A Genetically Encoded Probe for Live-Cell Imaging of H4K20 Monomethylation

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Abstract

Eukaryotic gene expression is regulated in the context of chromatin. Dynamic changes in post-translational histone modification are thought to play key roles in fundamental cellular functions such as regulation of the cell cycle, development, and differentiation. To elucidate the relationship between histone modifications and cellular functions, it is important to monitor the dynamics of modifications in single living cells. A genetically encoded probe called mintbody (modification-specific intracellular antibody), which is a single-chain variable fragment tagged with a fluorescent protein, has been proposed as a useful visualization tool. However, the efficacy of intracellular expression of antibody fragments has been limited, in part due to different environmental conditions in the cytoplasm compared to the endoplasmic reticulum where secreted proteins such as antibodies are folded. In this study, we have developed a new mintbody specific for histone H4 Lys20 monomethylation (H4K20me1). The specificity of the H4K20me1-mintbody in living cells was verified using yeast mutants and mammalian cells in which this target modification was diminished. Expression of the H4K20me1-mintbody allowed us to monitor the oscillation of H4K20me1 levels during the cell cycle. Moreover, dosage-compensated X chromosomes were visualized using the H4K20me1-mintbody in mouse and nematode cells. Using X-ray crystallography and mutational analyses, we identified critical amino acids that contributed to stabilization and/or proper folding of the mintbody. Taken together, these data provide important implications for future studies aimed at developing functional intracellular antibodies. Specifically, the H4K20me1-mintbody provides a powerful tool to track this particular histone modification in living cells and organisms.

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Introduction

Histone proteins are subjected to a variety of post-translational modifications that are thought to play crucial roles in gene regulation and genome integrity [1,2]. However, the precise role of specific histone modifications in transcription, replication, repair, and chromosome segregation remains to be elucidated. In addition, it is unclear how histone modifications are inherited or altered over time. Addressing the dynamic regulation of histone modifications has been limited because most studies conducted thus far have utilized...
fixed cells, thus preventing single-cell assessment of modification changes. To overcome the limitation of using fixed cells, we have developed two live-cell imaging systems based on the use of modification-specific antibodies [3]. These approaches include Fab-based live endogenous modification labeling (FabLEM [4]) and a genetically encoded system using modification-specific intracellular antibody probes (mintbodies [5–7]). The latter approach is particularly useful for investigations aimed at monitoring histone modifications in living animals.

Histone H4 Lys20 monomethylation (H4K20me1) is a key histone modification that regulates DNA replication [8–10] and repair [11] as well as chromosome compaction [12]. The level of H4K20me1 is regulated by three methyltransferases, PR-Set7 [13–15], Suv420H1, and Suv420H2 [16], and a demethylase, PHF8 [17,18]. Chromatin immunoprecipitation combined with deep sequencing (ChIP-seq) and immunofluorescence analyses have shown that H4K20me1 is generally distributed in euchromatin [16]. In addition, H4K20me1 enrichment in inactivated X chromosomes (X) and centromeres have also been reported [16,19–21]. However, the spatiotemporal regulation of H4K20me1 and its impact on DNA metabolism remain largely uncharacterized.

In this study, we report the generation of H4K20me1-specific mintbody. X-ray crystallography and mutation analyses identified key amino acids that improved mintbody stability and/or folding. We demonstrate that the H4K20me1-mintbody specifically recognizes the target histone modification and is useful to monitor the oscillations of H4K20me1 during the cell cycle and to visualize dosage-compensated X chromosomes in living mammalian cells and during Caenorhabditis elegans development.

Results

Generation and characterization of the H4K20me1-specific mintbody

To generate a new mintbody specific for H4K20me1, we cloned cDNA encoding variable regions of heavy and light chains from 15F11/CMA421 hybridoma cell line [22], and constructed mintbody expression vectors by fusing the single-chain variable fragment (scFv) to EGFP at the C-terminus (Fig. 1a). When expressed in HeLa cells, H4K20me1-mintbody was diffuse in the cytoplasm but concentrated in nuclei and chromosomes, where it co-localized with a DNA staining Hoechst33342 dye (Fig. 1b). This suggests that the probe binds to nuclear targets in living cells, as designed.

To determine if the H4K20me1-mintbody specifically recognizes H4K20me1, like its parental IgG [22], we performed both in vitro and in vivo analyses. We assessed binding in vitro using recombinant histone H4 proteins that harbor methylation-mimic modifications [22] as well as synthetic peptides arrays. When these were incubated with bacterially expressed and purified H4K20me1-scFv (Supplementary Fig. S1a), only H4 harboring monomethylation-mimic modifications and synthetic H4K20me1 peptides were bound by the scFv (Supplementary Fig. S1b–d). These results suggest that the specificity of the original IgG was well retained in the scFv format.

