

## JMJD6 Hydroxylates Histone Lysyl Residues

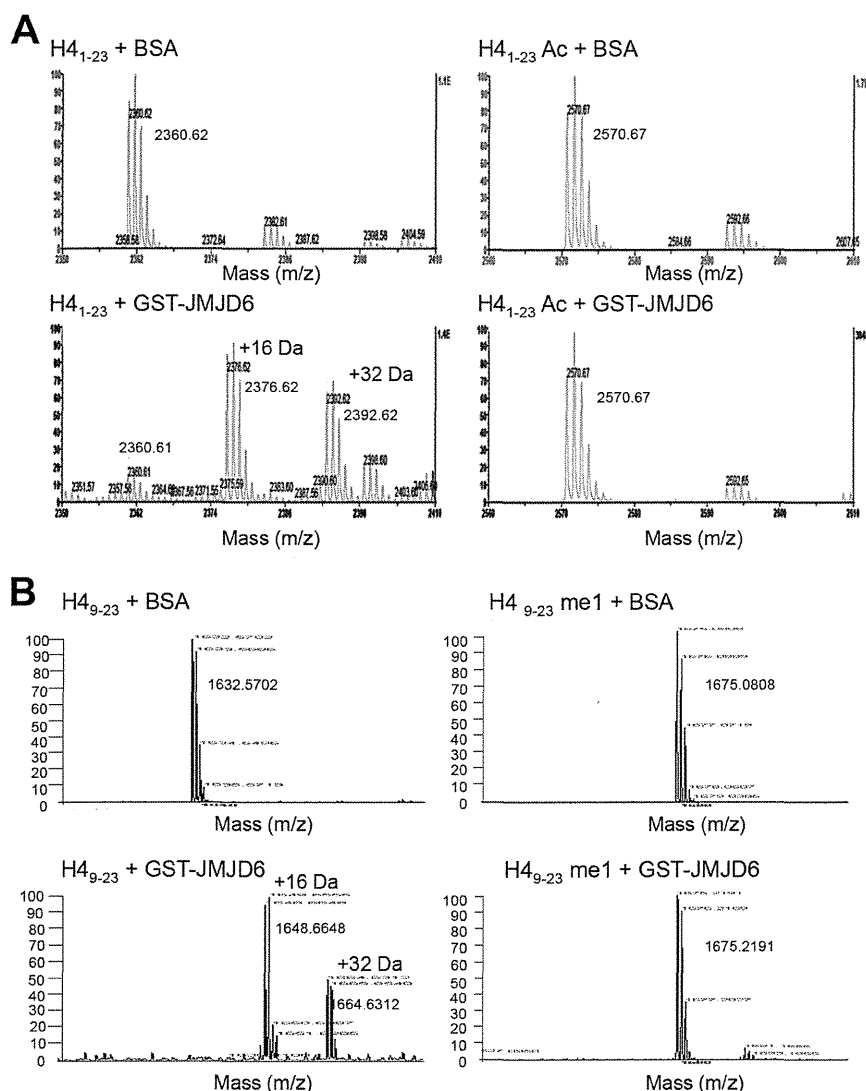


FIGURE 7. *N*-Acetylation and *N*-methylation of lysyl residues impairs 5-hydroxylation by JMJD6 *in vitro*. *A*, the *in vitro* hydroxylation assay was performed using GST-JMJD6 (10  $\mu$ M) and 85  $\mu$ M of control H4<sub>1-23</sub> peptides or *N*-acetyl-lysine-containing peptides. *B*, the *in vitro* hydroxylation assay was performed using GST-JMJD6 (10  $\mu$ M) and 85  $\mu$ M control H4<sub>1-23</sub> peptides or *N*-monomethyl-lysine-containing peptides. BSA was used as a negative control. 5-Hydroxylation by JMJD6 was detected by MS analysis.

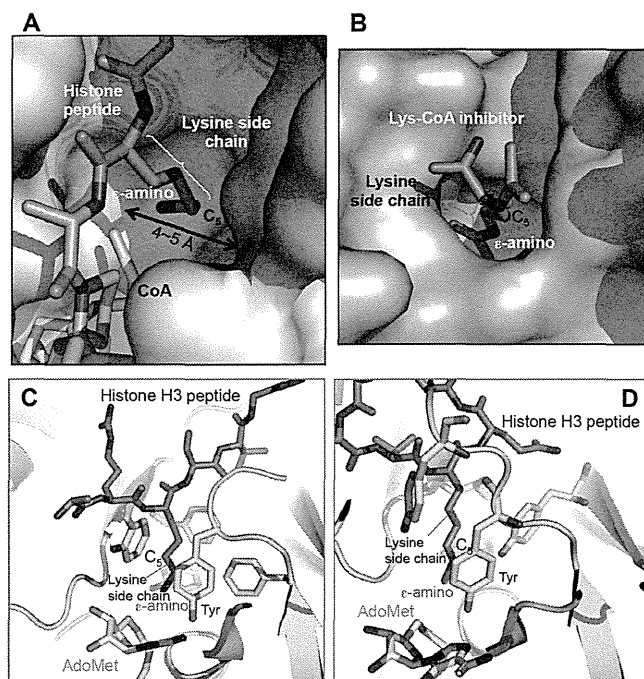
We also examined the effect of lysyl 5-hydroxylation on the histone methyltransferase activity of SMYD3, which catalyzes lysyl *N*-methylation of histone H3 (13) and also H4 (data not shown) through its SET (su(var) 3-9 enhancer-of-zeste trithorax) domain by the histone methyltransferase assay. The results showed that 5-hydroxylation at lysyl residues almost completely inhibited *N*-methylation catalyzed by SMYD3 (Table 2 and Fig. 6, *D-F*);  $V_{\max}$  values of the reactions with the control peptides (H4<sub>1-23</sub>) and the 5-hydroxylysine-containing peptides (H4<sub>1-23</sub>OH) as substrates were  $10.90 \pm 0.92$  and  $0.48 \pm 0.26$  nm/min, respectively. Similar to the HAT assay,  $K_m$  values of the two reactions were  $\sim 80.63 \pm 16.26$  and  $75.26 \pm 9.60$   $\mu$ M, respectively.

Subsequently, we performed reciprocal experiments using H4<sub>1-23</sub> or H4<sub>9-23</sub> peptides, in which all the lysines are either unmodified, *N*-acetylated, or *N*-monomethylated. JMJD6 effectively hydroxylated the control peptides (Fig. 7, *A* and *B*, *left*

*panels*); however, *N*-acetylation and *N*-monomethylation at the  $\epsilon$ -amino group of the lysines completely blocked 5-hydroxylation by JMJD6 (Fig. 7, *A* and *B*, *right panels*).

## DISCUSSION

We found a novel histone modification, 5-hydroxylation, by JMJD6. JMJD6 reportedly hydroxylates a splicing factor, U2AF65 (1). That study and another report (1, 14) stated that evidence of histone lysyl hydroxylation was not found by MS-based analysis *in vivo*. In the present study, we developed an alternative method, amino acid composition analysis, to detect 5-hydroxylation of histone lysyl residues. As reported previously, we have not detected clear evidence of 5-hydroxylation of histone lysyl residues by MS-based analysis. We think that there are several causes for this. 1) The amount of 5-hydroxylysine is too small to detect by MS-based analysis. 2) Artificial methionine oxidation during preparation of samples for MS analysis



**FIGURE 8. Structure around the active site of HAT domains and SET domains, a lysine side chain, and an S-adenosyl methionine (AdoMet).** *A*, spatial localization among the HAT domain of GCN5 (gray), a lysine side chain (magenta), and CoA (cyan) (Protein Data Bank code 1QSN). The 5-hydroxyl group may locate close to acetyl-CoA, indicating that this may work as a steric barrier and prevent effective *N*-acetylation by the catalytic domain. *B*, structure of the HAT domain of p300 (gray) in complex with inhibitor, Lys-CoA (magenta) (Protein Data Bank code 3BIY). The 5-hydroxyl group may restrict the conformation of lysine side chain in the catalytic pocket of p300. The figures were prepared by using program PyMOL. *C*, spatial localization among the SET domain of *N. crassa* Dim-5 (green), a histone H3 peptide (magenta), and AdoMet (cyan) (Protein Data Bank code 1PEG). *D*, spatial association among the SET domain of human SETD7/9 (green), a histone H3 peptide containing monomethylated Lys-4 (magenta), and AdoMet (cyan) (Protein Data Bank code 1O9S). A side chain of a lysine residue locates in tightly hydrophobic pocket of the SET domains (C and D). Hydroxylation at position C<sub>5</sub> of the chain may prevent a lysine side chain to locate in the pocket, causing inhibition of *N*-methylation by SET domains.

makes detection of 5-hydroxylysine difficult. 3) 5-Hydroxylysine could be an intermediate form as it is in collagen, and a further unknown modification(s) such as glycosylation could be added; the final product of collagen is glucosylgalactosyl hydroxylysine (4). The collagen hydroxylase, PLOD3, possesses galactosyltransferase and glucosyltransferase activities. Unlike PLOD3, JMJD6 does not appear to possess any other enzymatic activities by domain search; therefore, it is difficult to assume possible further modification(s) by its protein structure. We may have been able to detect 5-hydroxylation in histone lysyl residues by amino acid composition analysis but not by the MS-based analysis because many modifications such as glycosylation or galactosylation could be removed during the hydrolysis process of amino acid composition analysis. By this analysis, we detected both *SS/RR*- and *SR/RS*-hydroxylysine in JMJD6-treated histone peptides and also in JMJD6 wild-type E14.5 embryos, ES cells, and the Dox-inducible JMJD6 stable HEK293 cells. Because the relationship between *RR* and *RS* and also between *SS* and *RS* is a diastereomer, we were able to distinguish them. However, because a relationship between *SS* and *RR*, and also between *RS* and *SR* is an enantiomer, we were not

able to separate them by this method. Despite this, these two peaks are most likely *SS*- and *RS*-hydroxylysine because JMJD6 is reported to generate *SS*-hydroxylysine (11). The *RS*-hydroxylysine could be generated from *SS*-hydroxylysine through the lactone derivative, 3-amino-6-(aminomethyl)oxan-2-one, which is unstable and difficult to be quantified. Because of this difficulty, we only quantified *SS/RR*- and *RS/SR*-hydroxylysine in this report. Therefore, actual quantity of 5-hydroxylysine in the samples examined here could be a little higher.

Because we detected 5-hydroxylysines in the UHRF1 KO ES cells (data not shown), UHRF1 is not required for 5-hydroxylation of histone lysyl residues by JMJD6. Therefore, biological significance of the interaction between UHRF1 and JMJD6 remains unclear. Further analysis is also required to determine the biological significance of 5-hydroxylation of histone lysyl residues. *In vitro* experiments suggest that 5-hydroxylation can inhibit *N*-acetylation and *N*-methylation by p300 and SMYD3. The active site structure of the p300 and general control of amino acid synthesis 5 (GCN5) HAT domains showed that the side chain of the 5-hydroxylysine can invade the catalytic pocket; however, the 5-hydroxyl group may disturb active form formation of the substrate (Fig. 8, A and B). The catalytic site of SET domains of *Neurospora crassa* Dim-5 and human SETD7, which are structurally similar to SMYD3 (15), suggested that the side chain of 5-hydroxylysine can invade the catalytic pocket; however, the 5-hydroxyl group may disturb an active form formation of the substrate (16, 17) similar to that in HAT domains (Fig. 8, C and D). Therefore, 5-hydroxylation could be important in the context of the histone code. It is known that histones H2A and H2B move more dynamically between the nucleosome and nucleoplasm. 5-Hydroxylation of these histones may have some effects for the movement because the modification was detected more in histones H2A/H2B than in histones H3/H4. The expression pattern of JMJD6 is also interesting. JMJD6 may play important role(s) in the testis, such as a role in histone-protamine exchange. We believe that our present finding provides a novel insight into epigenetic regulations of gene transcription and/or chromosomal rearrangement.

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# Quantitative Structural Characterization of Local N-Glycan Microheterogeneity in Therapeutic Antibodies by Energy-Resolved Oxonium Ion Monitoring

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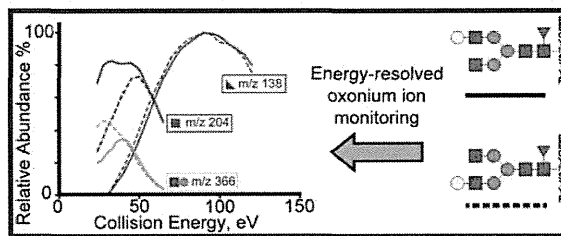
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## Supporting Information

**ABSTRACT:** Site-specific characterization of glycoform heterogeneity currently requires glycan structure assignment and glycopeptide quantification in two independent experiments. We present here a new method combining multiple reaction monitoring mass spectrometry with energy-resolved structural analysis, which we termed “energy-resolved oxonium ion monitoring”. We demonstrated that monitoring the yields of oligosaccharide-derived fragment ions (oxonium ions) over a wide range of collision induced dissociation (CID) energy applied to a glycopeptide precursor exhibits a glycan structure-unique fragmentation pattern. In the analysis of purified immunoglobulin glycopeptides, the energy-resolved oxonium ion profile was shown to clearly distinguish between isomeric glycopeptides. Moreover, limit of detection (LOD) of glycopeptide detection was 30 attomole injection, and quantitative dynamic range spanned 4 orders magnitude. Therefore, both quantification of glycopeptides and assignment of their glycan structures were achieved by a simple analysis procedure. We assessed the utility of this method for characterizing site-specific N-glycan microheterogeneity on therapeutic antibodies, including validation of lot-to-lot glycoform variability. A significant change in the degree of terminal galactosylation was observed in different production lots of trastuzumab and bevacizumab. Cetuximab Fab glycosylation, previously known to cause anaphylaxis, was also analyzed, and several causative antigens including Lewis X motifs were quantitatively detected. The data suggests that energy-resolved oxonium ion monitoring could fulfill the regulatory requirement on the routine quality control analysis of forthcoming biosimilar therapeutics.



