Introduction

DNA stability is continuously compromised by light exposure, chemical reactants and the molecular machineries involved in transcription and replication. DNA lesions including DNA double-strand breaks (DSBs) trigger a signaling cascade, the DNA damage response (DDR), which results either in DNA repair or apoptosis. In eukaryotes, repair processes are complicated by the presence of chromatin. Nucleosomes, the basic units of chromatin, organize ~147 bp of DNA around an octamer of the histone proteins H2A, H2B, H3 and H4. Strings of nucleosomes can form compact higher order structures, including segments of chromatin fibers. A fundamental ordering principle of chromatin fibers are tetranucleosome units, where four consecutive nucleosomes form two stacks connected by DNA in a zig-zag (two-start) pattern (Scheme 1a). Such chromatin organization restricts access to DNA, and thus hinders repair processes. DDR processes relieve this inhibition and prime the damaged chromatin region for repair through structural remodeling and chromatin expansion early in the cascade, e.g. as observed by fluorescence imaging in live cells. The control of local chromatin structure involves histone post-translational modifications (PTMs). An early hallmark of DDR signaling is the ataxia-telangiectasia mutated (ATM) kinase-mediated phosphorylation of the histone variant H2A.X on serine 139 (H2A.X S139ph, also known as γH2A.X). This PTM is followed by ubiquitylation, i.e. the attachment of the ~8 kDa protein ubiquitin to the ε-amino group of lysines 13 or 15 (K13/15ub) of γH2A.X or canonical H2A (Scheme 1a). Nucleosomes carrying such combinatorial PTMs, e.g. phosphorylated and ubiquitylated γH2A.X K15ub, function as recruitment cues for downstream DDR effectors, such as 53BP1 and BRCA1. Changes in chromatin structure, e.g. caused by PTMs on H2A.X, contribute to the recruitment mechanism of downstream effectors, further determining the subsequent repair pathway. As dysregulation of PTMs on H2A.X is implicated in radiosensitivity, immunodeficiency and cancer, it is thus important to understand how chromatin structure is remodeled by DDR-associated PTMs.

Chemically modified histones, e.g. prepared by expressed protein ligation (EPL), enable direct testing of the individual effects of each PTM on chromatin structure and dynamics. To study γH2A.X K15ub function, H2A.X has to be modified on both N- and C-termini. Multistep total synthesis approaches yield access to combinatorially modified proteins, using
Results and discussion

EPL involves the reaction between a C-terminal thioester and an N-terminal cysteine (or related thiol-containing amino acid), resulting in the formation of a native peptide bond.\textsuperscript{26,37} To synthesize $\gamma$H2A.X K15ub, we decided to disconnect H2A.X at two alanine residues at positions 21 and 135 (Scheme 1b). After ligation of both the ubiquitylated N-terminal peptide and the phosphorylated C-terminal fragment to the H2A.X core, the non-native cysteines C21 and C135 required for ligation are desulfurized to alanine, restoring the native histone sequence.\textsuperscript{38,39} In our semisynthetic strategy (Scheme 1c), we decided to produce the core of H2A.X, residues 21–134, recombinantly, thus simplifying the synthetic endeavor. We envisioned using both a genetically encoded N-terminal protection strategy as well as a recombinant intein-based crypto-thioester in this fragment. At the C-terminus of the H2A.X fragment, we decided to add the N-terminal half of the split intein from Nostoc punctiforme (Npu).\textsuperscript{40} Upon addition of the C-terminal intein half (NpuC) and in the presence of suitable thiols this allows the installation of a thioester.\textsuperscript{40} For protection of the N-terminal cysteine residue, we settled upon a genetic fusion of the H2A.X fragment to small ubiquitin-like modifer (SUMO). After a first ligation introducing the phosphorylated C-terminus, the SUMO protecting group can efficiently be removed by the highly specific ubiquitin-like protease 1 (Ulp1). This is then followed by the ligation of a convergently assembled ubiquitylated N-terminus.\textsuperscript{41,42} We further envisioned that most steps of $\gamma$H2A.X K15ub production, including thioester activation and N-terminal deprotection by Ulp1 can be performed in a one-pot reaction (Scheme 1c).