We next evaluated the specificity of H4K20me1-mintbody in living cells using fission yeast Schizosaccharomyces pombe, which can be genetically manipulated to delete the target modification. Targeting of the H4K20me1-mintbody to the crescent-shaped chromatin compartment in the nucleus can also be assessed readily in S. pombe (Supplementary Fig. S2a) [23,24]. We generated yeast strains expressing H4K20me1-mintbody in the wild type and set9Δ background. As Set9 is the sole H4K20 methyltransferase in S. pombe [25], no H4K20me1 was expected in set9Δ strains. Indeed, immunofluorescence and immunoblotting using the specific antibody showed no H4K20me1 in set9Δ strains (Fig. 1c and Supplementary Fig. S2b). Note that similar results were obtained using H4K20me1-scFv, instead of the specific IgG, which further validates that the scFv retained the original specificity (Supplementary Fig. S2c and d).

To assess H4K20me1-mintbody localization in the wild type and set9Δ background strains, growing yeast cells were incubated with Hoechst33342 for DNA labeling [26]. In the wild-type background, the H4K20me1-mintbody was concentrated in the chromatin domain, as represented by Hoechst staining, and excluded from the nucleolar domain in the nucleus.
(Fig. 1d and e and Supplementary Fig. S2a). In contrast, in the \textit{set9}Δ strain, the H4K20me1-mintbody diffused throughout the cell and the concentration in the chromatin domain was substantially reduced. Although H4K20me1-mintbody was still slightly enriched in the nucleus of the \textit{set9}Δ strain, GFP alone also exhibited the same slight enrichment, probably due to their free diffusion throughout the cell.

\begin{figure}[h]
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To further assess the specificity of the mintbody, we generated a strain that harbors a single H4 gene by deleting two out of three H4 genes in *S. pombe* (H4.2 wt), and another strain that harbors a single mutant H4 gene in which lysine 20 is substituted with alanine (H4.2 K20A). As expected, H4K20me1-mintbody was enriched in the chromatin domain of the H4.2 wt, but not in the H4.2 K20A strain (Fig. 2d and e). These results indicate that H4K20 methylation is required for H4K20me1-mintbody’s chromatin targeting.

To examine if H4K20me1-mintbody specifically recognizes H4K20me1 over H4K20me2 and H4K20me3, we used a mammalian cell line harboring multiple H4K20 methyl transferases with selective activities (Fig. 2a); PR-Set7/SET8/SETD8/KMT5A is known to monomethylate unmodified H4K20, and SUV420H1/ KMT5B and SUV420H2/KMT5C mediate further methylation (to me2 and me3) of H4K20me1 [16]. A demethylase PHF8 is also known to demethylate H4K20me1 [17,18]. We anticipated that overexpression of these enzymes would alter the balance among different methylation states in living cells. To screen the effect of overexpression on H4K20 methylation, HeLa cells were transfected with HaloTag-fusion enzymes and then fixed and stained with the specific antibodies directed against H4K20me1, H4K20me2, and H4K20me3. HaloTag-fusion enzymes were labeled with tetramethylrhodamin-conjugated Halo ligand and Hoechst33342. Optical sections were collected for quantifying the nuclear and cytoplasmic intensities of H4K20me1-mintbody and the nuclear intensity of Halo-SUV420H1 (Fig. 2e). The nucleus to cytoplasm intensity ratios of H4K20me1-mintbody in SUV420H1-expressing cells were indeed lower compared to untransfected cells, and more effects were seen in higher expressing cells (Fig. 2e, right), similar to the H4K20me1 antibody staining (Fig. 2c, left). This result indicates that H4K20me1-mintbody can be used in living cells to selectively monitor H4K20me1, as opposed to H4K20me2 or H4K20me3.

To further evaluate the specific binding of H4K20me1-mintbody to H4K20me1 in HeLa cells, a simple co-localization analysis was employed by comparing the distribution of H4K20me1-mintbody with those of different modifications, including H4K20me1, H4K20me2, H4K20me3, H3K4me1, H3K9me1, and H3K27me1 (as detected with specific antibodies). After collecting confocal fluorescence images of H4K20me1 and each of the antibodies, we performed a co-localization analysis to obtain co-localization plots and correlation coefficients. As expected, H4K20me1-mintbody was most highly correlated with H4K20me1, even though H4K20me1-mintbody signals had higher background than the antibody stains, probably due to the presence of unbound mintbody (Supplementary Fig. S4). Taken together, these data support the view that H4K20me1-mintbody specifically binds to H4K20me1 in living cells and is useful as a probe to monitor the level and localization of H4K20me1.
Monitoring changes in H4K20me1 levels during the cell cycle