Foreseeing the patent expiry of several therapeutic antibodies that sold more than \$30 billion worldwide in 2009,<sup>1</sup> there is growing interest over how biosimilar substitutes could win United States Food and Drug Administration (FDA) approval in the near future.<sup>2</sup> The first draft guidance on the evaluation of biosimilarity was published by the FDA in Feb 2012,<sup>3</sup> in which emphasis was placed on the importance of evaluating minor structural differences that can significantly affect the potency and safety of biopharmaceuticals, with specific reference to glycosylation patterns. This implicates the need for precise and routine structural characterization of biopharmaceuticals. With regard to therapeutic antibodies which have a common N-glycosylation consensus sequence at Asn<sub>297</sub> in the conserved (Fc) region of heavy chain, some specific features of N-glycosylation have been characterized to affect their potency and safety. For example, the absence of core fucosylation showed enhanced antibody-dependent cellular cytotoxicity by 10-fold.<sup>4</sup> Moreover, recent studies revealed that nonhuman oligosaccharide motifs such as N-glycolylneur-

aminic acid (Neu5Gc)<sup>5</sup> and galactose- $\alpha$ 1,3-galactose ( $\alpha$ -Gal epitope)<sup>6,7</sup> are immunogenic and can cause anaphylaxis in patients expressing specific IgE.<sup>8</sup> Further characterization elucidated more specifically that immunogenicity of  $\alpha$ -Gal epitope was primarily attributed to an extra N-glycan occurring within the antigen-binding (Fab) region.<sup>9</sup> These findings have raised the issue of antibody glycosylation to the level that a global picture of its heterogeneity and biological impact is urgently needed.

Therefore, we developed and describe herein a novel mass spectrometric (MS) method for analyzing the local microheterogeneity of antibody glycosylation. The method involves tryptic digestion of the antibody followed directly by MS analysis at the glycopeptide level, which enables simultaneous

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and independent characterization of multiple glycosylation sites. The common drawbacks experienced in MS analysis of glycopeptides are the lack of sensitivity of glycopeptides in a mixture with nonglycosylated peptides<sup>10</sup> and, importantly, the difficulty in verifying the glycan structures by MS/MS analysis of glycopeptides, which in most cases can only determine the oligosaccharide composition. To address these problems, we modified the methodology of multiple reaction monitoring (MRM) using a triple-quadrupole mass spectrometer into a novel concept which we termed "energy-resolved oxonium ion profiling". Oxonium ions are a series of oligosaccharide-derived fragment ions generated by collision induced dissociation (CID).<sup>11,12</sup> We iteratively scanned oxonium ions at various collision energy and plotted the ion yield against the collision energy. This "energy-resolved" approach is known to strengthen structural interpretation of analytes based on differential activation energy of fragmentation,<sup>13–15</sup> with potential application to characterization of oligosaccharide structures.<sup>16,17</sup> Hence, we examined whether the pattern of energy-resolved oxonium ion profile might be unique to the glycan structure of glycopeptides by acquiring the oxonium ion profiles of well-characterized standard glycopeptides. Next, we showed the lot-to-lot heterogeneity in trastuzumab (Herceptin) and bevacizumab (Avastin) as a worked example of routine analysis of therapeutic antibodies for quality control. Moreover, energy-resolved oxonium ion monitoring enabled simultaneous characterization of cetuximab glycoforms at both Fc and Fab glycosylation sites, demonstrating putative anaphylaxis-inducing glycan structures of nonhuman origin.

## EXPERIMENTAL SECTION

**Reagents.** Trastuzumab (Herceptin) and bevacizumab (Avastin) were purchased from Chugai Seiyaku (Osaka, Japan). Cetuximab (Erbix) was purchased from Merck Serono (Tokyo, Japan). Human immunoglobulin G (IgG), iodoacetamide, iodoacetic acid, and ammonium bicarbonate were purchased from Sigma-Aldrich (Saint Louis, MO). Dithiothreitol, urea, and PD-Miditrap desalting column was purchased from GE Healthcare (Buckinghamshire, UK). Trypsin Gold and ProteaseMAX was supplied by Promega (Madison, WI). All other unspecified reagents were products of Wako Pure Chemicals (Osaka, Japan).

**Preparation of Therapeutic Immunoglobulin.** Eighty micrograms of trastuzumab, bevacizumab, or cetuximab was dissolved in 33  $\mu$ L of 8 M urea, and dithiothreitol and ammonium bicarbonate were added to a final concentration of 5 and 50 mM, respectively. After a 30 min incubation in 55 °C, reduced cysteine residues were alkylated in 50 mM iodoacetic acid at room temperature for 30 min. The product was diluted 10 times in 2.5 mM dithiothreitol/25 mM ammonium bicarbonate before addition of 4  $\mu$ g of mass spectrometric grade endoproteinase Lys-C (Wako Pure Chemicals) for digestion at 37 °C for 2 h. The Lys-C digest was further digested with addition of 4  $\mu$ g of Trypsin Gold and allowed to react for an additional 4 h. The digest was desalted with OASIS HLB cartridge and eluted with 15% acetonitrile, and the eluate was diluted 5 times with 0.1% acetic acid for analysis by nanoHPLC-MRM.

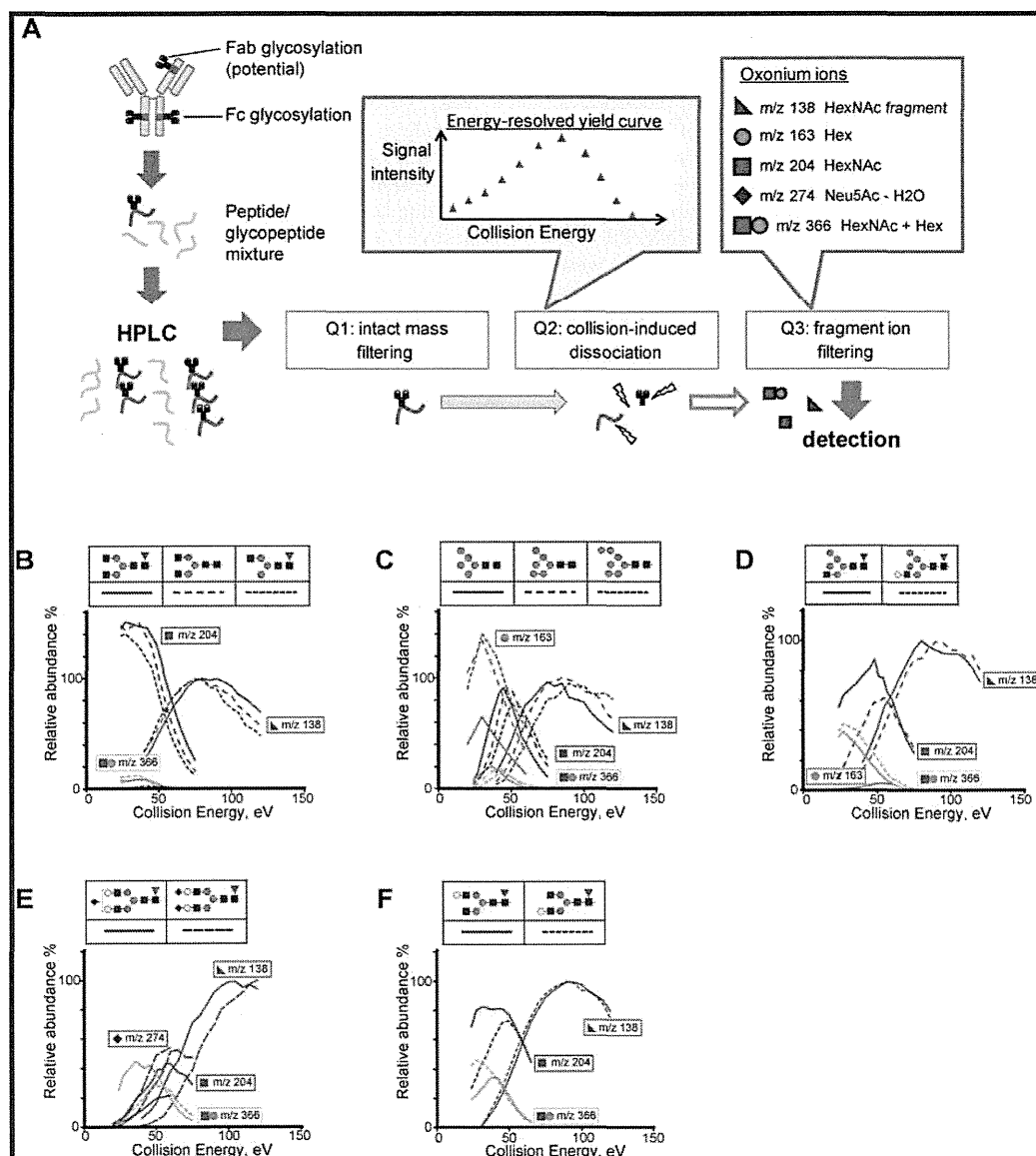
**Preparation of Glycopeptide Standards.** For the preparation of glycopeptide standards, lyophilized powder of IgG was dissolved at 1.5 mg/mL in 50 mM Tris-HCl, pH 7.5, and 8 M urea. The solution was reduced and alkylated prior to trypsin digestion by 10 mM dithiothreitol followed by 50 mM

iodoacetamide. The mixture was desalted by a PD-Miditrap desalting column to 100 mM ammonium bicarbonate. Twenty micrograms of Trypsin Gold was added to every 1 mg of IgG, and the mixture was incubated at 37 °C for 12 h. Tryptic digest was aliquoted after desalting by OASIS HLB cartridge (Waters Corporation, Milford, MA), and the aliquots were dried by SpeedVac for long-term storage. Homogeneous glycopeptides were prepared by two-dimensional HPLC fractionation using the Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan). The first dimension was performed with 4.6 mm  $\times$  500 mm Cadenza CD-C18 column (Imtakt Corporation, Kyoto, Japan). Using 0.2% trifluoroacetic acid (TFA, Tokyo Chemical Industry, Tokyo, Japan) as solvent A and 75% acetonitrile/0.1% TFA as solvent B, IgG tryptic digest was separated by a 5–20% solvent B gradient in 90 min at a flow rate of 0.6 mL/min. Collected fractions were dried, reconstituted in 0.1% TFA, and further fractionated by a 150 mm  $\times$  4.6 mm SunShell C18 column (Chromanik Corporation, Osaka, Japan). Solvent A was 6% acetonitrile with 0.1% TFA, and Solvent B was 12% acetonitrile with 0.1% TFA; separation was performed over a 10–90% solution B gradient.

**Fluorescent HPLC.** A portion of each glycopeptide fraction was treated with 0.5 U of N-glycosidase F (Roche Diagnostics, Basel, Switzerland) dissolved in 0.01% ProteaseMAX solution and incubated at 37 °C for 8 h. Released glycans were dried in a SpeedVac and derivatized with 2-aminopyridine (2-AP) by reductive amination as described elsewhere. 2-AP labeled oligosaccharides were purified by a MassPREP HILIC  $\mu$ Elution plate (Waters Corporation) according to the manufacturer's protocols but using 90% acetonitrile/100 mM triethylammonium bicarbonate for washing and 25% acetonitrile/100 mM triethylammonium bicarbonate for elution. The separation of 2-AP labeled oligosaccharides was carried out by RP-HPLC with a Shimpack CLC-ODS 0.6  $\times$  150 mm column (Shimadzu Corporation). Solvent A was 10 mM sodium phosphate buffer pH 3.8, and Solvent B was the same buffer containing 0.5% 1-butanol. After equilibrating the column with 20% solvent B, the oligosaccharides were eluted by raising the concentration of solvent B to 50% in 75 min. 2-AP labeled oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively. The retention times were normalized and expressed as glucose units using PA-glucose oligomer (Takara Bio Inc., Shiga, Japan), and glycan structures were verified according to the HPLC mapping data as described elsewhere.<sup>18</sup>

**Quantification of Glycopeptide Standards.** Glycopeptide fractions, which were successfully purified at high yield, and structural homogeneity were next subjected to quantification by UV absorbance following HPLC separation. The accuracy of quantification was guaranteed using lyophilized synthetic peptide EEQYNSTYR as the standard for calibration curve. Two species of glycopeptides were successfully quantified and were used as calibration standards for assessing the quantification by oxonium ion monitoring (Figure 2b). Standard concentration was adjusted to 100, 33.3, 11.1, 3.70, 1.23, 0.41, 0.14, 0.045, 0.015, and 0.005 fmol/ $\mu$ L with 0.1% TFA containing 50 fmol/ $\mu$ L (final concentration) of EEQYNSTYR as the internal standard and analyzed by nanoHPLC-MRM as described in the next subsection.