To establish the synthetic strategy, we first synthesized the single-modified $\gamma$H2A.X. We thus expressed and purified the fragment H2A.X (1–134)-Npu\textsuperscript{c} (1a) (Fig. S1a–c\textsuperscript{+}). We further synthesized a peptide encompassing the C-terminus of H2A.X several protecting groups or kinetically controlled activation of thioesters.\textsuperscript{31–36} Semisynthetic methods can be more convenient, due to a reduced synthetic load. However, they can be difficult to implement, as orthogonal protection schemes in recombinant fragments are required. Here we report the facile convergent semisynthesis of $\gamma$H2A.X K15ub, employing a recombinantly produced central H2A.X fragment, containing an N-terminal protected cysteine and a C-terminal crypto-thioester. Semisynthetic dual-modified $\gamma$H2A.X K15ub (and singly-modified variants) are subsequently incorporated into synthetic chromatin fibers, which are further engineered to carry a FRET donor and acceptor dye pair in the DNA of neighboring nucleosomes at precise positions. Employing a single-molecule FRET approach, we demonstrate that S139ph does not alter chromatin structure, whereas K15ub disrupts inter-nucleosomal stacking and opens tetranucleosome units. Together, this shows that K15ub in H2A.X directly opens chromatin structure, providing chromatin access for repair proteins.

![Scheme 1](image1)

**Scheme 1** Semisynthetic strategy to produce $\gamma$H2A.X K15ub. (a) Hierarchical chromatin structure: (i) chromatin fiber. (ii) An individual tetranucleosome unit. (iii) Nucleosome structure (PDB code: 1KX5). H2A is shown in yellow with indicated positions of K15 (red) and S139 (green). (b) Amino acid sequence of human H2A.X, showing the disconnections and EPL reactions to produce $\gamma$H2A.X K15ub. (c) Scheme of the semisynthesis of $\gamma$H2A.X K15ub: (i) recombinant expression of H2A.X with truncated N- and C-termini (H2A.XNpuN, H2A.XNpuC), N-terminally fused to SUMO and C-terminally fused to the N-terminal part of a split intein (IntN). (ii) Split-intein mediated thioester conversion (in the presence of the C-terminal intein fragment, IntC and small molecule thiols). (iii) Ligation to the phosphorylated H2A.X C-terminal octapeptide. (iv) Enzymatic N-terminal deprotection by SUMO protease Ulp1. (v) Ligation to semisynthetic, ubiquitylated H2A.X N-terminal fragment. (vi) Final desulfurization to yield $\gamma$H2A.X K15ub. Steps (ii–iv), as well as (v and vi) were performed in one-pot.

![Fig. 1](image2)

**Fig. 1** One-pot semisynthesis of $\gamma$H2A.X. (a) Synthetic scheme for the production of $\gamma$H2A.X. (b) HPLC analysis of reaction progress for the ligation between 1a and 2 in the presence of MESNa, MTG and Npu\textsuperscript{c} peptide. After 6 h of metal-free desulfurization in the same pot, $\gamma$H2A.X 3 is purified. (c) Mass spectrometry analysis of purified 3. Observed mass is 15 095.0 Da. Expected mass is 15 093.3 Da.
(residues 135–142, S139ph, 2) by Fmoc SPPS (47% isolated yield) (Fig. S1d–f). We then proceeded to produce full-length γH2A.X by one-pot thioester conversion, ligation and desulfurization. Due to the poor solubility of 1a, all reactions were performed in 2 M urea, which prevented aggregation while still allowing intein function. Upon addition of the C-terminal intein half NpuC (mutated to prevent trans-splicing, Fig. S1g–i†) and mercapto-ethanesulfonate (MESNa), thioester conversion of 1a was initiated.40,41 We then directly added peptide 2 in the presence of the non-aromatic thiol catalyst methyl thiglycolate (MTG),44 which resulted in simultaneous thioester conversion and ligation (Fig. 1b). In the same mixture, cysteine 135 at the ligation site was finally desulfurized to the native alanine residue, employing radical-based chemistry.39,43 In summary, γH2AX (3) was synthesized in a three-step one-pot reaction (ca. 42% isolated yield) (Fig. 1b and c).