To investigate whether the H4K20me1-mintbody could be used as a live-cell probe without disturbing cell function, we compared cell growth and cell cycle progression in HeLa cells that stably expressed H4K20me1-mintbody versus wild-type cells. Both the growth curve and cell cycle profile—based on 5-ethynyl-2'-deoxyuridine (EdU) incorporation and DNA content—were very similar between the mintbody-expressing and wild-type cells (Fig. 3a and b). These data suggest that H4K20me1-mintbody does not affect cell cycle progression.
Fig. 3 (legend on next page).
We then investigated whether H4K20me1-mintbody can monitor the oscillations of H4K20me1 levels during the cell cycle in living cells. A number of studies have reported that H4K20me1 increases during late G2 and M, and decreases after cell division and throughout G1 and S [9,15,27,28]. We confirmed this oscillation in HeLa cells expressing mCherry-tagged proliferating cell nuclear antigen (PCNA), a marker of DNA replication factories [29], by time-lapse imaging followed by immunofluorescence with the H4K20me1-specific antibody (Fig. 3c and Supplementary Fig. S5a). This trend remained the same after normalization using DNA density (Hoechst33342 intensity) (Fig. 3d). We then used HeLa cells that stably express both H4K20me1-mintbody and mCherry-PCNA. After time-lapse imaging to identify the cell cycle position, the nuclear and cytoplasmic intensities of H4K20me1-mintbody at the final image were measured to obtain the nuclear/cytoplasm ratio. This ratio is expected to reflect H4K20me1 levels, as shown before. The nuclear/cytoplasm ratio increased in G2 and M compared to G1 and S (Fig. 3e and Supplementary Fig. S5b). A few examples of cells imaged for a long period (over 45 h) are shown in Fig. 3f and Supplementary Fig. S6. These live-cell data are qualitatively in good agreement with the previous studies that utilized Western blotting and mass spectrometric approaches [27,28]. This result demonstrates that the H4K20me1-mintbody enables monitoring of changes in H4K20me1 levels in living cells. We noticed, however, that the nuclear/cytoplasm ratio during interphase should not be directly compared with the chromosome/cytoplasm ratio during M phase. Although the nuclear intensity of H4K20me1-mintbody peaked in M phase, it dropped to a minimum when normalized with Hoechst33342 intensity due to chromosome condensation (Supplementary Figs. S7 and S8). In fact, the nuclear intensity includes both the bound and free H4K20me1-mintbody molecules, and free molecules disperse into the cytoplasm after the nuclear membrane breaks down; a similar minimization in Fab-probe nuclear/cytoplasm ratio during M phase was shown previously [30]. Therefore, a careful interpretation is needed when cells undergo dynamic changes in shape.

**Visualization of Xi using the H4K20me1-mintbody in living cells**

H4K20me1 has been described as one of the epigenetic marks associated with the Xi in cultured cells, in addition to H3 trimethylated at lysine 27 (H3K27me3) [16,19,20]. To investigate whether Xi can be visualized with the H4K20me1-mintbody, we used mouse embryonal carcinoma cell line MC12 that harbors Xi fused to an autosome fragment [31]. Immunofluorescence using fixed cells revealed that when the H4K20me1-mintbody was expressed in MC12 cells, the probe was enriched on Xi together with H3K27me3 and H4K20me1 (detected by the specific antibodies) (Fig. 4a). Using metaphase chromosome spreads, we also observed that the H4K20me1-mintbody was enriched on the arm of Xi, but not on Hoechst-dense pericentromeric major satellite regions (Fig. 4b). Time-lapse imaging of H4K20me1-mintbody indeed allowed us to visualize in single cells the dynamic behavior of Xi during the cell cycle, including mitosis (Fig. 4c). Enrichment of the H4K20me1-mintbody on Xi was also observed in hTERT-RPE1 cells, a human pseudo-diploid female cell line immortalized with telomerase (Supplementary Fig. S9). In addition, accumulation of H4K20me1 on centromere was visualized using the H4K20me1-mintbody (Supplementary Fig. S9), as previously reported [21].

Making use of the detectability of Xi by the H4K20me1-mintbody, we monitored the exact timing of Xi replication by time-lapse imaging using MC12 staining.
cells that stably express both the H4K20me1-mintbody and mCherry-PCNA (Fig. 4d). In early S phase, numerous PCNA foci were observed in euchromatic regions and were excluded from Xi. As cells progressed through mid-S phase, PCNA foci appeared preferentially around the nuclear periphery and were also enriched on Xi. A few hours later, we observed the appearance of larger PCNA foci and their separation from Xi. Replication of Xi often began at precisely the middle of S phase.

Fig. 4. Visualization of Xi in living cells. (a) H4K20me1-mintbody was expressed in mouse embryonal carcinoma MC12 cells, which harbor one or two Xi. The cells were fixed and stained with antibodies against H3K27me3 (Cy3) and H4K20me1 (Cy5), and with Hoechst33342. IF, immunofluorescence. (b) Chromosome spreads. Metaphase chromosomes were prepared by a cytospin and stained with Hoechst33342. (c) Time-lapse images of mitosis. Elapsed times are indicated (hh:mm). (d) Representative images of MC12 cells expressing both H4K20me1-mintbody and mCherry-PCNA. The timing of Xi replication was judged by the co-localization of H4K20me1-mintbody and mCherry-PCNA. Arrowheads indicate Xi, which replicated in the middle of S phase (Mid-S). (e) The timing and length of Xi replication in S phase. The period of PCNA foci appearance (“S phase,” red bars) and the timing of co-localization with Xi foci (“Xi replication,” yellow bars) are shown (nine individual cells and the averages with the standard deviations). The scale bars represent 10 μm.
and was complete in 2–3 h, allowing ~2 additional hours to complete S phase (Fig. 4e).