**Multiple Reaction Monitoring of Oxonium Ions.** The energy-resolved oxonium ion profiling and glycopeptide quantification by  $m/z$  138 oxonium ion monitoring was performed using a 4000QTRAP triple quadrupole mass



**Figure 1.** The energy-resolved oxonium ion monitoring and profiling. (a) An overview of glycoform analysis by multiple reaction monitoring (MRM) mass spectrometry. Glycoprotein sample (immunoglobulin) is digested by trypsin into peptide/glycopeptide mixture and is directly subjected to MS analysis. In the mass spectrometer, the first quadrupole (Q1) isolates the glycopeptide ions with unique intact mass, which are subsequently guided into the second quadrupole (Q2) where they undergo collision-induced dissociation. The kinetic energy of the precursor ions, which governs the energy and rate of dissociation, can be controlled by changing the collision energy applied in the Q2 cell. The third quadrupole (Q3) filters the product ions to selectively detect oligosaccharide-derived oxonium ions (inset) at high-sensitivity. (b–f) The energy-resolved oxonium ion profiles of representative glycopeptides displayed as yield curves. Similar profiles are observed for glycoforms sharing the same motifs, such as nongalactosylated type (b), high-mannose type (c), hybrid type (d), and sialylated type (e). The monogalactosylated glycopeptide isomers (f) have been purified to >90% by HPLC. Blue square, GlcNAc; green circle, mannose (Man); yellow circle, Gal; red triangle, Fuc; purple diamond, Neu5Ac. The peptide portion EEQYNSTYR has been omitted from structure display for simplicity.

spectrometer (AB Sciex, Foster City, CA) with Agilent 1200 nano-HPLC system (Agilent Technologies, Palo Alto, CA). Glycopeptide samples were separated by a 75  $\mu\text{m}$   $\times$  200 mm electrospray ionization (ESI) sprayer tip packed with 3  $\mu\text{m}$  C18 resin (Nikkkyo Technos, Tokyo, Japan). The compositions of solvents A and B were 0.1% formic acid and 70% acetonitrile/0.1% formic acid, respectively, and the flow rate was 250 nL/min. The column was equilibrated with 20% solvent B, and glycopeptides were separated isocratically for 3 min, after which

the remainder was eluted by a 5 min gradient from 20% to 95% solvent B. The fixed instrumental settings were as follows: 2200 V ionization spray voltage; 12 psi curtain gas ( $\text{N}_2$ ); CAD = 4; 70 V declustering potential; 10 V entrance potential; Q1 resolution, HIGH; Q3 resolution, LOW; and 2 ms pause in between. For energy-resolved data acquisition, analysis transitions were designed such that there were several transitions for the same Q1 and Q3 mass combination, differing only by collision energy settings (in steps of 3 or 5

eV). Dwell times were adjusted to the total cycle time of 1–1.5 s. For relative quantification of glycopeptide microheterogeneity, precursor ion  $m/z$  was derived in-silico, covering all possible glycan compositions, and a single quantitative transition was monitored for each precursor ion (Q3  $m/z$  = 138, collision energy (CE) = predicted optimum collision energy, as described in text). Only for generating the calibration curves for determining the sensitivity and dynamic range of detection (Figure 2b), synthetic peptide EEQYNSTYR was coinjected, and the following internal standard transitions were monitored for normalization: Q1  $m/z$  = 595.4, Q3  $m/z$  = 640.4, CE = 38; Q1  $m/z$  = 595.4, Q3  $m/z$  = 526.4, CE = 30.

**Data Analysis.** The acquired raw data was processed with MultiQuant version 2.02 (AB Sciex, Foster City, CA). The mass chromatogram of each transition was integrated after Gaussian Smoothing ( $\sigma = 1$  point) and quantified by peak area. For energy-resolved oxonium ion profiling, the data set for each glycopeptide was normalized with respect to the highest intensity of Q3  $m/z$  = 138 and expressed as percentage. Analyses were performed in triplicates, and average normalized percentage was computed. Optimum collision energy was defined as the voltage setting to achieve the maximum percentage, unless a series of 98–100% was observed as a plateau, in which case the average collision energy was regarded as optimum. For relative quantification of glycopeptide microheterogeneity, the percentage composition of each component was calculated with respect to the sum of all components combined. The data for plotting the calibration curves (Figure 2b) was generated by normalizing the glycopeptide peak area by the average of two internal standard transitions.

## RESULTS AND DISCUSSION

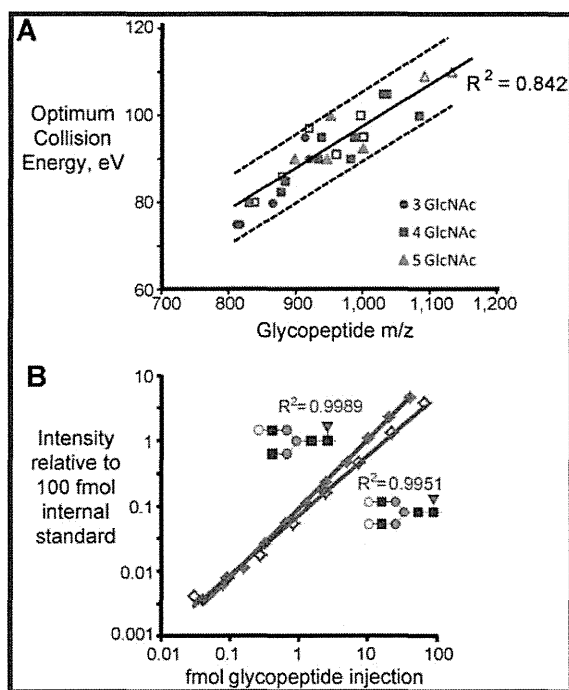
**Energy-Resolved Oxonium Ion Profile.** Figure 1a shows the scheme to acquire an energy-resolved yield curve of oxonium ions using a triple quadrupole mass spectrometer. Briefly, the collision energy applied in the Q2 cell was changed in stepwise fashion in repetitive measurements of short time intervals for each mass filter settings. We termed simultaneous acquisition of oxonium ion yield curves as energy-resolved oxonium ion monitoring technology and examined whether this profile could be utilized for determination of glycan structures on a particular glycopeptide. We first prepared a series of glycopeptides from human IgG by chromatographic separation. The isolated glycopeptides shared the same amino acid sequence EEQYNSTYR but bore different glycan structures. For each glycopeptide, energy-resolved yield curves of 5 selected oxonium ions were simultaneously acquired and displayed on the same plot, namely,  $m/z$  138 (internal fragment of *N*-acetylglucosamine, GlcNAc),  $m/z$  204 (GlcNAc),  $m/z$  366 [Hexose (Hex) + GlcNAc],  $m/z$  163 (Hexose), and  $m/z$  274 (*N*-acetylneuraminic acid, Neu5Ac – H<sub>2</sub>O). The results as displayed in Figure 1b–f convey an interesting feature of the oxonium ion profiles, that they represent the glycan structure as a whole rather than simple summation of possible bond breakage events. The non-galactosylated glycoforms (Figure 1b) are characterized by an explicitly higher yield of  $m/z$  204 (GlcNAc) than other glycoforms bearing the same number of GlcNAc. Moreover, the high yield of oxonium ion  $m/z$  163, which is unique to the high-mannose type (Figure 1c), cannot be explained by the number of mannosyl linkages alone because hybrid type (Figure 1d) structures hardly produce  $m/z$  163. A possible

explanation of this is that mannose or galactose, which is in proximity to GlcNAc, lacks the labile proton needed for oxonium ion formation.

Most striking was the different profile acquired from independent analysis of isomeric glycopeptides with glycan composition 4[Hex]4[GlcNAc]1[Fuc] (where Fuc is fucose; structure validation by fluorescent HPLC supplied in Figure S1, Supporting Information), differing by terminal galactose linked either to  $\alpha$ 1-3 branch or  $\alpha$ 1-6 branch (Figure 1f). These glycans share the same types of glycosidic linkages but exhibited distinct energy-resolved patterns, suggesting that galactose present on  $\alpha$ 1-6 branch contributed to higher yield of  $m/z$  366 oxonium ion at low collision energy than when present on  $\alpha$ 1-3 branch. Previous examples of energy-resolved mass spectrometry suggested that the maximum of yield curve indicates the ease of CID event to generate the product ion.<sup>15</sup> Extending this view, the different maxima observed in  $m/z$  366 curves (Figure 1f) are indicative of one bond being more stable than the other, despite that the bonds responsible for  $m/z$  366 production, GlcNAc  $\beta$ (1-2)Man, are locally identical in both structures.

From these results, we conclude that bond stability in glycans is governed by the overall spatial arrangement, and the energy-resolved oxonium ion profile reflects the shape of the glycan rather than its composition. The notion of precise glycoform identification by relative quantification of fragment ions is not a novel one,<sup>19</sup> but its application has been limited to detached N-glycans. Here, we showed that energy-resolved oxonium ion monitoring can identify glycoforms attached to peptides by focusing on low molecular weight oxonium ions, which occur at the same  $m/z$  for any glycopeptides, and differentiating between structures by collision energy-resolved profiles. Multiple reaction monitoring by triple-quadrupole mass spectrometer complies with this purpose because it does not exert a low-mass cutoff effect which otherwise alters the signal yield, depending on the size of intact glycopeptides. Therefore, we expect to observe the same energy-resolved oxonium ion profiles for the same glycoform regardless of the peptide amino acid sequence. Furthermore, this energy-resolved approach can potentially be used to estimate the percentage composition of isomeric glycopeptides without requiring chromatographic separation. The energy-resolved yield curves of 1:1 and 1:2 mixtures of the two isomers showed curve shapes that fell between the two reference curves (Figure S2, Supporting Information).

**High-Sensitivity Quantification of Glycopeptides.** A notable observation from various energy-resolved profiles was that the yield curve shapes of oxonium ion  $m/z$  138 were similar among the tested glycan structures. We speculated that  $m/z$  138 originates from the GlcNAc of the core structure which was conserved among all N-glycans and, therefore, oxonium ion  $m/z$  138 under optimum collision energy would serve as an ideal reporter ion for quantifying glycopeptides of various glycan structures. To clarify this, we plotted the collision energy at which  $m/z$  138 was maximal against the  $m/z$  of various glycopeptides (Figure 2a). The result suggested a strong linear correlation ( $R^2 = 0.842$ ), which was independent of the number of GlcNAc in the glycan composition or the peptide amino acid sequence. From these results, it seemed adequate to assume that the collision energy to yield maximum  $m/z$  138 signal can be predicted from the  $m/z$  of glycopeptide and that  $m/z$  138 signal acquired in this way would best represent the quantity of the glycopeptide. Furthermore, since the relative intensity of  $m/z$  138 at its maximum was generally

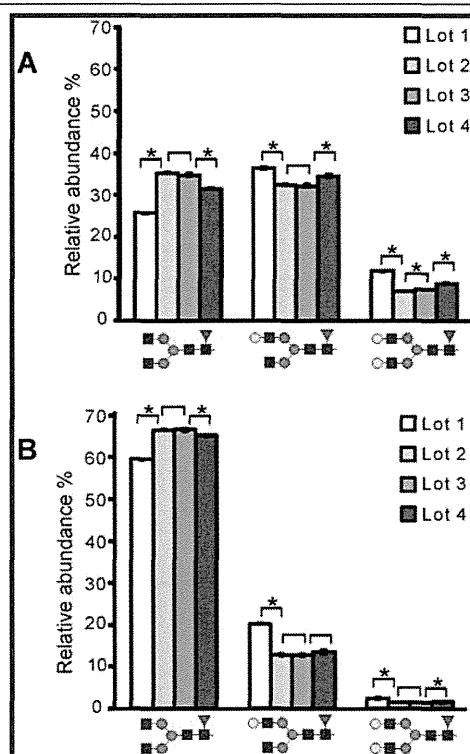


**Figure 2.** Monitoring oxonium ion  $m/z$  138 for high-sensitivity quantification of glycopeptides. (a) The energy-resolved oxonium ion profiles were acquired for 28 glycopeptides, and the collision energy that yielded highest signal intensity of  $m/z$  138 was plotted against the  $m/z$  of precursor glycopeptide. Blue circle, glycopeptide containing 3 GlcNAc; red square, containing 4 GlcNAc; green triangle, containing 5 GlcNAc; filled points, peptide sequence EEQYNSTYR; open points, peptide sequence MNSLQSNDAIYYCAR. Correlation coefficient  $R^2$  is 0.842, and all the points fall within 95% confidence interval (broken lines). (b) Two purified homogeneous glycopeptides, 4[Hex]4-[HexNAc]1[Fuc] and 5[Hex]4[HexNAc]1[Fuc] (where HexNAc is *N*-acetylhexosamine), were serially diluted and subjected to sensitivity determination.  $m/z$  138 was monitored at the optimum collision energy. Signal was normalized to 100 fmol of internal standard EEQYNSTYR (synthetic peptide with no glycosylation). The quantitative range of detection was determined to be 30 amol to 30 fmol injection with  $R^2 = 0.9989$  and  $0.9951$ .

higher than other oxonium or peptide-derived fragment ions, it was expected that monitoring  $m/z$  138 would enhance the sensitivity of glycopeptide detection. This was demonstrated by the standard curve of two glycopeptide standards, each composed of different and homogeneous glycan structure and concentration determined by UV absorbance (Figure 2b). The linear quantitative range of the glycopeptides were 30 amol to 30 fmol injection, spanning 4 orders of magnitude. Surprisingly, the sensitivity of glycopeptide detection was about 10 times more than the synthetic nonglycopeptide EEQYNSTYR which was coinjected as an internal standard. This result is contrary to the common notion that glycopeptides have relatively low ionization efficiency<sup>20</sup> and implicates the overwhelming capability of our technology in detecting glycopeptides at high sensitivity and quantitatively in mixture with nonglycopeptides. Moreover, the yield of  $m/z$  138 is maximized at much higher collision energy than conventionally used for MRM.<sup>21</sup> Under this optimum condition, production of other oxonium ions or peptide backbone fragmentation is suppressed,

which effectively reduces the background signal and further contributes to selective detection of glycopeptides.

