after release from nocodazole arrest. Cells were collected by centrifugation, resuspended in cold PBS and fixed with 75% ethanol prior to propidium iodide staining. Stained samples were counted on an Becton Dickinson FACSort and analyzed using FCS Express Version 3.0 and ModFit LT.

3. Results

3.1. RAG1 promotes ubiquitylation of H3

A fragment of RAG1 (amino acids 1–389) including the RAG1 RING finger E3 domain was combined in various amounts with the E2 enzyme UbcH2, E1 enzyme and PK-ubi, along with mixed histones purified from cultured RAG1-negative pro-B lymphocytes (Fig. 1). The mixed histones were expected to include all histone variants and post-translational modifications present in cycling lymphocytes. Products were separated on a denaturing gel, blotted and probed with a polyclonal anti-H3 antibody that recognizes all H3 variants (Fig. 1, lanes 1–5) and with anti-ubiquitin–protein conjugate antibody (Fig. 1, lanes 6–9). In the complete reaction, a product of the molecular weight consistent with mono-ubiquitylated H3 was detected. Additional products consistent with di-, tri- and higher order ubiquitylation were also seen as the concentration of RAG1 was increased. When the products of these reaction were probed with anti-ubiquitin conjugate antibody, the putative H3 ubiquitylated species were detected (note that the intensity of the bands detected with anti-ubiquitin increases with the number of ubiquityl moieties). This reaction was dependent on the presence of UbcH2. Neither CDC34, which promotes auto-ubiquitylation of RAG1, nor ubcH5b, which has been implicated in RAG1-dependent H3 ubiquitylation previously, were able to support ubiquitylation of H3 in this system (data not shown).

Ubiquitylation of H3 was dependent on an intact RAG1 RING finger ubiquitin ligase domain. We previously demonstrated that the RAG1 P326G substitution eliminates RAG1 E3 functional interaction with E2 enzymes but does not disrupt the folded structure of this domain [21]. RAG1[1–389] P326G did not support ubiquitylation of H3 beyond background (Fig. 2A). Regions upstream

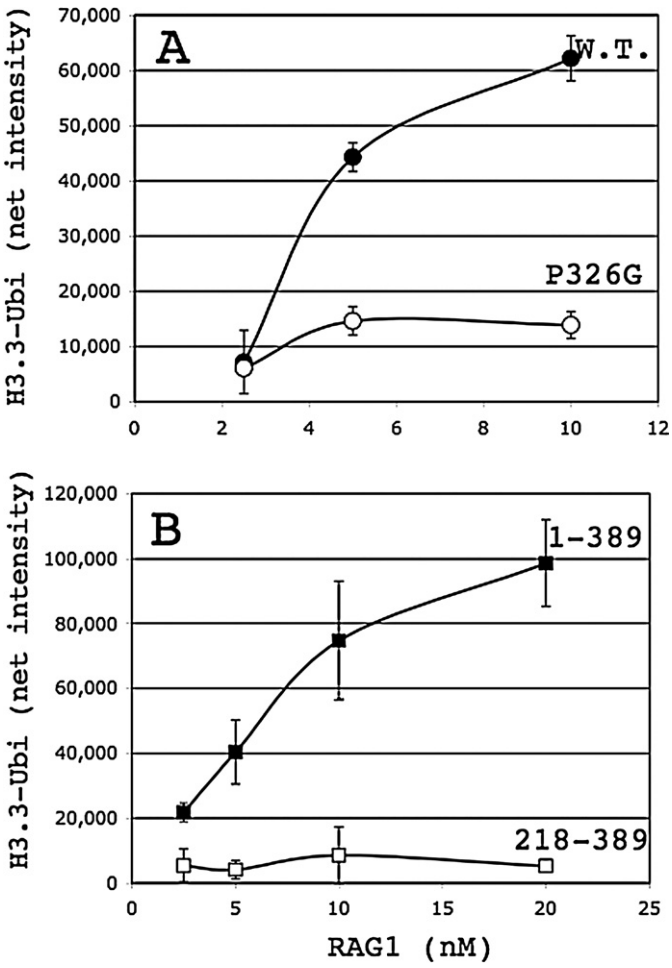

Fig. 2. Ubiquitylation of H3 by RAG1[1–389] P326G and RAG1[218–389]. Ubiquitylation reactions were performed as described in Section 2.2, using (A) RAG1[1–389] (W.T.) or RAG1[1–389] P326G or (B) RAG1[1–389] or RAG1[218–389]. Results are the average of three independent trials; standard deviation is indicated by error bars.