**Visualization of Xi during early development in C. elegans**

Finally, to demonstrate that the H4K20me1-mintbody can also be utilized in living multicellular organisms, we used C. elegans as a model. In XX hermaphrodite worms, X-linked gene expression is twofold repressed by a dosage compensation complex to equalize gene dosage with XO males [32]. H4K20me1 has been reported to be enriched in X chromosomes in hermaphrodites and to participate in the repression [33]. We generated a transgenic nematode line that expressed the H4K20me1-mintbody. The worms developed normally and were fertile, indicating that the mintbody expression did not have inhibitory effects. We found that the H4K20me1-mintbody was present in nuclei with subtle concentration patterns in the 120-cell and Bean stages (Fig. 5; 1 and 2). After the Comma stage, however, two bright foci were clearly observed in each nucleus (Fig. 5; 3–6). The transition in H4K20me1 localization during development has been demonstrated in a previous study using an H4K20me1-specific antibody [33]. It is therefore likely that the change in mintbody localization is not due to differences in accessibility of the mintbody to the chromosomes, but instead reflects true localization of H4K20me1. This result demonstrates the utility of our new H4K20me1 probe as a tool to visualize changes in chromosome structure and dosage-compensated X chromosomes in C. elegans.

**Structural and mutational analysis of the H4K20me1-specific mintbody**

Previous studies [34–36] have demonstrated that small differences in amino acid sequence can affect the folding and/or stability of functional scFv expressed in living cells. In addition to the clone 15F11/CMA421 used to generate the H4K20me1-mintbody, we also obtained from the same immunized mouse two other hybridoma clones, 12C8 and 22G3. The deduced amino acid sequences of V\(_H\) and V\(_L\) were highly similar among these clones, and the diversity was probably due to somatic hypermutation (Supplementary Fig. S10), as previously reported [5]. When expressed in HeLa cells in scFv-GFP form, these proteins failed to accumulate in nuclei (Fig. 6a and Supplementary Fig. S12a), suggesting that these two scFv-GFP proteins are less stable and/or more prone to aggregate than the H4K20me1-mintbody derived from 15F11/CMA421.

To identify structure–function relationships that impact intracellular scFv expression, we performed mutational and structural analyses. H4K20me1-mintbody and 12C8 scFv-GFP differed in only five amino acids, four of which were located in the framework regions (FRs; dark blue and pink in Fig. 6b) and the remaining one in a complementarity determining region (light blue in Fig. 6b). We then investigated the functional impact of these amino acids in living cells by introducing point mutations and assessing effects on chromatin binding, or nucleoplasmic enrichment. We replaced each of the five 12C8-specific amino acids with the corresponding residues present in the 15F11

**Fig. 5.** Visualization of dosage-compensated X chromosomes in C. elegans with H4K20me1-mintbody. H4K20me1-mintbody was stably expressed in C. elegans. Representative images at various developmental stages, from Bean to threefold, are shown (maximum projection images of 12 sections and single sections). The scale bar represents 10 μm. Magnified images of six different positions (numbered boxes) are also shown. The scale bar in magnified images represent 5 μm.
When expressed in HeLa cells, only the 12C8 G99A mutant exhibited nucleoplasmic concentration in the transfected cells (Fig. 6c), pointing to a key role for A99 in the function of 15F11. However, a reciprocal 15F11 mutant (A99G) was still in the nucleoplasm, although it diffused more into the cytoplasm (Fig. 6d). We then generated double mutants that combined A99G and I95M mutations (I95M-A99G) and found that this 15F11 mutant was no longer concentrated in the nucleoplasm (Fig. 6d). Thus, both I95 and A99 are critical for the intracellular function of the 15F11-derived H4K20me1-mintbody.