**Lot-to-Lot Heterogeneity of Trastuzumab and Bevacizumab.** We next evaluated the applicability of the oxonium ion monitoring technology, taking advantage of the highly quantitative detection of glycopeptides, to routine lot-to-lot quality control of therapeutic antibodies by analyzing four lots of trastuzumab (Herceptin) and bevacizumab (Avastin). The glycoform heterogeneity was quantified, and the relative abundances of three most abundant glycoforms were presented in Figure 3 to illustrate the difference among the production



**Figure 3.** Evaluation of lot-to-lot heterogeneity of trastuzumab and bevacizumab. The glycoform heterogeneity of four lots of each product was quantified by  $m/z$  138 oxonium ion monitoring, and the relative abundances of agalactosylated, monogalactosylated, and digalactosylated structures are displayed for (a) trastuzumab and (b) bevacizumab. The lots are numbered in chronological order of production, and the time-course variation was evaluated by comparing two consecutive lots. Error bars are standard deviation of triplicate runs. Asterisks represent where the difference between consecutive lots was statistically significant ( $p < 0.05$  by Student's *t* test,  $n = 3$ ).

lots. The quantification results of other glycoforms were given in Tables S1 and S2, Supporting Information. Analysis was performed in three independent preparations for each lot, and statistical significance of the difference between two consecutive lots was evaluated by Student's *t* test. The mean coefficient of variation of triplicate analysis was 4.7% for all measurements with more than 0.1% relative abundance, and the quantitative linearity over 4 orders of magnitude enabled the detection of minor glycoforms of as low as 0.05% relative abundance. As a result, the frequency of galactosylation onto the biantennary GlcNAc was shown to be prone to significant lot-to-lot variation in both trastuzumab and bevacizumab (Figure 3a,b,



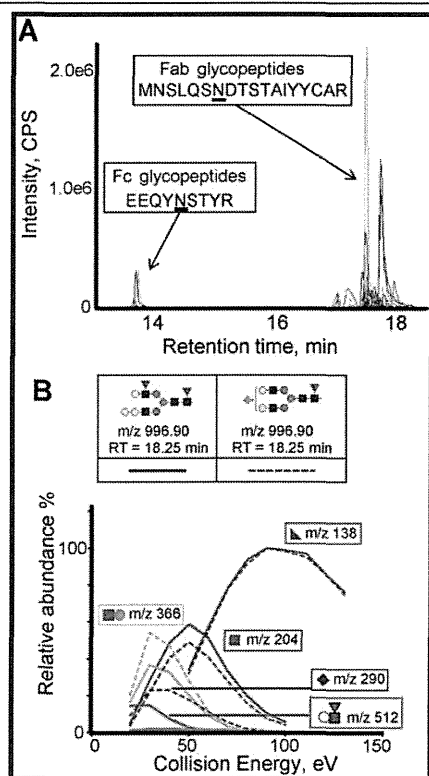
respectively,  $P < 0.05$  by Student's  $t$  test). Convincingly, the observed variations in glycoform composition were interrelated in the way that the decrease in galactosylated form was compensated by the increase in agalactosylated form. The smallest statistically significant variation observed here was 1.2-fold between Lot 3 and Lot 4 of bevacizumab in the abundance of digalactosylated form (1.29% and 1.51%, respectively,  $P < 0.05$  by Student's  $t$  test). Surprisingly, we observed that bevacizumab contained a fraction of nonglycosylated form (EEQYNSTYR detected without glycosylation) and that this fraction also varied among lots. These results outline the continual lot-to-lot variation of glycoform heterogeneity in both trastuzumab and bevacizumab and demonstrate that  $m/z$  138 oxonium ion monitoring can accurately evaluate whether the glycoforms of therapeutic antibodies between lots are identical.

**Analysis of Immunogenic Glycoforms on Fc and Fab of Cetuximab.** We next applied energy-resolved oxonium ion monitoring for site-specific glycoform characterization of therapeutic antibody cetuximab for identification of potentially immunogenic glycoforms. Figure 4a shows the MRM chromatogram of cetuximab digest, monitoring  $m/z$  138 for

the quantification of all possible glycoforms. A series of glycopeptides derived from Fc region (EEQYN<sub>297</sub>STYR) or Fab region (MNSLQSN<sub>88</sub>DTAIYYCAR) were eluted at 13–14 min or 17–19 min, respectively. Glycoforms on both sites were thus characterized simultaneously in a single analysis. The most major glycoform in Fc glycan was agalactosylated biantennary structure, and relatively high level of hybrid and high-mannose structures were also detected (results summarized in Figure S3, Supporting Information). Focusing on the characterization of its Fab glycan, which has been reported as the primary cause of anaphylaxis by cetuximab,<sup>9</sup> we quantified all theoretically available combinations of glycoforms with potentially immunogenic glycan motifs and verified their structures by energy-resolved oxonium ion profiling. As a result, we discovered that a Lewis X antigen was also present on cetuximab along with previously reported  $\alpha$ -Gal and Neu5Gc antigens (Figure 4b). The Lewis X motif was verified by the energy-resolved yield curves including the structure-specific oxonium ion of  $m/z$  512 (Gal-GlcNAc-Fuc), which clearly discriminated the two almost isomeric glycopeptides with Lewis X or Neu5Gc motifs. The result of quantification was summarized in Table 1, showing the panel of biantennary and triantennary glycan structures with Lewis X,  $\alpha$ -Gal, and/or Neu5Gc modification. Since the total number of modification never exceeded the number of antennae, it is most likely that addition of fucose to antennal GlcNAc (Lewis X), addition of galactose to terminal galactose ( $\alpha$ -Gal), and addition of Neu5Gc to terminal galactose (Neu5Gc) occur in a competitive fashion, resulting in the occurrence of all possible combinations of the antigenic motifs. Considering the fact that 1–2% of patients administered with cetuximab experience severe hypersensitivity reactions<sup>22</sup> and potentially at much higher rate,<sup>23</sup> it is important to fully elucidate the cause of this hypersensitivity and strictly regulate it to minimize the risk. The implications of the variety of immunogenic glycoforms which has previously been overlooked would require revisiting.

## CONCLUSIONS

We developed the new technology that combined multiple reaction monitoring mass spectrometry and energy-resolved structural analysis. It focuses on how the yields of commonly observed fragment ion signals change under various fragmentation conditions and uses this information as a new cue to replace the conventional notion of finding structure-specific signature ions for glycoform identification.<sup>24,25</sup> The advantage of this technology over other methods is 2-fold. First, the collision energy-resolved oxonium ion profiles uniquely represent glycan structures on tryptic glycopeptides. It is therefore suited for site-specific analyses for characterizing glycoproteins with multiple glycosylation sites. Moreover, the depth of structural information covers differentiation of isomeric structures, which currently requires glycan detachment. By accumulating more reference data of glycopeptides with validated glycan structures, it should be possible in the near future to program a matching system to assign unknown data with the glycan structure. Second, detection of glycopeptides by monitoring oxonium ion  $m/z$  138 at optimum collision energy showed superior sensitivity and quantitative range to all currently available methods. This feature enables analysis using minute sample quantity or detection of very minor glycoforms to unravel glycoform microheterogeneity, as demonstrated herein by the analysis of therapeutic antibodies. The energy-resolved oxonium ion monitoring technology thus



**Figure 4.** Characterization of glycoform heterogeneity of cetuximab. (a) Overlaid MRM chromatogram showing the quantification of glycoform heterogeneity in cetuximab digest by monitoring product ion  $m/z$  138 for 40 different glycan structures. Fc glycopeptides elute at 14 min and Fab glycopeptides elute at 18 min, with minor variation in retention time by glycoforms. (b) The energy-resolved oxonium ion profiles of glycopeptides with  $m/z$  996.90 and  $m/z$  996.65 were acquired for verification of their structures, represented by solid lines and broken lines, respectively. The deduced structures are shown in the inset. Blue square, GlcNAc; green circle, Man; yellow circle, Gal; red triangle, Fuc; blue diamond, Neu5Gc. The peptide portion MNSLQSN<sub>88</sub>DTAIYYCAR is omitted for simplicity.

Table 1. Combinations of Potentially Immunogenic Glycan Motifs Found on Cetuximab<sup>a</sup>

	composition			% abundance	
	$\alpha$ -Gal	Lewis X	Neu5Gc	biantennary	triantennary
antenna occupancy = 1	1			6.4	1.1
		1		0.7	0.2
			1	7.1	0.9
antenna occupancy = 2	2			25.9	1.7
	1	1		2.5	1.0
	1		1	18.5	1.3
		2		0.2	0.1
		1	1	1.0	0.2
antenna occupancy = 3			2	3.3	0.3
	3				2.5
	2	1			1.0
	2		1		1.8
	1	2			0.2
	1	1	1		0.5
	1		2		0.6
			3		0.0
		2	1		0.1
		1	2		0.2
		3		N.D.	

<sup>a</sup>Composition indicates the number of  $\alpha$ -Gal, Lewis X, and Neu5Gc motifs in the glycan structure. N.D.: no signal detected.

shows novelty in both qualitative and quantitative aspects of glycoprotein analysis. It can potentially alleviate a burden on multiple, orthogonal analyses for site-specific characterization of glycan microheterogeneity. Primarily, it is applicable to evaluation of biosimilar pharmaceuticals at a routine basis; however, it can potentially be applied to any glycoproteins, with or without prior enrichment of the target.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Supplementary Figures S1–S3 and Supplementary Tables S1 and S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### 📄 Notes

The authors declare the following competing financial interest(s): A.T. and T.A.S. are employees of Shimadzu Corporation, see grant information below.

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ARTICLE

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# Regulation of histone modification and chromatin structure by the p53–PADI4 pathway

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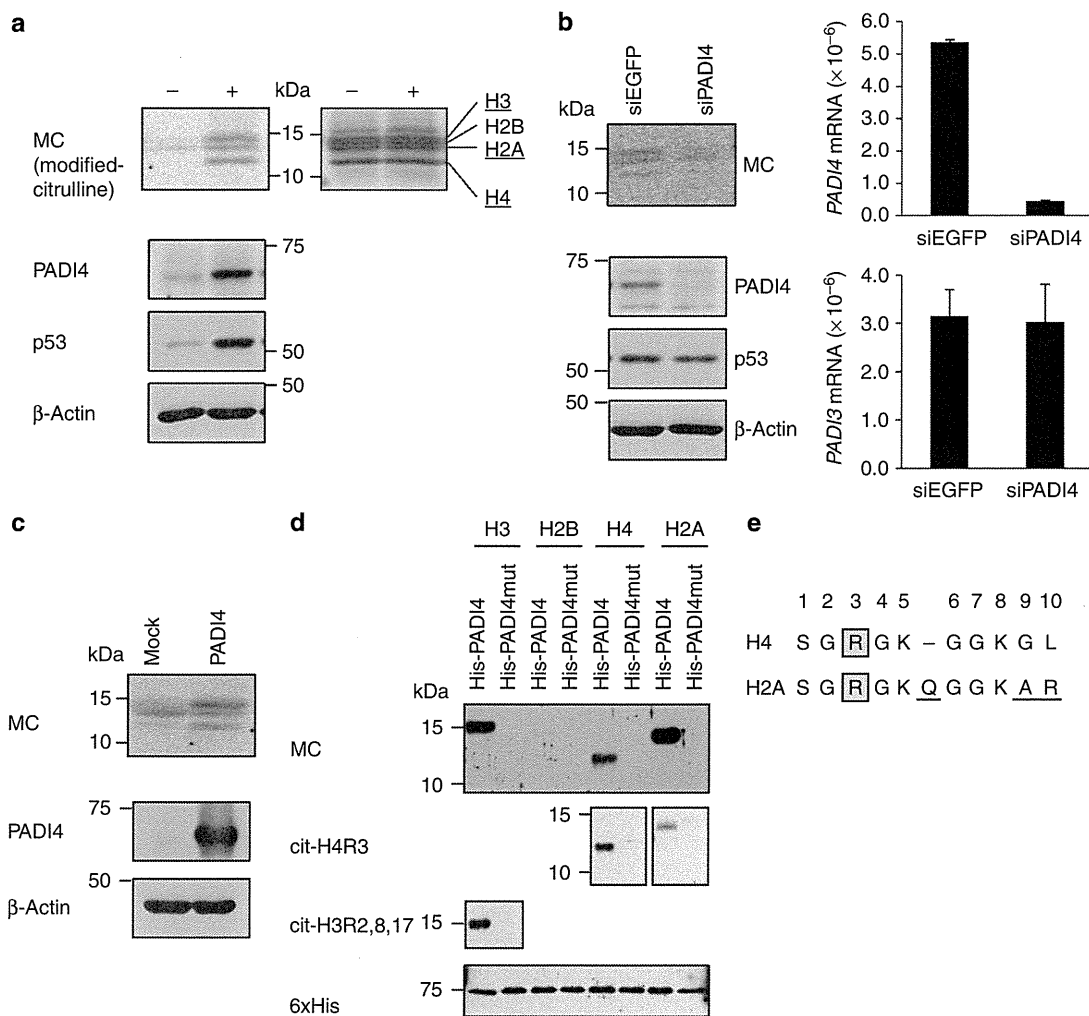
Histone proteins are modified in response to various external signals; however, their mechanisms are still not fully understood. Citrullination is a post-transcriptional modification that converts arginine in proteins into citrulline. Here we show *in vivo* and *in vitro* citrullination of the arginine 3 residue of histone H4 (cit-H4R3) in response to DNA damage through the p53–PADI4 pathway. We also show DNA damage-induced citrullination of Lamin C. Cit-H4R3 and citrullinated Lamin C localize around fragmented nuclei in apoptotic cells. Ectopic expression of PADI4 leads to chromatin decondensation and promotes DNA cleavage, whereas *Padi4*<sup>-/-</sup> mice exhibit resistance to radiation-induced apoptosis in the thymus. Furthermore, the level of cit-H4R3 is negatively correlated with p53 protein expression and with tumour size in non-small cell lung cancer tissues. Our findings reveal that cit-H4R3 may be an ‘apoptotic histone code’ to detect damaged cells and induce nuclear fragmentation, which has a crucial role in carcinogenesis.

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In response to DNA damage, various protein kinases phosphorylate p53 at its amino terminus leading to the stabilization and accumulation of p53 protein<sup>1,2</sup>. As a guardian of the genome, p53 transactivates a number of its target genes to induce cell-cycle arrest or apoptosis and subsequently eliminates cells with risk of malignant transformation<sup>3</sup>. Morphological changes such as chromatin condensation, DNA laddering, nuclear lamina breakdown and nuclear fragmentation are characteristic of apoptotic cell death, although the role of p53 during these dynamic nuclear events has not been elucidated.