of the RING domain were also required for interaction with H3. RAG1[218–389] is competent in auto-ubiquitylation and ubiquitylation of KPNA1 [5,8], but the protein did not support ubiquitylation of H3 (Fig. 2B). This result indicates that the binding site for H3 must reside within the first 217 residues of RAG1.

3.2. RAG1 interacts with the modified amino-terminal H3 tail

Regions within the H3 amino-terminal tail were required for its RAG1-dependent ubiquitylation. Full length H3 underwent hydrolysis to a stable ~13 kDa species after overnight incubation at 37 °C. A panel of antibodies against epitopes in the amino terminus was used to discern the approximate breakpoint (Fig. 3A). Polyclonal antisera raised against intact H3 recognized both the full-length and truncated (H3Δ) species, while a monoclonal raised against a peptide at the extreme amino terminus of H3 recognized only the intact protein (Fig. 3A, cf, panels 1 and 2), indicating that the truncated species resulted from loss of the amino terminal tail. Polyclonal antibodies against H3 acetylated on lysines 9, 18 or 23 (H3K9a, 18a, or 23a, respectively), all recognized both full length and H3Δ (Fig. 3A, panels 3–5), and an antibody against H3.3 phosphorylated on serine 31 (H3.3S31p) recognized primarily the lower band (Fig. 3A, panel 6). Along with the size difference between the two species, these data suggest that the break point is somewhere between lysine 23 and serine 31 (or A31 on H3.1).