While the I95 and A99 residues are clearly important for the robust stability and/or folding of the H4K20me1-specific mintbody in living cells, they may differentially affect binding to H4K20me1. Therefore, we measured the binding affinities of different H4K20me1-specific antibodies in their IgG and scFv forms, using surface plasmon resonance (SPR). In their native IgG form, 15F11 and 12C8 showed similar profiles and affinities (52 and 33 pM, respectively, by two-state fitting), whereas 22G3 was much weaker (15 nM) (Supplementary Fig. S11). To purify scFv versions of each, His6-tagged constructs were expressed in Escherichia

Fig. 6. Mutational and structural analysis of H4K20me1-mintbody. (a) Intracellular localization of H4K20me1-mintbody. H4K20me1-mintbody (derived from 15F11) and 12C8-scFv-EGFP were expressed in HeLa cells. 12C8-scFv-EGFP was not functional in the cells. (b) The position of amino acid substitutions between 15F11 and 12C8. (c) The localization of 12C8-scFv-EGFP mutants. Each of the five amino acids in 12C8 was changed to the corresponding amino acid in 15F11 (R32S, M95I, G99A, D105H, and A150T) and expressed in HeLa cells. The nuclear localization of G99A mutant suggests that this mutation significantly improved the stability and/or folding in cells. (d) The localization of H4K20me1-mintbody mutants. Either I95M or A99G mutation alone did not abolish the nuclear localization, although the nuclear signal was relatively weakened. The combinatorial mutation was not concentrated in nuclei. (e) The crystal structure of 15F11-scFv at 1.94 Å resolution. VH and VL are shown in light blue and pink, respectively. The amino acids that differ in 12C8 are indicated in yellow. Space-filling models of two important residues (I95 and A99; letters shown in red) are blown-up to the side. The oxygen and nitrogen atoms are colored in red and dark blue. The scale bars represent 10 µm.
coli. Although a substantial fraction of 15F11-scFv was recovered in the cytosolic extract, most 12C8-scFv and 22G3-scFv remained in the insoluble pellet (Supplementary Fig. S12b), suggesting that 12C8 and 22G3 are more prone to aggregate than 15F11 in bacteria, as in mammalian cells. We then purified scFvs from pellets by denaturation and renaturation. 15F11 and 12C8 were successfully purified (Supplementary Fig. S12c), although 22G3 aggregated during renaturation. According to SPR, the affinities of 15F11 and 12C8 in scFv forms were again similar to each other (250 nM and 100 nM, respectively, by two-state fitting), although they were more than 3 orders of magnitude lower than those of the counterpart IgG forms. These results are consistent with the view that the difference between 15F11 and 12C8 is their stability and/or folding inside cells rather than their affinity to target modifications.

To investigate the role of these amino acids in the structure of H4K20me1-mintbody, we determined the crystal structure of recombinant 15F11 scFv, which were purified from the soluble fraction of E. coli lysate (Supplementary Fig. S1a). The crystal structure of the scFv was solved at a resolution of 1.94 Å (Fig. 6e). Structural analysis revealed that both I95 and A99 are located inside the immunoglobulin fold. I95 is in hydrophobic contact with Y97 and is located near Q41, G116, T117, and T118 (Fig. 6e). Interestingly, the side chain of I95 is exposed to the solvent. In contrast, A99 is buried in the hydrophobic core composed of V39, F110, and W113 (Fig. 6e). When A99 is replaced with glycine, this hydrophobic interaction is likely loosened due to absence of the side-chain methyl-group (Supplementary Fig. S13). These observations suggest that A99 plays a key role in stabilizing the H4K20me1-mintbody.

**Discussion**

In this study, we developed an H4K20me1-mintbody, a new genetically encoded antibody-based probe specific for the detection of H4K20me1. From various in vitro and in vivo analyses, we concluded that the H4K20me1-scFv and H4K20me1-mintbody retain the original IgG’s specificity to H4K20me1. The H4K20me1-mintbody allowed us to monitor changes in the level of H4K20me1 during the cell cycle and localization of Xi. Furthermore, dosage-compensated X chromosomes were observed in living transgenic nematodes that express the H4K20me1-mintbody.