We have isolated a number of p53-target genes including p53AIP1, p53R2, p53RDL1 and XEDAR<sup>4-9</sup>, and implicated the molecular mechanisms by which p53 regulated the cell fate, death or survival, by regulating the expression levels of these genes. We recently reported a p53-regulated posttranscriptional modification

of many proteins through the transcriptional regulation of PADI4 (ref. 10) that converts an arginine residue in proteins to a citrulline residue, known as citrullination or deimination<sup>11</sup>. This reaction is mediated by the Ca<sup>2+</sup>-dependent enzyme peptidylarginine deiminase. Citrullination of proteins causes a loss of positive charge and results in a significant biochemical effect. Bacterial infection has been indicated to stimulate PADI4-mediated hypercitrullination of histones in neutrophils and subsequently promoted highly decondensed extracellular chromatin structure called NET (neutrophil extracellular trap) that could capture and kill microorganisms<sup>12,13</sup>. On the other hand, citrullinated peptides/proteins are recognized as non-self proteins and subsequently activate immune systems. Citrullinated myelin basic protein in the brain is considered as a putative autoantigen associated with multiple sclerosis<sup>14</sup>. In addition, autoantibodies against citrullinated peptides derived



**Figure 1 | Citrullination of core histones in the damaged cells.** (a) Histones extracted from U-2 OS cells 48 h after ADR treatment (lane 2) were immunoblotted with anti-modified citrulline (anti-MC) antibody (left panel). U-2 OS cells without ADR treatment were used as a negative control (lane 1). CBB stain is shown in the right panel to ensure equal loading. Whole-cell extracts were subjected to immunoblotting with anti-PADI4, anti-p53 or anti- $\beta$ -actin antibody. (b) siRNA against PADI4 was transfected 10 h before DNA damage treatment. siEGFP was used as a control. Purified histones were immunoblotted with anti-MC antibody (upper left panel). Whole-cell extracts were subjected to immunoblotting with anti-PADI4, anti-p53 or anti- $\beta$ -actin antibody (lower left panel). Quantitative PCR analysis of *PADI4* or *PADI3* expression (right panel). Error bars represent range ( $n=2$ ). *GAPDH* was used for normalization of expression levels. (c) Histones were extracted from PADI4-introduced or mock-transfected HEK293T cells, and immunoblotted with anti-MC antibody. Whole-cell extracts were subjected to immunoblotting with anti-PADI4 or anti- $\beta$ -actin antibody. (d) *In vitro* citrullination followed by western blotting with anti-MC, anti-citrullinated histone H4R3 (cit-H4R3), anti-citrullinated histone H3R2,8,17 (cit-H3R2,8,17), or anti-His-tag (6 $\times$ His) antibody. Each recombinant histone was incubated with wild-type or mutant PADI4 protein in the presence of  $10^{-3}$  M of calcium at 37 °C for 1 h. (e) Alignment of amino-acid sequences of the N-terminal regions of histone H2A and H4.

from flaggrin or fibrin have been established as specific markers for rheumatoid arthritis<sup>15–17</sup>. Furthermore, we have revealed that a functional haplotype of the *PADI4* gene is associated with rheumatoid arthritis<sup>18</sup>. Thus, activated PADI4 function has been shown to be associated with the pathogenesis of rheumatoid arthritis through induction of the antigenicity of proteins. Here we investigate the physiological role of p53–PADI4 signalling in the DNA damage response by using PADI4 deficient mice as well as clinical cancer tissues. In this study, we revealed the crucial role of PADI4 in the apoptotic pathway and human carcinogenesis.

## Results

### Citrullination of core histones in response to DNA damage.

As histones H3 and H4 were shown to be citrullinated by PADI4 (refs 19,20), we assessed whether core histones are citrullinated in response to DNA damage. Acid-extracted histones from adriamycin (ADR)-treated U-2 OS cells were immunoblotted with an antibody against a chemically modified form of citrulline (anti-MC antibody). Western blot analysis revealed the citrullination of three of four core histones in a PADI4-dependent manner (Fig. 1a,b). Ectopic expression of PADI4 also induced the citrullination of at least two core histones (Fig. 1c). To further characterize this modification, purified histones were treated with recombinant PADI4. Antibodies against specific citrulline residues (cit-H3R2,8,17 or cit-H4R3) as well as anti-MC antibody could detect PADI4-treated histones H3 and H4, respectively (Fig. 1d). Interestingly, anti-cit-H4R3 antibody cross-reacted with citrullinated H2A, probably owing to the high similarity between histones H4 and H2A (Fig. 1d,e).

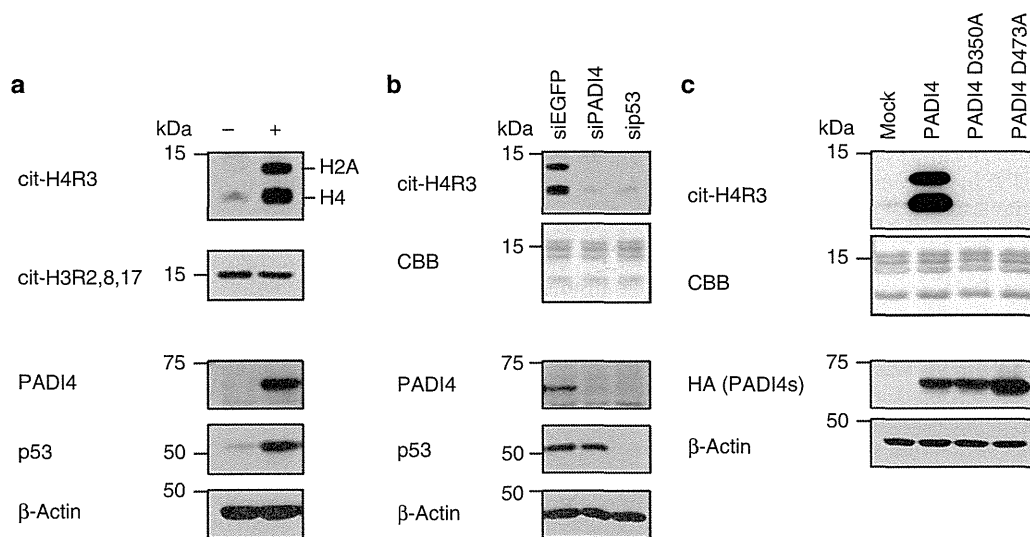
**p53–PADI4 citrullinates histone H4R3.** Western blotting of core histone proteins purified from ADR-treated U-2 OS cells indicated citrullination of H4R3 by DNA damage (Fig. 2a), but the citrullination was blocked by small interfering RNA (siRNA) against p53 or PADI4 (Fig. 2b). Concordantly, ectopic introduction of PADI4 into cells induced the citrullination of H4R3 (Fig. 2c). Although PADI4 was reported to function as demethylase of mono-methyl-H4R3

*in vitro*<sup>19,20</sup>, PADI4 activation did not affect H4R3 methylation status in our experimental system (Supplementary Fig. S1).

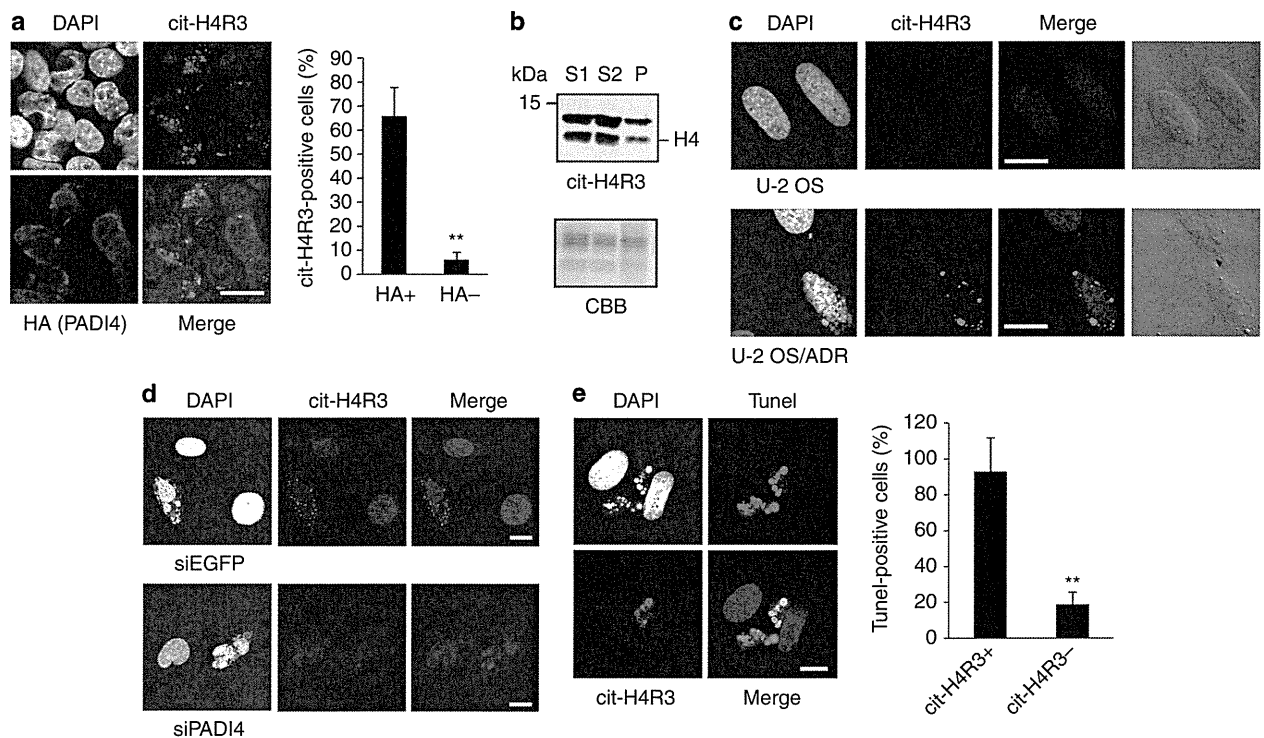
We also found the citrullination of histone H3 at its N terminus in non-treated U-2 OS cells and HEK293T cells (Supplementary Fig. S1), but these modifications were hardly detectable with anti-MC antibody, probably because of the low sensitivity of this antibody to the citrullination at its N terminus of histone H3 (Fig. 1a,c). Although citrullination of H3 was slightly increased by ectopic expression of PADI4 or by ADR treatment, siPADI4 did not affect H3-citrullination level (Supplementary Fig. S1). Because citrullination of H3 was observed in HEK293T and U-2 OS cells without any stresses, we suspect that other PADI family members might also be involved in H3 citrullination in these cells.

### Localization of citrullinated histone H4 at fragmented nuclei.

To investigate the physiological role of PADI4-mediated histone citrullination, we examined chromatin structure and subcellular localization of cit-H4R3 in PADI4-introduced HEK293T cells. Immunocytochemical analysis revealed the weak and irregular staining of nuclei with 4,6-diamidino-2-phenylindole (DAPI), a characteristic feature of chromatin decondensation (Fig. 3a). In contrast, cit-H4R3 displayed a dot-like staining pattern around the decondensed nuclei in cells expressing HA-PADI4 (Fig. 3a). Fractionation of chromatin obtained from PADI4-introduced HEK293T cells<sup>21</sup> suggested that cit-H4R3 was enriched in S1 (soluble in divalent metal; transcriptional active) and S2 (soluble in EDTA; transcriptional inactive) fractions, compared with P fraction (insoluble compact chromatin) (Fig. 3b). As apoptosis is associated with dynamic changes of nuclear structure, we examined the localization of cit-H4R3 in apoptotic cells. At 48 h after treatment with ADR, cit-H4R3 was localized around the fragmented nuclei in U-2 OS cells, but it was not detected in the cells, additionally treated with siRNA against PADI4 (Fig. 3c,d). Similar results were observed in ADR-treated MCF7 cells (Supplementary Fig. S2a,b). In addition, cit-H4R3 expression was significantly correlated with the level of apoptosis induction demonstrated by the TdT-mediated dUTP nick



**Figure 2 | Citrullination of histone H4R3 in the damaged cells.** (a) Western blotting for histones extracted from ADR-treated U-2 OS cells (lane 2) using antibodies against citrullinated histone H4R3 (cit-H4R3) or citrullinated histone H3R2,8,17 (cit-H3R2,8,17). Whole-cell extracts were subjected to immunoblotting with anti-PADI4, anti-p53 or anti- $\beta$ -actin antibody. U-2 OS cells without ADR treatment were used as a control (lane 1). (b) siRNA against PADI4 or p53 was transfected 10 h before ADR treatment. siEGFP was used as a control. Extracted histones were immunoblotted with anti-cit-H4R3 antibody. Whole-cell extracts were subjected to immunoblotting with anti-PADI4, anti-p53 or anti- $\beta$ -actin antibody. CBB staining was shown to ensure equal loading. (c) Histones were extracted from HEK293T cells transfected with plasmid-expressing HA tagged-wild-type PADI4, mutant PADI4 (D350A or D473A), or mock and immunoblotted with anti-cit-H4R3 antibody. Whole-cell extracts were subjected to immunoblotting with anti-HA or anti- $\beta$ -actin antibody.

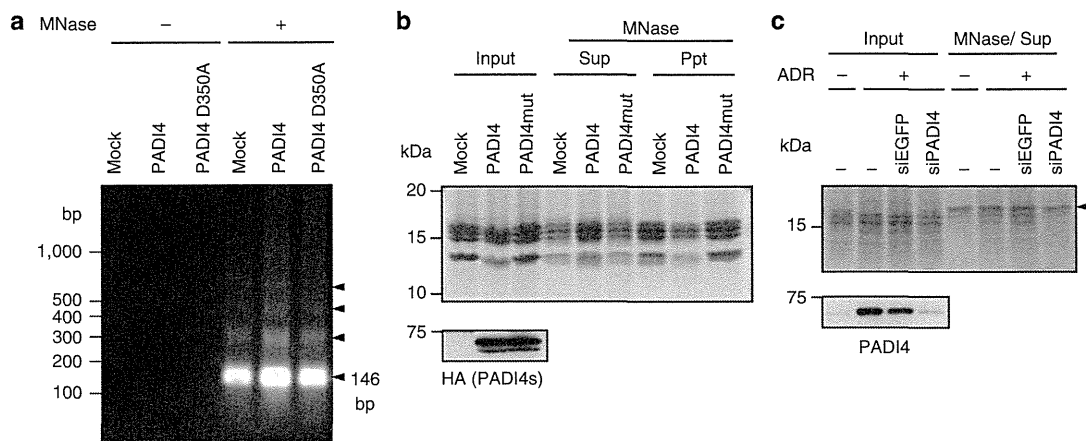


**Figure 3 | Localization of cit-H4R3 around the fragmented nuclei during apoptosis.** (a) Representative image of HA-PAD14-transfected HEK293T cells stained with anti-citrullinated histone H4R3 (cit-H4R3) antibody (Alexa Fluor 488) and anti-HA antibody (Alexa Fluor 594) (left panel). The proportion of cit-H4R3-positive cells stratified by HA-PAD14 expression was indicated (right panel). Error bars represent s.d. ( $n = 4$ ).  $**P < 0.01$  by Student's *t*-test. Scale bars, 20  $\mu\text{m}$ . (b) Chromatin fractionation followed by western blotting. Isolated nuclei from HEK293T cells transfected with PAD14-expressing plasmids were digested with MNase. Digested nuclei were fractionated into three fractions: transcriptionally active (S1), transcriptionally inactive (S2) and insoluble compact chromatin (P). Aliquots from S1, S2 and P fractions were separated by SDS-PAGE and then analysed by western blotting using anti-cit-H4R3 antibody. (c) Representative image of ADR-treated U-2 OS cells stained with anti-cit-H4R3 antibody (Alexa Fluor 488). Cells were treated with  $2 \mu\text{g ml}^{-1}$  of ADR for 2 h and fixed 48 h later. Scale bars, 20  $\mu\text{m}$ . (d) Representative image of ADR-treated U-2 OS cells stained with anti-citrullinated histone H4R3 antibody (Alexa Fluor 488). Each cell line was treated with  $2 \mu\text{g ml}^{-1}$  of ADR for 2 h and fixed 48 h later. Each siRNA was transfected 10 h before the ADR treatment. siEGFP was used as a control. Scale bars, 20  $\mu\text{m}$ . (e) Co-staining of U-2 OS cells with TUNEL (FITC) and anti-cit-H4R3 antibody (Alexa Fluor 594) at 48 h after treatment with  $2 \mu\text{g ml}^{-1}$  of ADR for 2 h (left panel). The proportion of TUNEL positive cells stratified by cit-H4R3 expression was indicated (right panel). Error bars represent s.d. ( $n = 7$ ).  $**P < 0.01$  by Student's *t*-test. Scale bars, 20  $\mu\text{m}$ .

end labeling (TUNEL) assay in ADR-treated U-2 OS cells (Fig. 3e). These findings indicated that cit-H4R3 was associated with the decondensed soluble chromatin in apoptotic cells.