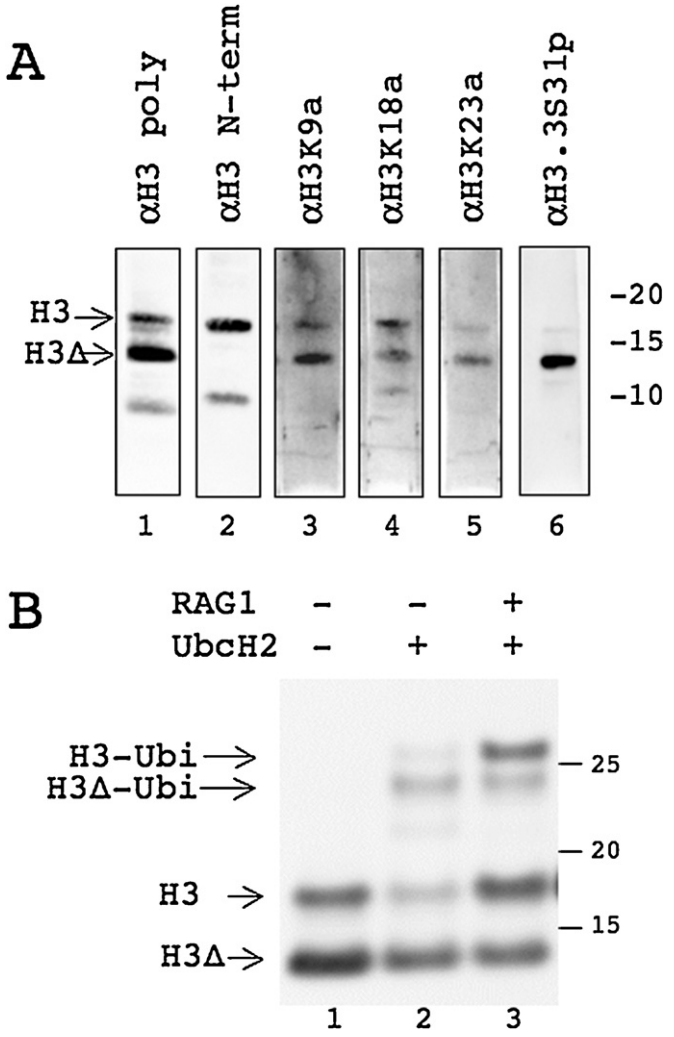

Fig. 3. N-terminal truncation of H3. (A) H3 was allowed to undergo spontaneous hydrolysis as described in Section 2.1. Aliquots from this reaction were separated by gel electrophoresis and subjected to Western blot. Individual lanes were probed with various antibodies as indicated. The positions of intact H3 and amino-terminally truncated H3 (H3Δ) are indicated. (B) A mixed pool on intact H3 and H3Δ was subjected to ubiquitylation as described in Section 2.2. Positions of substrates and products are indicated.

The ubiquitylation reaction was repeated with a mixed population of full length and truncated H3 (Fig. 3B). Under these conditions, a RAG1-independent mono-ubiquitylated species was observed that migrated at a slightly lower apparent molecular weight than the RAG1-dependent product (Fig. 3B, lane 2), corresponding in molecular weight to ubiquitylation of truncated H3 (H3Δ-Ubi). The addition of RAG1 had no effect on this product, but strongly stimulated generation of the full length ubiquitylated species (Fig. 3B, lane 3). Thus RAG1 specifically promoted ubiquitylation of full length H3 but not H3Δ, suggesting that it requires elements within the H3 amino terminal tail. Furthermore, truncated H3 could undergo promiscuous, RAG1-independent ubiquitylation.

Mono-ubiquitylated H3 generated in the presence of RAG1 was excised from the gel and subjected to mass spectrometry (MS) to identify (1) which lysine residues had been modified, (2) what other modifications were present on the ubiquitylated H3 species, and (3) whether H3 variants were present. These data are summarized in Table 1. Five different H3 lysine residues were found to undergo mono-ubiquitylation under these conditions. These included lysines in the amino terminal histone tail, the region most commonly post-translationally modified, and those in the histone core. This suggests that the higher molecular weight ubiquitylated species observed were the result of multiple mono-ubiquitylation steps. When the RAG1-independent product was analyzed, three positions of ubiquitylation within the histone core were detected (K36, K79, and K122). It should be noted that RAG1-independent H3 ubiquitylation may occur even in the presence of RAG1, and that this MS technique is not quantitative. Thus it is not possible to tell from these data whether RAG1 targets one particular lysine residue for modification.

Table 1
Mass spectrometric analysis of H3 modifications.

Fragment	Position
With RAG1	
R.KSAPS*p*TGGVK.K (H3.3)	S31-phos
R.K#STGGK@APR.K	K9-Ubi
R.K#QLATK@AAR.K	K18-Ubi
R.K@QLATK#AAR.K	K23-Ubi
R.EIAQDFK#TDLR.F	K79-Ubi
K.RVTIMPK#DIQLAR.R	K122-Ubi

#: ubiquityl; @: acetyl; *p*: phosphoryl. Trypsin cleavage sites are indicated with a period (.).

The RAG1-dependent H3-ubi product was found to contain phosphorylated H3.3 (H3.3 S31p), while no phosphorylated species were detected in the RAG1-independent product (Table 1). The MS results also demonstrated that the RAG1-dependent ubiquitylated H3 product had undergone extensive acetylation (Table 1 and data not shown). In fact we detected individual fragments that were both ubiquitylated and acetylated. It is not possible to tell with this technique whether the acetylated products are also H3.3 S31p, as the residues that distinguish H3.1 from H3.3 are not present in the acetylated fragments. However, acetylation and other H3 modifications associated with active chromatin are hypothesized to occur primarily on H3.3 [14,15].