We thus turned to the task of establishing a convergent route to synthesize the N- and C-terminally modified histone γH2A.X K15ub. Following our envisioned route (Scheme 1b and c) the H2A.X core (21–134) A21C was expressed and purified as an N-terminal fusion to SUMO and as a C-terminal fusion with NpuN (1b, Fig. S2a–c†), enabling selective thioester conversion and N-terminal deprotection. We then initiated thioester formation and protein ligation by addition of the NpuN peptide, MESNa, MTG and phosphorylated H2A.X C-terminal peptide 2, in the presence of 2 M urea (Fig. 2a–b). After 16 h, the reaction was deemed complete by RP-HPLC and MS analysis, yielding ligation product 4a (Fig. S2d–f†). We then continued with the deprotection of the N-terminal cysteine using Ulp1, which proceeded to yield H2A.X (21–42) A21C S139ph (4b) in 90 minutes (Fig. 2a and b).

At this point, we purified 4b by semipreparative RP-HPLC for subsequent ligation. In our convergent strategy, the ubiquitylated N-terminal peptide H2A.X (1–20) K15ub (5b) is prepared by ligation of ubiquitin thioester to H2A.X (1–20), 5a, carrying a cysteine residue attached to the ε-amino of K15.46 To allow subsequent attachment of 5b to the H2A.X core, we incorporated a C-terminal hydrazide, which can be converted into a thioester by oxidation with NaNO2 followed by the addition of thiols45 (Fig. 2c). We thus synthesized the hydrazide 5a, which was ligated to recombinant Ub (1–75) thioester in the presence of MTG (Fig. S2g–p†). Test reactions (using H2A instead of H2A.X) of hydrazide thioester conversion and subsequent ligation did however not result in reaction progress (Fig. S3a–d†). Instead we observed a loss of ~32 Da (Fig. S3e–f†). This likely resulted from an intramolecular cyclization reaction between the C-terminal thioester and the cysteine residue remaining at the ubiquitin-H2A.X peptide ligation junction, a side reaction, which, to our surprise, irreversibly blocked further reaction progress (Fig. S3g†). Therefore, we decided to convert the offending cysteine at the ubiquitin ligation junction to alanine by desulfurization before purification of the intermediate, resulting in 5b (Fig. 2c).

With both 4b and 5b in hand, we proceeded to carry out the final ligation. We thus converted the hydrazide in 5b into a thioester and performed the ligation to 4b in the presence of MTG (Fig. 2d). After 22 h, the ligation was complete and the product was desulfurized, converting the two remaining non-native cysteines at positions 21 and 135 into the native alanine residues. The final product, γH2AX K15ub (6), was subsequently purified by semi-preparative RP-HPLC (Fig. 2d and e, isolated yield 24%). Finally, we prepared H2A.X K15ub, following the same general strategy (Fig. S4†). Together, we thus produced dual modified γH2AX K15ub (as well as the single-modified variants) in a convergent synthesis from four fragments, employing a central recombinant precursor containing genetically encoded orthogonal protection groups.

We then set out to determine the effects of H2AX modifications on the modulation of chromatin organization. Some histone PTMs, including ubiquitylation of H2B at K120,16,47,48 have been shown to result in a loss of chromatin compaction. Recent structural studies identified the tetraneucleosome as the fundamental structural unit of chromatin fibers.49,50 We thus decided to use a single-molecule FRET approach51 to measure the ability of nucleosomes containing modified H2AX to form stacked tetraneucleosome units within a chromatin fiber context (Fig. 3a). To this end, we employed a DNA template composed of a 12-mer tandem repeat of the 177 bp 601 nucleosome positioning sequence.52 To introduce FRET dyes into the ~2 kilobase long chromatin template, we resorted to a multistep DNA ligation approach. Connecting recombinantly produced DNA
fragments to PCR generated fluorescently labeled fragments, the two FRET dyes Alexa568 and Alexa647 (resulting in a Förster radius of 82 Å) were positioned within the DNA of nucleosomes 5 and 7 in the center of the 12-nucleosome containing chromatin fiber (Fig. S5f). Based on the crystal-structure of a tetranucleosome unit,4 dye positions were chosen such that they are separated by an interdye distance $R_{DA}$ of 46–64 Å within a compact tetranucleosome unit.47,49 We then proceeded to assemble histone octamers containing either unmodified H2A.X, γH2A.X, H2A.X K15ub or dual-modified γH2A.X K15ub (Fig. S6f). These histone octamers were used to reconstitute chromatin fibers (Fig. 3b).