**PAD14 promotes DNA fragmentation.** In neutrophils, histone hypercitrullination is known to mediate NET formation that is associated with highly decondensed chromatin structures<sup>22</sup>. As chromatin decondensation was shown to increase the accessibility of DNase, we hypothesized that PAD14 promotes nuclear fragmentation. Micrococcal nuclease (MNase), which can digest chromatin at the linker DNA region into solubilized poly- and mono-nucleosome-size fragments, was used to evaluate the accessibility of DNA as well as chromatin compaction. MNase treatment of purified chromatin produced fragmented-nucleosomes more effectively in cells transfected with wild-type PAD14 than those with mutant PAD14 or mock (Fig. 4a). This result was confirmed by SDS-PAGE in which core histones from soluble nucleosome were stained with Coomassie brilliant blue (CBB) (Fig. 4b). ADR treatment also enhanced DNA cleavage (evaluated by histones in soluble nucleosome), but pretreatment with the siPAD14 remarkably suppressed MNase-mediated DNA digestion (Fig. 4c). Taking these observations into account, our findings revealed that PAD14 increased the accessibility of DNA and subsequently promoted the DNA fragmentation.

**p53-PAD14 citrullinates Lamin C at its carboxy terminus.** Next, we introduced PAD14 into HEK293T cells and performed immunocytochemical analysis using anti-MC antibody. The results clearly indicated the citrullination of nuclear membrane proteins in cells expressing HA-PAD14 (Fig. 5a). Lamin family proteins are the main components of the nuclear lamina and are essential for various nuclear processes including apoptosis. Western blotting using nuclear extracts from PAD14-introduced HEK293T cells revealed the band shift of Lamin C protein, but not those of other Lamin proteins (Fig. 5b). Furthermore, we detected the citrullination of immunopurified Lamin C protein from PAD14-introduced HEK293T cells using anti-MC antibody (Fig. 5c). Because Lamins A and C share 566 amino acids of their N-terminal portions, six carboxy-terminal amino acids (VSGSRR) specific for Lamin C is likely to contain a target(s) of citrullination (Supplementary Fig. S3a). Hence, we constructed plasmids expressing various types of mutant Lamin C and examined the citrullination of each mutant protein. Western blotting with anti-MC antibody revealed that substitution of both 571st and 572nd arginine residues caused a significant reduction of protein citrullination (Fig. 5d). Accordingly, we raised an antibody against citrullinated Lamin C at these two arginine residues (Supplementary Fig. S3b). This antibody clearly recognized the citrullinated Lamin C in PAD14-introduced HEK293T cells as



**Figure 4 | Regulation of DNA cleavage by PADI4.** (a) Chromatin was isolated from HEK293T cells transfected with PADI4 expressing plasmid. Chromatin extracted from equal numbers of cells was digested with MNase at 37 °C for 2 min. Soluble DNA released from MNase-treated chromatin were subjected to agarose gel electrophoresis. Arrow heads denote mono- and poly-nucleosomal DNAs. (b) Chromatin was isolated from HEK293T cells transfected with PADI4-expressing plasmid. Chromatin extracted from equal numbers of cells was digested with MNase at 37 °C for 2 min. Soluble core histones released from MNase-treated chromatin were subjected to SDS-PAGE. CBB staining for input chromatin is shown as controls. Input chromatin was subjected to immunoblotting with anti-HA antibody. (c) Chromatin was isolated from U-2 OS cells treated with ADR. Each siRNA was transfected 10 h before DNA damage treatment. siEGFP was used as a control. Chromatin extracted from equal numbers of cells was digested with MNase at 37 °C for 2 min. Soluble core histones released from MNase-treated chromatin were subjected to SDS-PAGE. CBB staining for input chromatin is shown as controls. Input chromatin was subjected to immunoblotting with anti-PADI4 antibody. Arrow head indicates MNase.

well as ADR-treated U-2 OS cells (Fig. 5e). In addition, immunocytochemistry revealed that the citrullinated Lamin C was localized predominantly around the fragmented nuclei in the damaged U-2 OS (Fig. 5f) and MCF7 cells (Supplementary Fig. S4a). Furthermore, cit-Lamin C expression was significantly correlated with the apoptotic cells in ADR-treated U-2 OS cells (Supplementary Fig. S4b), suggesting the involvement of citrullination of Lamin C in the nuclear fragmentation during apoptosis. Taken together, PADI4 could mediate the critical steps of apoptotic cell death through the citrullination of the core histones and Lamin C.

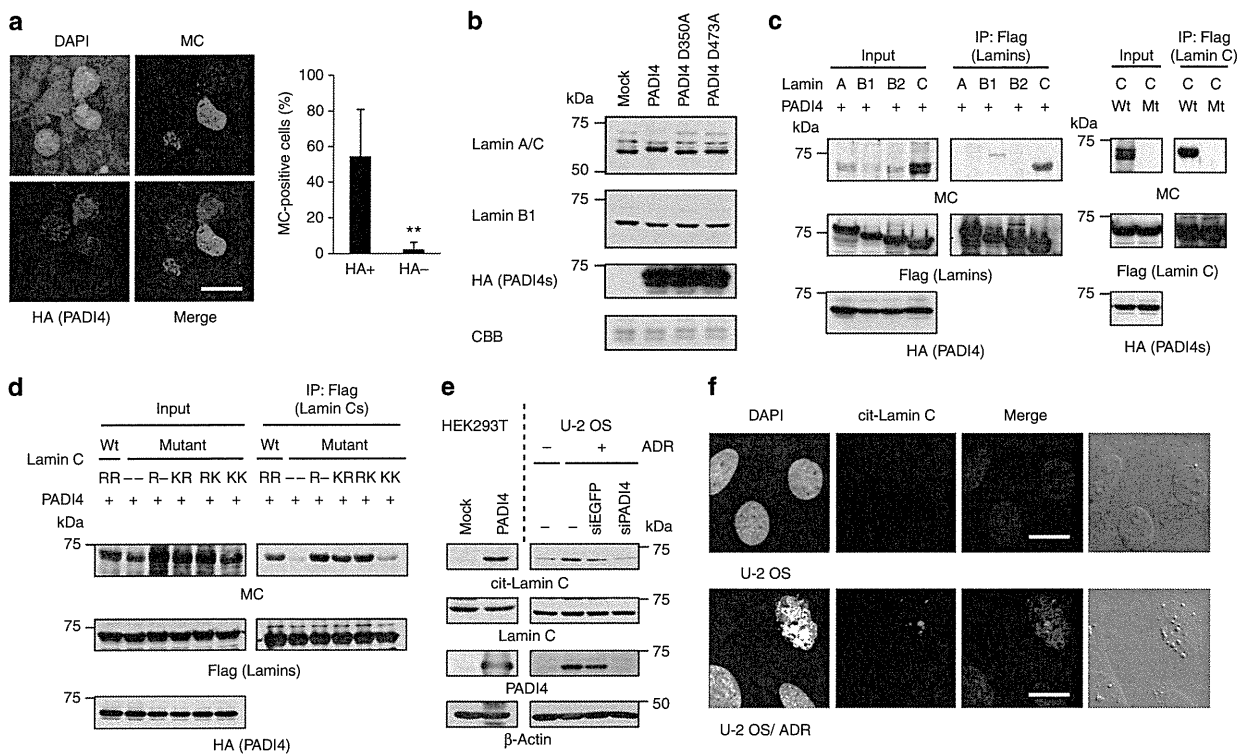
***Padi4*<sup>-/-</sup> mice exhibits apoptosis resistance.** To further investigate the physiological function of Padi4, we generated *Padi4*<sup>-/-</sup> mice (Supplementary Fig. S5). *Padi4*<sup>-/-</sup> mice were grossly normal in terms of survival, physical appearance and organ morphology. Then, we prepared messenger RNAs of thymuses from  $\gamma$ -ray-irradiated mice. The result of quantitative real-time PCR demonstrated that *Padi4* mRNA was significantly increased after DNA damage in wild-type mice but not in *Padi4*-deficient mice, compared with *p21*<sup>Waf1</sup> as a positive control (Fig. 6a). We also detected the  $\gamma$ -ray-induced PADI4 expression in U-2 OS cells in a dose-dependent manner (Supplementary Fig. S6a). Immunohistochemical analysis of *in vivo* citrullination of H4R3 in *Padi4*<sup>+/+</sup> mice exhibited the significant signal of cit-H4R3 in thymus at 24 h after  $\gamma$ -ray irradiation, although cit-H4R3 was not detected in *Padi4*<sup>-/-</sup> mice (Fig. 6b). Concordantly, the numbers of TUNEL-positive and cleaved caspase-3-positive-cells were decreased in *Padi4*<sup>-/-</sup> mice (Fig. 6c,d). We also found that X-ray irradiation induced expressions of mouse *Padi4* and human *PADI4* in a p53-dependent manner (Supplementary Fig. S6b-d). Subsequently, we examined by western blotting, the level of cleaved caspase-3 in X-ray-irradiated thymus and found the significant reduction of cleaved caspase-3 in *Padi4*<sup>-/-</sup> mice (Fig. 6e), further supporting a critical role of Padi4 in the apoptotic process.

**Frequent missense mutations of *PADI4* in cancer cell lines.** To verify the biological and clinical significance of PADI4 in human carcinogenesis, we performed DNA sequencing of the *PADI4* gene

in 22 breast, 21 colorectal and 37 lung cancer cell lines (Supplementary Table S1). We identified six missense mutations in six cell lines, among which the wild-type *PADI4* allele was lost in three cell lines (Supplementary Fig. S7a). As these mutations were not likely to affect the mRNA expression level (Supplementary Fig. S7b), we constructed plasmid vectors expressing either of six mutant PADI4 proteins and transfected either of these plasmids into HEK293T cells. Although T79 mutation did not affect enzymatic activity, five of six mutations lead to the remarkable decrease in enzymatic activity (Fig. 7a). Interestingly, T79 was not strictly conserved, while other five mutations were highly conserved among 14 mammalian species (Supplementary Table S2). These findings suggested the complete loss of PADI4 activity in MDA-MB-435S and SK-BR-3 cells. In concordance with this result, ectopic expression of p53 using adenovirus vector expressing p53 (Ad-p53) in MDA-MB-435S cells did not induce H4R3 citrullination, while H4R3 citrullination was observed in MDA-MB-231 cells in which wild-type PADI4 was retained (Fig. 7b). Moreover, citrullination of H4R3 was significantly associated with apoptosis induction of Ad-p53-infected MDA-MB-231 cells (Supplementary Fig. S7c), indicating a critical role of PADI4-mediated histone H4 citrullination in a p53-related pathway. Similarly, Ad-p53 infection enhanced citrullination of Lamin C only in cells in which both *PADI4* alleles are intact (Supplementary Fig. S7d). Because PADI4 was expected to function as a homodimer<sup>23</sup>, our findings suggested that mutant PADI4 would exhibit dominant-negative activity. Although *PADI4* was mutated in LoVo cells, citrullination of Lamin C was remarkably increased in these cells. We assume that *PADI1*, *PADI2* and *PADI3* belonging to the PADI family might mediate the citrullination of Lamin C in LoVo cells because of relatively high levels of expression of these three members (Supplementary Fig. S7e).