Several lines of evidence indicated that modification (acetylation and/or phosphorylation) of H3 was required for RAG1-dependent ubiquitylation. Bacterially expressed H3.1 and H3.3 were poor substrates for RAG1-dependent ubiquitylation (Fig. 4, lanes 3 and 6), while both underwent RAG1-independent ubiquitylation (Fig. 4, lanes 2 and 5). We used a panel of antibodies against H3 modifications identified in our MS analysis to determine if any were required for ubiquitylation. Since the pool of histones used as substrates in this reaction was purified from actively dividing lymphocytes, it should contain histones modified in a wide variety of ways as well as unmodified. An antibody

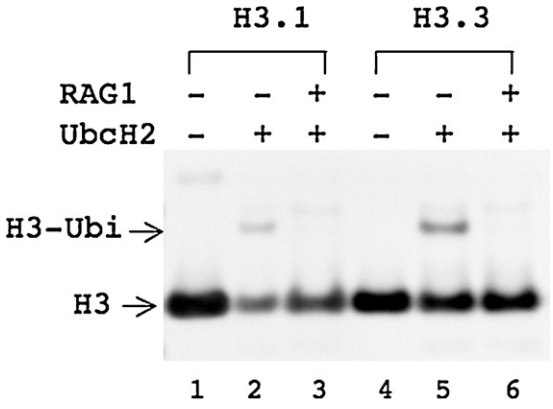

Fig. 4. Ubiquitylation of recombinant H3 variants. Recombinant H3.1 or H3.3 were subjected to ubiquitylation by RAG1[1–389] as described in Section 2.2, and products were detected with anti-H3 antibody. The positions of unmodified H3 and mono-ubiquitylated H3 (H3-ubi) are indicated.

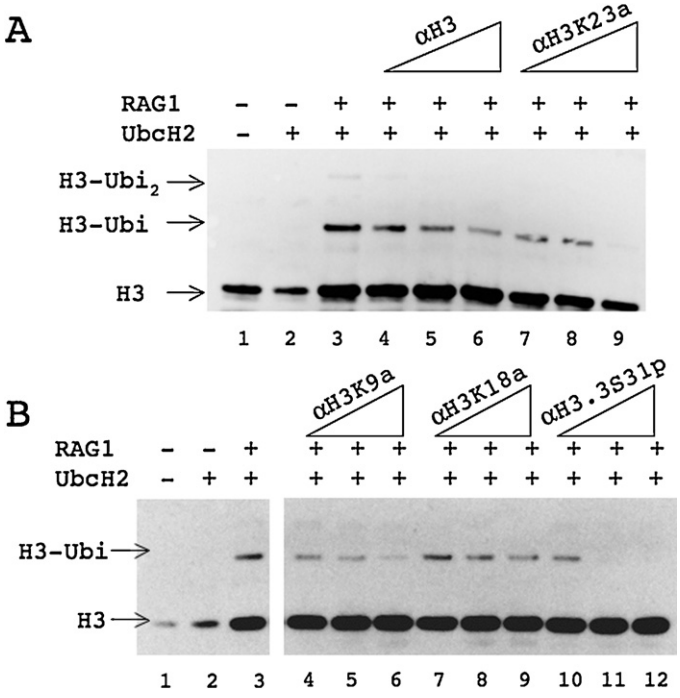

Fig. 5. Inhibition of H3 ubiquitylation by antibodies against specific H3 modifications. Mixed histones were subjected to ubiquitylation by RAG1[1–389] as described in Section 2.2, with the addition of increasing amounts (0.5, 1 or 2 μl) of various polyclonal antibodies as indicated. (A) Reactions included anti-H3 or anti-H3 acetylated on lysine 23 (H3K23a). (B) Reactions included anti-H3 acetylated on lysine 9 or 18 (H3K9a or H3K18a) or anti-H3.3 phosphorylated on serine 31 (H3.3S31p). Lanes between 3 and 4 have been excised for clarity. The position of mono-ubiquitylated H3 is indicated.

specific for a certain H3 modification should only inhibit the reaction if RAG1 must also recognize that modification, otherwise RAG1 could easily use other, differently modified H3 molecules as substrates. Therefore, if RAG1 has no preference for a specific modification, no individual antibody should inhibit the reaction. We found that the reaction was strongly inhibited by antibody specific for H3 acetylated on lysine 23 (αH3K23a) and H3.3 phosphorylated on serine 31 (αH3.3S31p) (Fig. 5A, lanes 7–9, and B, lanes 10–12), and to a lesser extent by antibodies against H3 acetylated on lysine 9 or 18 (Fig. 5B, lanes 4–9). The reaction was only mildly inhibited by polyclonal anti-H3 (Fig. 5A, lanes 3–5), which has epitopes throughout the protein. Taken together these results suggests that certain post-translational modifications, specifically acetylation and phosphorylation, activate H3 as a substrate for RAG1-dependent ubiquitylation.