**The structure of H4K20me1-specific variable fragments**

Cytoplasmic expression of antibody fragments has been challenging because the cytoplasmic environment differs from the endoplasmic reticulum, where antibodies are naturally folded and processed. This difference can cause low solubility and instability, often leading to aggregation [37]. In the present study, H4K20me1-mintbody (derived from 15F11) functioned properly in the cytoplasm, but 12C8-scFv-GFP and 22G3-scFv-GFP did not. The heavy-chain variable regions of the H4K20me1-specific antibodies were very similar to the human V_{H}3 type shown to be thermodynamically stable and less prone to aggregation by the systematic studies of human combinatorial antibody libraries [34,38,39]. Importantly, key amino acids present in the framework regions were conserved, including E8 (corresponding to amino acid H6 in the Kabat numbering system and H6 in the AHo numbering system [34,40], written as “E8(H6/6))”, F31(H29/31), V39(H37/44), R69(H66/77), D92(H85/100), and T117(H107/143) (Supplementary Fig. S10). In addition, R100(H94/108) and D111(H101/139) at the edges of complementarity determining region 3, which can make a salt bridge [34,37], were conserved. These features were common to all three H4K20me1-specific antibody clones, suggesting that these proteins had high potential as functional intracellular antibodies [36]. However, conservation of framework regions was not sufficient, as only 15F11 was functional in living cells. Crystallographic and mutational analyses revealed that A99(H93/97) is critical in the formation of a hydrophobic core with V39, F110(H100e/119), and W113(H103/139). Replacement of G99(H93/97) with alanine in 12C8 likely strengthened hydrophobic interactions and greatly improved functionality, as judged by the nucleoplasmic accumulation of the G99A mutant. A structure model of A99G reveals that hydrophobic contacts with the residue at position 99(H93/97) are indeed greatly reduced (Supplementary Fig. S13). Alanine at the H93/97 position is conserved in human V_{H}3, and the hydrophobic core involving this alanine may be important for the general stability of the scFv structure. However, substitution of A99(H93/97) with glycine in 15F11 did not completely prevent nucleoplasmic accumulation, although the signal was relatively weak and diffused. Hence, in addition to A99(H93/97), 15F11 functionality was assisted by I95(H89/103), which associates with Q41(H39/45), Y97(H91/105), G116(H106/142), T117(H107/143), and T118(H108/144). Although Q41(H39/45), Y97(H91/105), G116(H106/142), and T117(H107/143) are conserved amino acids in human V_{H}3, I95(H89/103) and T118(H108/144) are not. The I95(H89/103)-mediated intramolecular hydrophobic interaction may have a supporting function in the mintbody stability in living cells. It would be interesting to investigate how these variations affect the stability and folding of V_{H}3-type scFv.

An overall negative charge at cytoplasmic pH and low hydrophilicity has been proposed to improve the solubility of intracellular antibodies [41]. However, the net charge and hydrophilicity (GRAVY score [41]) of H4K20me1-scFvs are 0, −0.389, and −0.1 and...
In this study, we demonstrated that Xi can be visualized in living cells using our newly developed H4K20me1-mintbody. This strategy, combined with mCherry-PCNA imaging, allowed precise determination of Xi replication timing at the single-cell level. In mouse MC12 cells, Xi replication took place in mid-S phase, a period during which facultative heterochromatin was replicated, in agreement with a previous study in mouse C2C12 cells [42]. It has recently been suggested that replication of Xi is rapid, random, and less organized than that of actively transcribed genome regions [43], and that this feature may account for increased mutation rates. Enrichment of H4K20me1 on Xi may represent involvement of this modification in DNA damage repair. Future studies using H4K20me1-mintbody combined with probes targeting DNA damage repair proteins and their modifications (e.g., gamma-H2A.X) may reveal the relationship between H4K20me1 and DNA damage repair on Xi during DNA replication. Utilization of the H4K20me1-mintbody in differentiating cells is also anticipated to help visualize the process of Xi formation.

Two mintbodies have been developed to date, including the H4K20me1-mintbody described here and a previously reported H3K9ac-specific mintbody [5,6]. A recent in vivo study in transgenic frogs using the H3K9ac-mintbody has detected changes in acetylation during notochord regeneration [7], thus demonstrating the high potential of this genetically encoded system. Further development of additional mintbodies directed against diverse histone post-translational modifications will facilitate the identification of regulatory mechanisms that control epigenetic modifications in vivo.

Materials and Methods

Plasmid construction

To generate the H4K20me1-mintbody expression vectors, cDNAs encoding variable regions of heavy and light chains (VH and VL) of specific antibodies against H4K20me1 (CMA421/15F11, 12C8, and 22G3) were cloned as previously described [5]. VH and VL cDNAs were connected into a single chain through a linker encoding 3× GGGGS. The resulting DNA was subcloned into the pEGFP-N2 vector (Clontech). Nucleotide sequence data of scFvs (CMA421/15F11, 12C8, and 22G3) are available in several public databases (DDBJ/EMBL/GenBank) under the accession numbers LC129890, LC129891, and LC129892, respectively. Amino acid substitution mutants were generated using a Quick Change kit (Stratagene). For expression of enzymes that control H4K20 methylation, Halo-PHF8, Halo-PR-Set7 (SETD8), Halo-SUV420H1, and Halo-SUV420H2 (Kazusa DNA Research Institute; FlexiHaloTag clone FHC01149, FHC06789, FHC01413, and FHC26822) were used. For Figs. 3 and 4, a pMX-puro-based expression vector carrying mCherry-PCNA [44] was co-transfected with the H4K20me1-mintbody expression vector. For analyzing enrichment of H4K20me1-mintbody on centromeres, hTERT-RPE1 cells were co-transfected with expression vectors for CENP-A-TagRFP [45] and H4K20me1-mintbody.

Histone modification antibodies

Mouse monoclonal antibodies anti-H4K20me1 (CMA421), anti-H4K20me2 (CMA422), anti-H4K20me3 (CMA423) [22], anti-H3K4me1 (CMA302) [46], anti-H3K9me1 (CMA316) [11], anti-H3K27me1 (4C4), and anti-H3K27me3 (CMA323) [4] were used. For immunofluorescence, antibodies were labeled with Alexa Fluor 488 (Thermo Fisher Scientific), Cy3 (GE Healthcare), or Cy5 (GE Healthcare). For H3K9me1 (CMA316), unlabeled IgG and Cy5-conjugated goat anti-mouse Fc secondary antibodies (Jackson Immunoresearch) were used.