**PADI4 is a potential tumour suppressor.** We examined the citrullination of H4R3 by tissue microarrays consisting of 309 NSCLC tissues (Fig. 7c). Clinicopathological analysis revealed that cit-H4R3 was associated with smaller tumour size ( $P=0.0136$  by Student's *t*-test, Table 1). We also found that 5-year survival rate in patients with cit-H4R3 expression (58.1%) was slightly longer than that in





**Figure 5 | Lamin C is citrullinated during nuclear fragmentation.** (a) Representative image of HA-PADI4-transfected HEK293T cells stained with anti-modified citrulline (MC) antibody (Alexa Fluor 488) and anti-HA antibody (Aleza Fluor 594) (left panel). The proportion of MC-positive cells stratified by HA-PADI4 expression was indicated (right panel). Error bars represent s.d. ( $n = 9$ ).  $**P < 0.01$  by Student's *t*-test. Scale bars, 20  $\mu\text{m}$ . Cells were incubated with  $5 \mu\text{mol l}^{-1}$  of A23187 for 1 h before fixation. (b) Western blotting for nuclear extracts from HEK293T cells transfected with wild-type PADI4, mutant PADI4 (D350A or D473A), or mock plasmid using each anti-Lamin antibody. (c) HEK293T cells were transfected with the indicated plasmids. Cell extracts were immunoprecipitated using anti-Flag antibody, followed by immunoblotting with anti-MC, anti-Flag, or anti-HA antibody. (d) HEK293T cells were transfected with the indicated plasmids. Cell extracts were immunoprecipitated using anti-Flag antibody, followed by immunoblotting with anti-MC, anti-Flag, or anti-HA antibody. (e) Whole-cell extracts from PADI4-transfected HEK293T cells or ADR-treated U-2 OS cells were subjected to western blotting using antibody against citrullinated Lamin C at arginine 571 and arginine 572 residues (cit-Lamin C), Lamin C, PADI4, or  $\beta$ -actin. Each siRNA was transfected 10 h before DNA damage treatment. siEGFP was used as a control. (f) Representative image of ADR-treated U-2 OS cells stained with anti-cit-Lamin C antibody (Alexa Fluor 488). Scale bars, 20  $\mu\text{m}$ .

patients without cit-H4R3 expression (52.7%), although the difference was not statistically significant ( $P = 0.24$  by log-rank test; Fig. 7d). As many of p53 mutations lead to a prolonged half-life of p53 protein, immunohistochemistry of p53 is commonly used to detect p53 mutations in various cancers<sup>24,25</sup>. Interestingly, the positive p53 staining was inversely associated with cit-H4R3 staining ( $P = 0.0079$  by Student's *t*-test), indicating the regulation of chromatin modification by p53 in human carcinogenesis (Table 1).

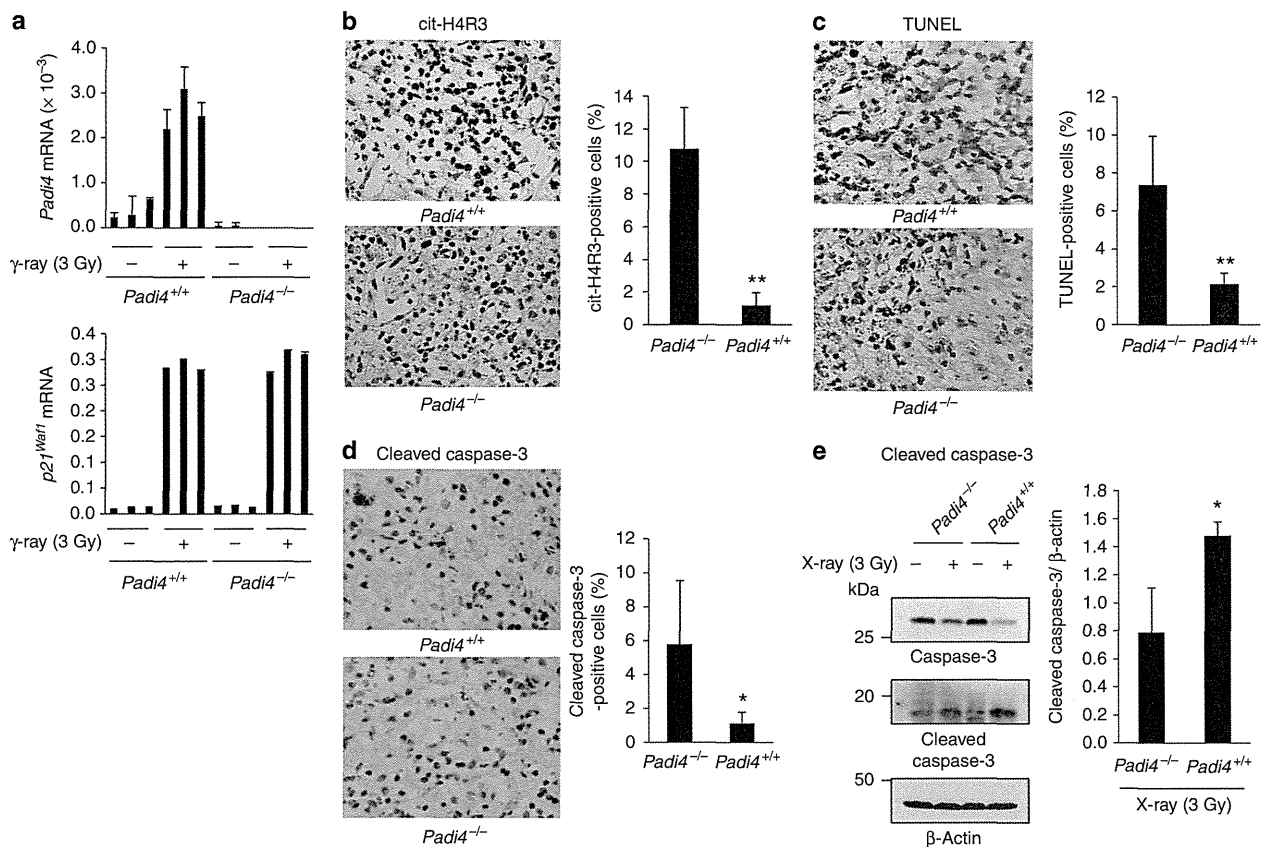
## Discussion

Accumulating evidence indicate that a combination of genetic and epigenetic alterations contribute to the development and progression of human cancers<sup>26</sup>. Among many genes altered in cancer tissues, the p53 gene was mutated in nearly half of all tumours<sup>27–29</sup>; however, the roles of p53 in histone modifications and chromatin structure have not been characterized. Here we clearly demonstrated the crucial roles of p53–PADI4 pathway in citrullination of H4R3 and Lamin C in response to DNA damage as well as in nuclear fragmentation.

A certain type or a combination of histone modifications termed as 'histone code'<sup>30</sup> are translated into a meaningful biological outcome such as gene expression and chromatin structure. Histone H2AX is phosphorylated at serine 139 residue ( $\gamma$ -H2AX) on external damage, and recruits DNA repair complex to promote chromatin remodelling<sup>31,32</sup>. Recently, phosphorylation of serine 14 residue

in H2B, which was induced on several apoptotic stimuli including DNA damage<sup>33</sup>, was proposed as a 'death code', but its physiological significance remains to be determined. Histone methylation, phosphorylation and acetylation are reversible processes that are regulated by histone-modifying enzymes<sup>34</sup>. In contrast, citrullination is a chemically stable modification, and decitullination enzymes have not yet been discovered<sup>35</sup>. Therefore, histone citrullination is considered to be an irreversible cellular process. As histone H4 citrullination is associated with apoptosis of damaged cells as well as neutrophilic death triggered by NET formation, histone H4 citrullination could be defined as a functional apoptotic histone code.

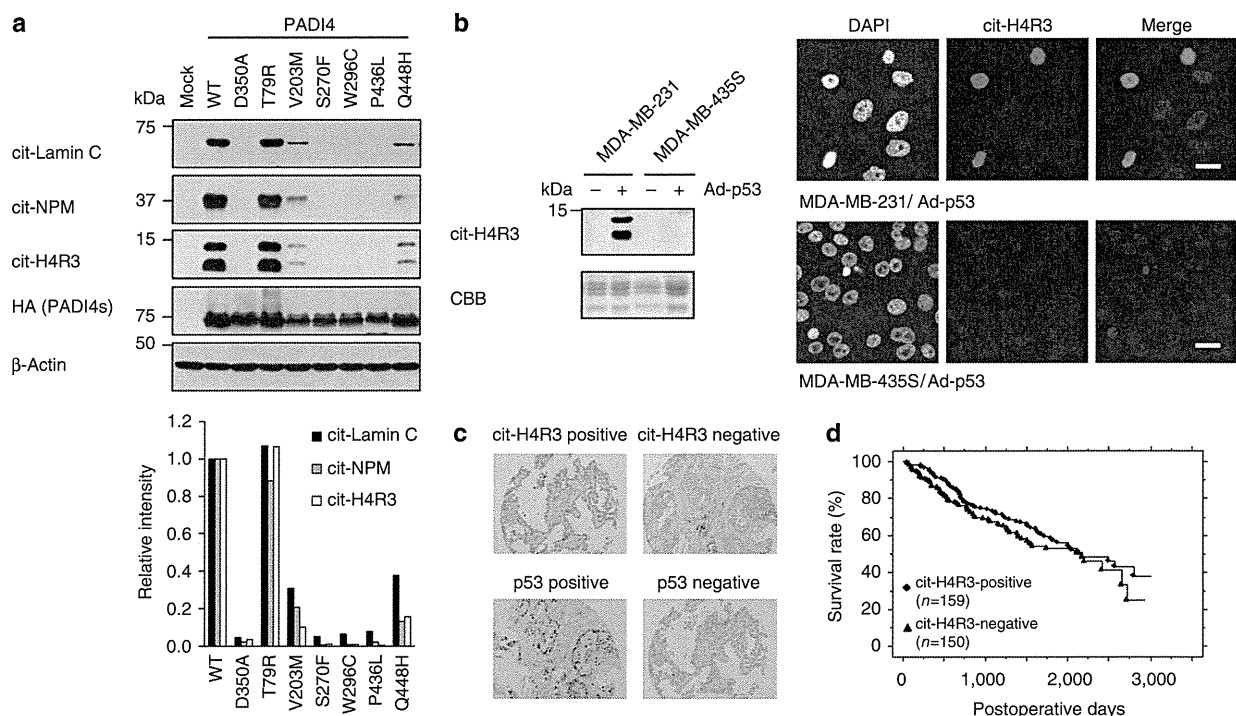
Li *et al.* previously reported that PADI4 negatively regulated the p21<sup>WAF1</sup> expression through the interaction with p53 at a p21<sup>WAF1</sup> promoter region in ultraviolet-irradiated U-2 OS cells<sup>36</sup>. In addition, PADI4 was shown to be transactivated in various cancer tissues<sup>37</sup>, and siPADI4 or PADI4 inhibitor (Cl-amidine) suppressed proliferation of U-2 OS cells<sup>36</sup>, suggesting PADI4 to function as an oncogene. However, the frequent inactivating mutations of PADI4 in cancer cell lines as well as apoptosis-resistance in *Padi4*-null mice implied the possible role of PADI4 as a tumour suppressor. This discrepancy could be partially due to the difference in the experimental conditions. We analysed the effects of PADI4 knockdown using irradiated mice or ADR-treated cells, whereas Li *et al.* analysed U-2 OS cells under the non-stress condition or after ultraviolet treatment.



**Figure 6 | The role of Padi4 in DNA damage induced apoptosis *in vivo*.** (a) Quantitative real-time PCR analysis of *Padi4* and *p21<sup>Waf1</sup>* expression in thymuses from  $\gamma$ -ray-irradiated *Padi4*<sup>-/-</sup> or *Padi4*<sup>+/+</sup> mice (3 Gy). Error bars represent range ( $n = 2$ ). *Gapdh* was used for normalization of *Padi4* expression levels. Mice were sacrificed 24 h after irradiation with 3 Gy of  $\gamma$ -ray. (b) Representative images of thymus sections from  $\gamma$ -ray-irradiated *Padi4*<sup>-/-</sup> or *Padi4*<sup>+/+</sup> mouse 24 h after irradiation with 3 Gy of  $\gamma$ -ray stained for citrullinated histone H4R3 (cit-H4R3) (left panel, X40). The proportion of positive cells in *Padi4*<sup>-/-</sup> or *Padi4*<sup>+/+</sup> mouse was indicated (right panel). Error bars represent s.d. ( $n = 4$ ). \*\* $P < 0.01$  by Student's *t*-test. (c) Representative images of TUNEL staining for thymus sections from  $\gamma$ -ray-irradiated *Padi4*<sup>-/-</sup> or *Padi4*<sup>+/+</sup> mouse 24 h after irradiation with 3 Gy of  $\gamma$ -ray (left panel, X40). The proportion of positive cells in *Padi4*<sup>-/-</sup> or *Padi4*<sup>+/+</sup> mouse was indicated (right panel). Error bars represent s.d. ( $n = 4$ ). \*\* $P < 0.01$  by Student's *t*-test. (d) Representative images of cleaved-caspase-3 staining in thymus sections from  $\gamma$ -ray-irradiated *Padi4*<sup>-/-</sup> or *Padi4*<sup>+/+</sup> mouse 24 h after irradiation with 3 Gy of  $\gamma$ -ray (left panel, X40). The proportion of positive cells in *Padi4*<sup>-/-</sup> or *Padi4*<sup>+/+</sup> mouse was indicated (right panel). Error bars represent s.d. ( $n = 4$ ). \* $P < 0.05$  by Student's *t*-test. (e) Whole-cell extracts of thymus from X-ray-irradiated *Padi4*<sup>-/-</sup> or *Padi4*<sup>+/+</sup> mice were subjected to immunoblotting with anti-cleaved caspase-3, anti-caspase-3 or anti- $\beta$ -actin antibody (left panel). Mice were sacrificed 48 h after irradiation with 3 Gy of X-ray. The ratio between cleaved caspase-3 and  $\beta$ -actin from independent samples ( $n = 4$  for *Padi4*<sup>-/-</sup> mice and  $n = 2$  for *Padi4*<sup>+/+</sup> mice) was indicated (right panel). \* $P < 0.05$  by Student's *t*-test.

Although ADR- or  $\gamma$ -ray-treatment remarkably increased PADI4 expression, ultraviolet-treatment did not induce PADI4 expression in U-2 OS cells (Supplementary Fig. S8a). In addition, expression levels of *p21<sup>Waf1</sup>* in thymus in the *Padi4*<sup>+/+</sup> and *Padi4*<sup>-/-</sup> mice after  $\gamma$ -ray irradiation were not significantly different (Fig. 6a). Similarly, siPADI4 treatment did not affect the expression of p53-target genes including *p21<sup>WAF1</sup>* in ADR-treated U-2 OS cells (Supplementary Fig. S8b). Moreover, siPADI4-treated U373MG cells exhibited resistance to p53-induced apoptosis (Supplementary Fig. S8c). In fact, we observed the citrullination of histone H4R3 in 51.5% of lung cancer tissues, suggesting the activation of PADI4 in a large proportion of cancer tissues, as suggested in the previous report<sup>37</sup>. However, cancer tissues are persistently exposed to oxidative stress compared with adjacent normal tissues<sup>38,39</sup>, activation of PADI4 would be related with cellular stress condition. Taken together, we assume that PADI4 could predominantly function as a tumour suppressor that mediates the apoptotic process of damaged cells.