3.3. H3.3 S31p is up-regulated during V(D)J recombination

H3.3 S31p has been associated with mitosis [16], and is not expected to be present during G1 when V(D)J recombination takes place [24]. A V(D)J recombination-inducible cell line was used to determine whether H3.3 S31p is present during recombination. 103/Bcl2/4 is a virally transformed pre-B cell line expressing a temperature sensitive v-abl protein [22]. At permissive temperature (34 °C), these cells cycle normally and V(D)J recombination is repressed. At non-permissive temperature (39.6 °C), cells arrest in G1 and undergo coding joint formation on the kappa and lambda loci [22,24]. Signal joint formation occurs after cells are shifted back to permissive temperature and re-enter the cell cycle [24]. 103Bcl2/4 cells were synchronized and H3.3 S31p levels were analyzed at various phases with a specific antibody (see Fig. 6A for experimental scheme). Initially, cells were arrested in M phase by nocodazole treatment. Under these conditions, a large accumulation of H3.3 S31p was detected by Western blot (Fig. 6B, lane 1), consistent with previously published data [16]. There was a dramatic drop in H3.3 S31p levels after release into G1, and levels remained low after 12 h of incubation at non-permissive temperature (Fig. 6B and C), during which time very little completed recombination could be detected (Fig. 6E). H3.3 S31p levels rose by a small but significant amount 24 h after induction of V(D)J recombination by transfer to non-permissive temperature (Fig. 6C), despite the fact that these cells are arrested in G1 phase (Fig. 6D). This corresponds to the period of time during which NHEJ DNA repair to produce coding joints occurs (Fig. 6E, left panel). There was a further dramatic increase in H3.3 S31p levels 24 h after shift back to permissive temperature, at which point NHEJ DNA repair to produce SJ occurs (Fig. 6E, right panel). FACS analysis indicated that these cells did not show an increase in G2 and were still in S phase (Fig. 6D), indicating that the spike in H3.3 S31p is not the one normally associated with mitosis. Thus the increase in H3.3 S31p levels correlated temporally with the DNA repair phase of V(D)J recombination.

4. Discussion

While great strides have been made in understanding the mechanism of DNA cleavage by RAG1 and RAG2, the role of the amino-terminal, so-called "non-core" region, of RAG1 has remained a mystery. The discovery of E3 activity in this region suggests many intriguing possibilities [5,6], and several potential substrates have been identified [7,8]. Recently it was demonstrated that RAG1 could promote ubiquitylation of H3 (variant unspecified). However, the resistance of this reaction to mutations that inactivate the RING finger domain—the catalytic core of RAG1 E3 activity—left open the possibility that the lysate used in the reaction was contaminated by another E3 protein possibly co-purifying with RAG2 [7]. RAG2 is known to interact with the Skp2-SCF E3 protein complex [25]. Nevertheless, these data suggested a functional interaction between RAG1 and H3. Our evidence suggests that RAG1 targets H3.3, the H3 variant known to be associated with recombining loci [9], and thus most likely to be encountered by RAG1 during V(D)J recombination. This reaction is absolutely dependent on an intact RAG1 RING domain, and requires regions of the far N-terminus of RAG1 where the H3.3 binding sight is likely to reside and regions within the H3 amino-terminal tail. We demonstrate using MS that several H3.3 lysines are subject to ubiquitylation, the first time the positions of H3 ubiquitylation have been identified. Furthermore, post-translational modification by acetylation and phosphorylation appears to activate H3.3 as a substrate, potentially allowing for more precise regulation of ubiquitylation. H3 acetylation is known to be vital for active V(D)J recombination [17]. Thus such specificity would theoretically prevent RAG1 from targeting the general pool of H3 and limit it's E3 activity to modified H3.3 present at the recombination loci proximal to where RAG1 is bound.