Protein purification and biochemical analysis

The DNA fragments encoding 15F11-scFv, 12C8-scFv, and 22G3-scFv were cloned into the pET15b vector (Novagen), which includes a His6 tag at the N-terminus. His6-15F11-scFv was expressed in E. coli BL21 (DE3) at 25 °C. Bacterial cells producing His6-scFv were harvested by centrifugation and then disrupted by sonication in 50 mM Tris–HCl (pH 7.5) buffer, containing 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol. After centrifugation, precipitates were denatured in 50 mM Tris–HCl (pH 8.0) buffer, containing 500 mM NaCl, 7 M Gdn–HCl, and 5% glycerol. After centrifugation, the supernatants were mixed with the Ni-NTA agarose (Qiagen) by rotating at 4 °C for 1 h. The beads were packed into an Econo-column (Bio-Rad) and washed with a 50-column volume of 50 mM Tris–HCl (pH 7.5) buffer, containing 500 mM NaCl, 5 mM imidazole, 6 M urea, 10% glycerol, and 2 mM 2-mercaptoethanol. His6-scFvs were eluted with a 50-column volume linear gradient of 5–500 mM imidazole in 50 mM Tris–HCl...
(pH 7.5) buffer, containing 500 mM NaCl, 6 M urea, 10% glycerol, and 2 mM 2-mercaptoethanol. The His6-scFvs were refolded during the dialysis against 20 mM Tris–HCl (pH 7.5) buffer, containing 1 mM EDTA, 200 mM NaCl, 2 mM 2-mercaptoethanol, and 10% glycerol at 4 °C. The resulting His6-scFv proteins were further purified by gel filtration column chromatography (Superdex 75 HiLoad 16/60 prep grade; GE Healthcare) and eluted with phosphate-buffered saline (PBS). The purity was checked by 16% SDS-PAGE (Supplementary Fig. S12c).

For preparation of 15F11-scFv for Western blot analysis, histone modification arrays, immunofluorescence of yeast cells, and crystallization, the E. coli cells producing His6-15F11-scFv were disrupted by sonication in 50 mM Tris–HCl (pH 7.5) buffer, containing 500 mM NaCl, 1 mM PMSF, and 10% glycerol. After removing cell debris by centrifugation, the supernatant was collected and mixed with Ni-NTA agarose by rotating at 4 °C for 30 min. The beads were washed with 50-column volume of 50 mM Tris–HCl (pH 7.5) buffer, containing 500 mM NaCl, 5 mM imidazole, 1 mM PMSF, 10% glycerol, 0.01% Triton X-100, and 2 mM 2-mercaptoethanol. His6-15F11-scFv was eluted with a 50-column volume liner gradient of 5–500 mM imidazole in 50 mM Tris–HCl (pH 7.5) buffer, containing 500 mM NaCl, 1 mM PMSF, 10% glycerol, 0.01% Triton X-100, and 2 mM 2-mercaptoethanol. The His6 tag peptide was cleaved by thrombin protease (GE Healthcare) during the dialysis against 20 mM Tris–HCl (pH 7.5) buffer, containing 1 mM EDTA, 200 mM NaCl, 2 mM 2-mercaptoethanol, and 10% glycerol at 4 °C. The resulting 15F11-scFv protein was further purified by gel filtration column chromatography (Superdex 75 HiLoad 26/60 prep grade; GE Healthcare) and eluted with PBS. The purity was checked by 16% SDS-PAGE (Supplementary Fig. S12c).

For Western blot analysis (Supplementary Fig. S1b), recombinant histone H4 proteins harboring mono-, di- and trimethylation of lysine K20 (Active motif; 10–20 ng/lane for antibodies, 100 ng/lane for scFv) were separated on 15% SDS-polyacrylamide gels. Proteins were transferred onto a polyvinylidene difluoride membranes in 100 mM Tris–HCl, 192 mM glycine, and 5% methanol, using a semidry transfer system. After blocking with Blocking One (Nacalai Tesque) for 20 min, membranes were incubated in H4K20me1-specific (15F11/CMA421; 1 μg/ml), H4K20me2-specific (CMA422; 1 μg/ml), and H4K20me3-specific (CMA423; 0.2 μg/ml) mouse monoclonal antibodies [22] for 1 h at room temperature, and then in horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Immunoresearch) for 1 h at room temperature. When probed with the 15F11-scFv, membranes were incubated in 5 μg/ml scFv at 4 °C overnight, and then in HRP-conjugated goat anti-mouse IgG. For Supplementary Fig. S1c and d, a peptide array (AbSurance Complete Core Histone Antibody Specificity Array; Millipore) was incubated in 15F11-scFv (2 μg/ml) overnight and then in HRP-conjugated goat anti-mouse IgG. Signals were detected using an enhanced chemiluminescent system (Immunostar LD; Wako Purechemicals).