Although the molecular mechanism of how PADI4 increases DNA accessibility was not fully elucidated, loss of positive charge by citrullination was shown to induce conformational changes of histone H4 N terminal<sup>40</sup>. DNA-histone interaction is charge-dependent, and changes in the charge of the histone tails are considered to weaken histone-DNA interaction<sup>41</sup>. Moreover, we found that the interaction between core histones and nucleophosmin was inhibited by citrullination (Supplementary Fig. S8d). Any or all of these changes can affect the structure and folding of individual nucleosome that could lead to a more open and permissive chromatin environment. The role of PADI4 in chromatin remodelling was shown in the previous papers<sup>42,43</sup>. We also reported that DNA damage induced PADI4 expression and the citrullination of various proteins in our previous study<sup>10</sup>. To our knowledge, this is the first report demonstrating the important role of histone H4R3 citrullination in human carcinogenesis and the p53-mediated apoptotic pathway.



**Figure 7 | Involvement of PADI4 in carcinogenesis.** (a) Whole-cell extracts from HEK293T cells transfected with wild-type or mutant PADI4 were subjected to western blotting using anti-citrullinated Lamin C (cit-Lamin C), anti-citrullinated nucleophosmin (cit-NPM), anti-citrullinated histone H4R3 (cit-H4R3), anti-HA, or anti- $\beta$ -actin antibody (upper panel). For quantification, band intensities were normalized to the signal in cells transfected with wild-type PADI4 (lower panel). (b) MDA-MB-231 and MDA-MB-435S cells were infected with adenovirus vector expressing p53 (Ad-p53). Histones were subjected to western blotting using anti-cit-H4R3 (left panel). Representative images of Ad-p53-infected cells stained for cit-H4R3 is shown in right panel. Scale bars, 20  $\mu$ m. (c) cit-H4R3 or p53 expression in NSCLC tissues (magnification X10). (d) Kaplan-Meier analysis of survival in patients with NSCLC according to the expression of cit-H4R3 ( $P=0.24$ , log-rank test).

## Methods

**Cell culture and transfections.** Cell lines were purchased from American Type Culture Collection, Lonza Biologics, or Japanese Collection of Research Bioresources. Cells were transfected with plasmids using FuGENE6 (Roche). Replication-deficient recombinant viruses Ad-p53 or Ad-LacZ, expressing p53 or LacZ, respectively, were generated and purified, as described previously<sup>5</sup>. siRNA oligonucleotides, commercially synthesized by Sigma Genosys, were transfected with Lipofectamine RNAiMAX reagent or Lipofectamine 2000 reagent (Invitrogen). Sequences of oligonucleotides are shown in Supplementary Table S3. For treatment with calcium ionophore, cells were incubated with 5  $\mu$ M of A23187 (Calbiochem) for 1 h at 37  $^{\circ}$ C.

**Plasmid construction.** Complementary DNA fragments of Lamin A, Lamin B1, Lamin B2 and Lamin C were amplified and cloned into pCAGGS vector. PADI4 expression plasmids were previously described<sup>10</sup>. Plasmids expressing mutant PADI4 or Lamin C were generated using the inverse PCR methods or by using the reverse primer containing point mutation at either of both of arginine 571 and 572 residues, respectively. The primer sequences for cloning and mutagenesis are shown in Supplementary Table S3.

**Knockout mice.** We amplified a 5,837 bp fragment containing exon 1 and exon 2 of *Padi4* using C57BL/6 genomic DNA as a template. The targeting vector was designed to replace exon 1 and 2, including the transcription initiation site, by mouse PGK-1 promoter and the neomycin-resistance gene (Supplementary Fig. S5a). We introduced the linearized targeting vector by electroporation into embryonic stem cells, and identified two independent targeted 129S1 embryonic stem cell clones by Southern blot analysis. We generated chimeric males by the aggregation method and crossed them with C57BL/6 females, and verified germline transmission by Southern blot analysis (Supplementary Fig. S5b,c). All *Padi4*<sup>-/-</sup> mice used in this study had been backcrossed for ten generations into the C57BL/6 background. We used RT-PCR to determine the presence of the *Padi4* transcript (Supplementary Fig. S5d). p53-deficient mice were provided from RIKEN BioResource Center (Ibaragi, Japan)<sup>44</sup>. Genotypes were confirmed by PCR analysis. The primer sequences are indicated in Supplementary Table S3. All mice were maintained under specific pathogen-free conditions and were handled in accordance with the Guidelines for Animal Experiments of the Institute of Medical Science (University of Tokyo, Tokyo, Japan).

**DNA damaging treatment.** For treatment with genotoxic stress, cells were continuously incubated with 2  $\mu$ g ml<sup>-1</sup> ADR for 2 h,  $\gamma$ -irradiated using a Cs-137 source (Gamma cell-40, Atomic energy of Canada), ultraviolet-irradiated using an ultraviolet cross-linker (XL-1500, Spectronics corporation), or X-irradiated using an X-ray irradiation system (MBR-1520R-3, Hitachi). Mice were also  $\gamma$ -irradiated using a Cs-137 source (Gamma cell-40, Nordion) or X-irradiated using an X-ray irradiation system (MBR-1520R-3, Hitachi).

**Quantitative real-time PCR.** Peripheral blood mononuclear cells were obtained by separating blood cells in a Ficoll gradient (Amersham). Peripheral blood mononuclear cells were incubated with phytohaemagglutinin for 72 h and exposed to an X-ray irradiation. Total RNA was isolated from mouse tissue or human cells using RNeasy spin column kits (Qiagen) according to the manufacturer's instruction. Blood RNA of irradiated mice was prepared using Mouse RiboPure-Blood RNA Isolation Kit (Ambion). Complementary DNAs were synthesized with the SuperScript Preamplification System (Invitrogen). Quantitative real-time PCR was conducted using the SYBR Green I Master on a LightCycler 480 (Roche). The primer sequences are indicated in Supplementary Table S3.

**Immunoprecipitation.** Cell extracts from HEK293T cells transfected with plasmids encoding Flag-Lamins and/or HA-PADI4s were prepared by adding HBST buffer (10 mM HEPES at pH 7.4, 150 mM NaCl and 0.5% TritonX-100). Extracts were precleared by incubation with protein G-Sepharose 4B (Zymed) and mouse IgG at 4  $^{\circ}$ C for 1 h. Precleared cell extracts were then incubated with anti-Flag affinity gel (Sigma) at 4  $^{\circ}$ C for 2 h. The beads were washed 4 times with 1 ml of ice-cold HBST buffer, and immunoprecipitated proteins were released from the beads by boiling in sample buffer for 2 min.

**Western blotting.** To prepare whole-cell extracts, cells or tissues were lysed in chilled HBST buffer for 30 min on ice and centrifuged at 16,000g for 15 min. Histones were purified in acidic condition as previously described<sup>45</sup>. Samples were subjected to SDS-PAGE and immunoblotting using standard procedures. To detect citrullinated proteins, blots of deiminated proteins were treated with medium for chemical modification at 37  $^{\circ}$ C for 3 h, and then, modified citrulline residues were detected with an anti-modified citrulline antibody (anti-MC antibody, Upstate). Bands were quantified by the Image J software.

**Table 1 | Association between citrullinated H4R3-positivity and clinicopathological parameters in NSCLC patients.**

	Total	cit-H4R3		P value
	n=309	Positive	Negative	Positive versus negative
		n=159	n=150	
<b>Gender</b>				
Male	214	100	114	0.0138**
Female	95	59	36	
<b>Age (years)</b>				
< 65	135	67	68	NS (0.6463)
≥ 65	174	92	82	
<b>Histological type</b>				
ADC	192	110	82	0.0099**
SCC	82	35	47	
Others	35	14	21	
<b>Smoking status</b>				
Never	90	60	30	0.0007**
Smoker	219	99	120	
<b>pT factor</b>				
T1	132	80	52	0.0136**
T2 + T3	177	79	98	
<b>pN factor</b>				
N0	204	104	100	NS (0.9044)
N1 + N2	105	55	50	
<b>p53</b>				
Positive	130	55	75	0.0079**
Negative	179	104	75	

Abbreviations: ADC, adenocarcinoma; NS, no significance; SCC, squamous-cell carcinoma; Others, large-cell carcinoma plus adenosquamous-cell carcinoma.  
\*\**P* < 0.05 (Fisher's exact test).

**Antibodies.** To develop an antibody against Cit571-572 Lamin C, a Lamin C peptide (LHHHHVSGCitCit) was chemically synthesized and used to immunize rabbits. The positive antisera were further purified by immune-affinity purification using a Lamin C Cit571-572 peptide. Anti-citrullinated nucleophosmin (NPM) antibody was prepared as previously described<sup>10</sup>. Polyclonal anti-MC antibody (17-347) and anti-citrullinated arginine 3 of histone H4 antibody (07-596) were purchased from Upstate. Anti-β-actin monoclonal antibody (A5441) and Anti-Flag monoclonal (F3165) and polyclonal (F7425) antibodies were purchased from Sigma. Anti-p53 monoclonal antibody (OP140) and anti-Lamin B antibody (NA12) were purchased from Calbiochem. Anti-HA monoclonal (sc-7392) and polyclonal (sc-805) antibodies were purchased from Santa Cruz Biotechnology. Anti-HA rat monoclonal antibody (3F10) was purchased from Roche. Anti-6×His monoclonal antibody (631212) was purchased from Clontech. Anti-PAD14 polyclonal antibody (ab50332), anti-citrullinated histone H3 antibody (ab5103) and anti-mono methyl histone H4 antibody (ab17339) were purchased from Abcam. Anti-Lamin A/C antibody (#2032 or #4777), anti-caspase-3 antibody (#9662) and anti-cleaved caspase-3 (#9661) were purchased from Cell signaling. List of antibodies and the concentrations used are shown in Supplementary Table S4.

**In vitro citrullination assay.** Recombinant histone proteins were purchased from Millipore. Glutathione S-transferase (GST) fusion PAD14 and NPM1 proteins were generated as previously described<sup>10</sup>. His-tagged proteins were generated by cloning of their coding sequences into pET21a or pET28a vector (Novagen). Proteins were expressed in *Escherichia coli* and purified on Ni-NTA agarose (Qiagen) by standard methods. Deimination reactions were carried out as previously described<sup>10</sup>.

**GST pull-down assay.** Core histones were purified from HEK293T cells using Histone Purification Kit (Active motif). GST-NPM (1 μg) and core histones (1 μg) were separately incubated with PAD14 (1 μg) or PAD14-D350A (1 μg) for 1 h at 37 °C with 1 mM of CaCl<sub>2</sub>. PAD14-treated NPM1 and histones were mixed and stored on ice for 1 h. The mixture was incubated at 4 °C for 2 h with 20 μl of a glutathione-Sepharose 4B bead suspension (Amersham Pharmacia). After the beads were washed extensively, proteins were eluted from the beads by incubation with SDS sample buffer, separated by SDS-PAGE, and visualized by silver staining.

**Immunocytochemistry.** Immunocytochemistry was performed as previously described<sup>10</sup>. Before incubating with anti-MC antibody, cells were treated with medium for chemically modifying citrulline residues at 37 °C for 3 h. For co-staining of TUNEL and citrullinated proteins, cells were immunostained followed by TUNEL reaction for 1 h at 37 °C using the kit (In Situ Cell Death Detection Kit, Fluorescein, Roche). Quantification was performed by counting around 100 cells from more than 4 independent fields.

**Immunohistochemistry.** Frozen sections of mouse thymus were used for immunohistochemistry and TUNEL staining. Immunohistochemistry was performed using the immunohistochemistry EnVision (Dako) method. TUNEL staining was performed using Apoptosis in situ Detection Kit (Wako) according to the manufacturer's instruction. Tissue microarrays were constructed in our laboratory, and staining and statistical analysis were performed as previously described<sup>46</sup>.

**Mutation analysis.** Genomic DNA was purified from 80 cancer cell lines by standard protocol<sup>8</sup>. The list of cell lines is shown in Supplementary Table S1. 16 coding exons of the *PAD14* gene were amplified, purified and sequenced. The sequences of primers used in this analysis are indicated in Supplementary Table S3.

**Chromatin fractionation.** S1, S2 and P chromatin fractions from HEK293T cell nuclei were prepared according to previously described procedures<sup>47</sup> with minor modifications. HEK293T cells were transfected with PAD14-expressing plasmid. The collected cells were incubated in cell homogenization buffer (10 mM Tris at pH 8.0, 10 mM MgCl<sub>2</sub>, 0.5% NP40 and 1 mM DTT) on ice for 10 min. The nuclear pellet was obtained by centrifugation and resuspended in MNase digestion buffer (15 mM Tris pH 7.4, 15 mM NaCl, 60 mM KCl, 0.25 M sucrose, 1 mM CaCl<sub>2</sub>). The pellet was then treated with MNase (New England Biolabs) for 2 min at 37 °C. The solution was centrifuged to obtain the supernatant (S1). The pellet was resuspended in 0.1 mM EDTA, incubated at 4 °C for 30 min, and centrifuged to obtain the chromatin fraction (P) and supernatant (S2). The proteins contained in each fraction were separated by 15% SDS-PAGE and detected by western blotting.

**MNase assay.** The nuclear pellet (Input) was treated with MNase as shown above. The reaction was terminated by adding the stop solution (0.5 mM EGTA, 25 mM EDTA). After centrifugation at 14,000g for 5 min, DNA was extracted from the aqueous phase and analysed on agarose gel. Histone proteins collected from the aqueous phase (Sup) and pellet (Ppt) were separated by SDS-PAGE.

**Cell death assay.** Cells were infected with 20 multiplicity of infection (MOI) of Ad-p53 at 7 h after transfection of siRNA oligonucleotide. 60 h after infection cells were incubated with TUNEL reaction mixture for 1 h at 37 °C using the kit (In Situ Cell Death Detection Kit, Fluorescein, Roche). Apoptotic cells were quantified by fluorescence-activated cell sorting analysis.

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