We can only speculate as to the potential role phosphorylation of H3.3 S31 plays in V(D)J recombination, as very little is currently known about its role in mitosis. H3.3 S31p occurs in very specific regions of the chromosome adjacent to the centromeres during late pro-metaphase and metaphase [16]. Unlike H3 S10p and S28p, it is not likely to be required for initiation of chromatin condensation, which has already occurred by that time. Instead, it has been suggested that H3.3 S31p in mitosis could prevent spreading of heterochromatin, presumably in conjunction with some transacting factor, helping to maintain the active status of certain genes. By analogy, phosphorylation of H3.3 could help prevent its replacement by H3.1 after the DNA repair phase of V(D)J recombination. Thus it would ensure maintenance of the active transcriptional status of the recombined locus.

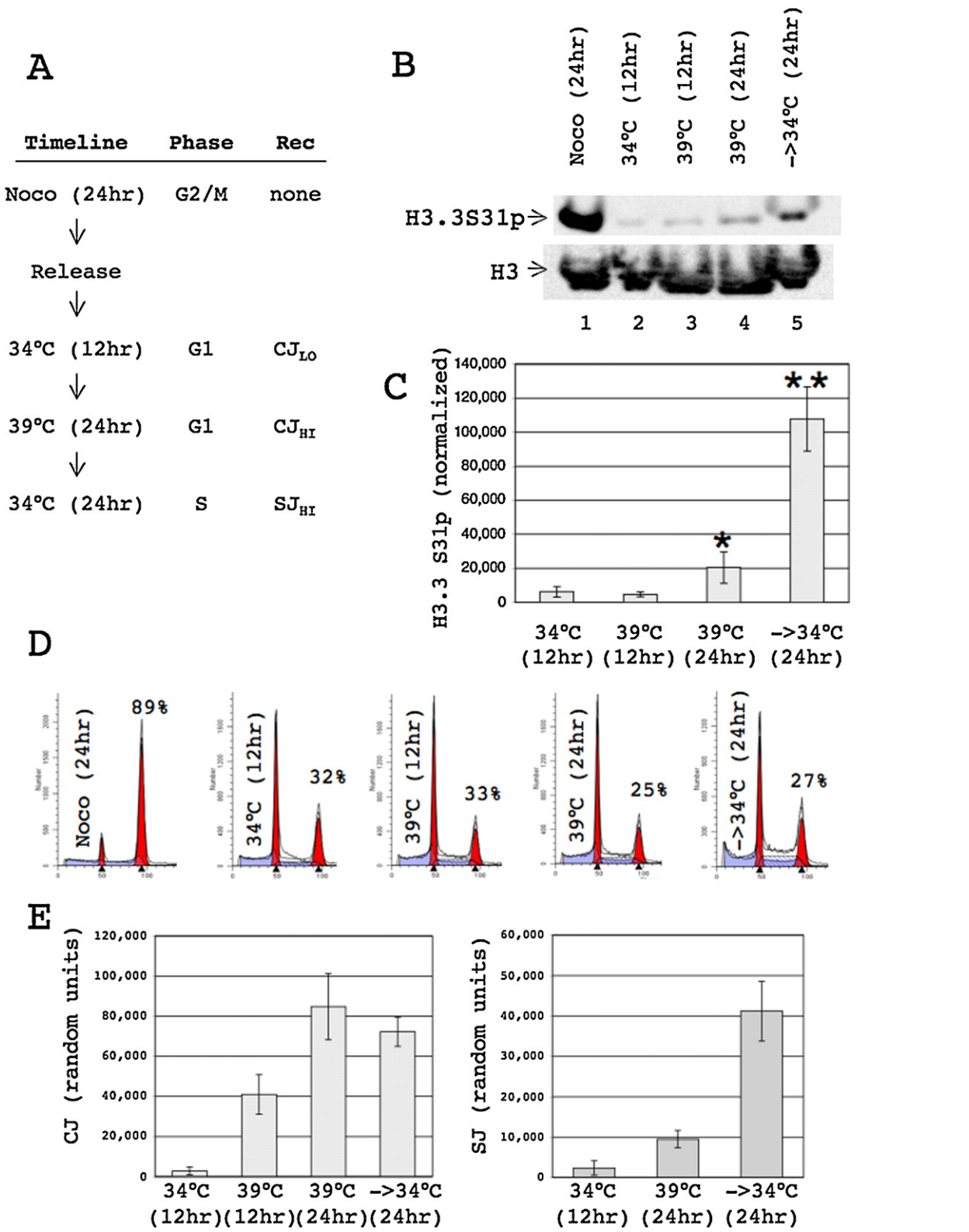

Fig. 6. H3.3 S31p accumulation in recombining cells. (A) Cells were arrested by 24 h treatment with nocodazole (Noco; 100 ng/ml), then released and incubated for an additional 12 h at permissive temperature, sampled, and then shifted to non-permissive temperature. Additional samples were taken 12 and 24 h after shift to non-permissive temperature, and 24 h after return to permissive temperature. Cell cycle phase and recombination status at the immunoglobulin kappa locus [24] are indicated. (B) H3.3 S31p detection by Western blot. Total H3 is shown in the bottom panel. (C) Average and standard deviation of three independent trials to detect H3.3 S31p. Levels have been corrected against β actin (not shown). Student's *t*-test was used to analyze differences; $^{*}p < 0.05$; $^{**}p < 0.001$. (D) Cells were sampled as described in (A), stained for DNA content, and counted as described in Section 2.5. Percentage of cells in G2 phase is indicated for each sample. (E) Coding joint and signal joint formation at various time points was determined as described in Section 2.3.