Tetranucleosome stacking, and thus chromatin folding, can be initiated by addition of bivalent cations (e.g. Mg$^{2+}$). Initial ensemble FRET experiments demonstrated energy transfer in the assembled chromatin fibers upon Mg$^{2+}$ addition, and indicated a reduction of nucleosome packing in chromatin fibers containing K15ub, independent of the presence of S139ph (Fig. S7f). However, due to sample heterogeneity, photobleaching and ensemble averaging, exact FRET efficiency values ($E_{FRET}$) are not accessible from those ensemble experiments. We thus used single-molecule imaging to measure chromatin fiber conformation on the single-fiber level. Using total internal reflection fluorescence (TIRF) imaging (Fig. 3c), we recorded $E_{FRET}$ time-traces from single chromatin fibers with a temporal resolution of 100 ms and Mg$^{2+}$ concentrations from 0 to 4 mM (Fig. 4a–f). No dynamic structural transitions were observed in the $E_{FRET}$ time-traces, independent on the chromatin modification state, indicating that chromatin dynamics were faster than our time-resolution. Using traces from donor and acceptor dye-containing chromatin fibers, we then constructed $E_{FRET}$ histograms (Fig. 4d–f) for each chromatin state. After inducing tetranucleosome stacking with 4 mM Mg$^{2+}$, both H2A.X and γH2A.X containing chromatin fibers exhibited a high FRET state with a $E_{FRET}$ distribution centered at 0.6 ± 0.001 (for H2A.X) and 0.59 ± 0.01 (for γH2A.X) (Fig. 4d and e). This indicates that these chromatin fibers form stacked tetranucleosome states, similarly to canonical H2A.47 A second, low $E_{FRET}$ state further reported on fibers in an unstacked conformation. The broad observed $E_{FRET}$ distributions indicate rapid dynamic processes beyond the time-resolution of our TIRF approach.47 γH2A.X K15ub however resulted in a significant reduction in $E_{FRET}$ both in the absence of Mg$^{2+}$ and at 4 mM Mg$^{2+}$, where the distribution was centered at $E_{FRET} = 0.41 ± 0.07$ (Fig. 4f). K15 ubiquitylation (but not S139ph) thus directly
disrupts tetranucleosome stacking. For chromatin fibers containing canonical H2A, we could previously identify the dynamically exchanging, underlying structural states that contribute to the observed $E_{\text{FRET}}$ value of $\sim$0.6 at 4 mM Mg$^{2+}$.

These states include stacking contacts between nucleosomes within a tetranucleosome unit ($R_{DA} = 64 \text{ Å}$) or between neighboring tetranucleosome units ($R_{DA} = 46 \text{ Å}$), populated to 35% and 23%, respectively. Assuming that ubiquitylation at K15 in γH2A.X reduces the molecular populations exhibiting close contacts, an overall $E_{\text{FRET}}$ value of $\sim$0.4 corresponds to a more than 50% reduction in compact states. Importantly, attachment of ubiquitin at H2A.X K15 places the ubiquitin moiety close to H2B K120. Ubiquitylation at this site has been shown to induce chromatin opening.$^{14}$ Within tetranucleosomes, the N-terminal helix of H2A and the C-terminus of H2B of two neighboring nucleosomes form a four-helix bundle, that is susceptible to disruption by ubiquitylation. This region on the nucleosomal surface is thus a hotspot for controlling chromatin structure by PTMs.

**Conclusions**

In summary, we have developed a general, genetically encoded protection strategy, by utilizing both SUMO as an N-terminal protection group and Npu$^\text{N}$ as a C-terminal crypto-thioester. This allowed us to demonstrate that S139 phosphorylation, the deprecisely positioned FRET pairs. This allowed us to demonstrate single-molecule data are given in ESI Table S2.$^\dagger$ Detailed descriptions of experimental procedures and reagents are provide in the ESI.

**Conflicts of interest**

There are no conflicts to declare.

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**Notes and references**

17. M. Simon, J. A. North, J. C. Shimko, R. A. Forties, M. B. Ferdinand, M. Manohar, M. Zhang, R. Fishel,