Histone ubiquitylation is one of the least well understood aspects of the histone code. H3 is a target for mono-ubiquitylation by the DDB complex, and this modification occurs after UV irradiation [20], suggesting that it is part of the DNA damage detection and repair pathway. The RAG1/2 recombinase is essentially a programmed DNA damage complex, introducing double stranded breaks in the DNA at defined locations. The response to these breaks shares components with the general NHEJ double strand break repair pathway [4]. For example, H2AX is phosphorylated at RAG1/2 induced breaks just as it is after ionizing radiation [19]. However, there are several aspects of RAG1/2 induced breaks that make them unique: (1) breakage occurs at pairs of sights simultaneously [26], (2) RAG1/2 remains tightly bound to the RSS ends following breakage [27,28], and (3) rejoining must occur between DNA ends that were originally distal from one another for recombination to occur successfully. These factors may influence how the damage DNA response is activated and how the NHEJ system is optimally deployed. In particular, the presence of the recombinase sequesters the cleaved RSS ends, and prevents access by the NHEJ repair proteins [28]. Likewise, the presence of the recombinase may prevent the ubiquitylation of H3 that would occur in response to other sources of DNA damage. Instead we propose ubiquitylation of H3.3 is promoted by RAG1, leading to recruitment of the repair complex and increasing accessibility of the ends. This model may represent a V(D)J specific mechanism for ubiquitylation of H3.3 associated with recombination loci that mirrors the general mechanism that occurs during the damage response elsewhere in the genome. This novel model for disassembly of the recombinase post-cleavage complex and transition to DNA repair, and more broadly for how histone ubiquitylation may potentiate DNA damage response, will be an active area of future inquiry.

5. Conclusions

RAG1 promotes ubiquitylation of histone 3 variant H3.3 that has been heavily acetylated and phosphorylated on serine 31 (acetyl-H3.3 S31p). H3.3 S31p, most commonly associated with mitotic chromosomes, is also up-regulated during V(D)J recombination. H3 is known to undergo ubiquitylation after UV irradiation, possibly as a mechanism for recruiting repair enzymes. RAG1-dependent ubiquitylation of H3.3 may be a V(D)J recombination-specific adaptation of this general DNA damage response.

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