SPR signals (Supplementary Figs. S11 and S12d) were measured using a Biacore X100 (GE Healthcare), as described previously [5]. H4K20me1 synthetic peptide (GKKGAKRHR(me1-K)VLRDNIQG) (Sigma-Aldrich) was conjugated to a CM5 sensor chip (GE Healthcare) (for scFv, 87.1 RU; for IgG, 16.9 RU) by amine coupling in PBS containing 0.005% Tween20 as a running buffer. For the binding assay, single cycle kinetics was performed, and different concentrations of antibodies (for scFv: 15.625, 31.25, 62.5, 125, and 250 nM; for IgG: 3.125, 6.25, 12.5, 25, and 50 nM) were successively injected at 30 μl/min. The resulting curves were analyzed with a two-state reaction model.

**Crystallization and structure determination**

Purified 15F11-scFv was concentrated to 30 mg/ml, dialyzed against 20 mM Bis–Tris–HCl (pH 6.0) buffer, containing 100 mM NaCl, and crystallized using the hanging drop vapor diffusion method. One microliter of purified 15F11-scFv was mixed with an equal volume of reservoir buffer (100 mM sodium acetate [pH 5.2], 7% poly-y-glutamic acid polymer, and 12% PEG2000) at 20 °C and crystal growth was observed in 2 weeks. Crystals were soaked into the cryoprotectant solution (100 mM sodium acetate [pH 5.2] and 30% PEG3000) and flash-cooled in a stream of N2 gas (100 K). The dataset was collected at the BL-1A, BL-5A, and BL-17A beamlines in the Photon Factory (KEK) and the BL41XU beamline in SPring-8. Diffraction data were indexed, integrated, and scaled using the HKL2000 program [47]. The structure of 15F11-scFv was solved by the molecular replacement method, using the Phaser program [48] and the PDB entry 2GHW as the search model [49]. Manual model building was performed using the COOT program [50]. Refinements of the models were performed using the PHENIX program [51]. The final structure contains no Ramachandran outliers, assessed by the MolProbity program (Supplementary Table I) [52]. All protein structure figures were prepared with PyMOL (Schrödinger®).

**Cell culture**

Cells were routinely grown in Dulbecco's modified Eagle's medium (Nacalai Tesque) with supplements (L-glutamine/penicillin/streptomycin [Sigma-Aldrich] and 10% fetal calf serum) at 37 °C under 5% CO2 atmosphere. Mouse embryonal carcinoma cell line MC12 was kindly provided by Dr. Nobuo Takagi [31].
Transfection of plasmid DNA was performed using Fugene HD (Promega), and cells stably expressing mintbody were selected in 1 mg/ml G418 (Nacalai Tesque). For live-cell imaging, cells were grown on a 35-mm glass bottom dish (MatTek) or a 24-well glass bottom plate (Iwaki), and the medium was replaced with FluoroBrite Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific) containing supplements.

**Growth curve and cell cycle profile**

HeLa cells stably expressing H4K20me1-mintbody and the parental cells were plated on a six-well dish (3 wells each) at a concentration of $2 \times 10^5$ cells/ml (2 ml). Cells were harvested in the original volume every 12 h and cell concentration was counted using an EVE automatic cell counter (Nano EnTek). Cell concentrations from 3 wells were averaged to obtain the result of a single experiment. Three independent experiments were performed to obtain the mean and the standard deviation. For cell cycle profiling, cells grown on a glass bottom dish were incubated in 10 μM EdU (Thermo Fisher Scientific) for 30 min, fixed with 4% formaldehyde (Electron Microscopy Sciences) in 250 mM Hepes–NaOH (pH 7.4; Wako Purechemicals) containing 0.1% Triton X-100 for 5 min, and permeabilized with 1% Triton X-100 in PBS for 20 min at room temperature. EdU was labeled with Alexa Fluor 647 azide for 30 min, using Click-iT EdU Imaging Kit (Thermo Fisher Scientific), and DNA was stained with 1 μg/ml Hoechst33342 in PBS for 20 min, before imaging using a wide-field fluorescence microscope (Ti-E; Nikon; operated by NIS-elements software), equipped with an EM-CCD (iXON+ DU897 X-3019, Andor), a 40× Plan Apo (NA 0.95) lens. Hoechst33342 and Alexa Fluor 647 were detected using DAPI-1160A (Semrock; Ex FF01-387/11; DM FF02-447/60; Em FF409-Di02) and Cy5-4040A (Semrock; Ex FF01-387/11; DM FF02-447/60; Em FF409-Di02) filter sets. The nuclear intensities of fluorescence signals were automatically defined by thresholding and fluorescence intensities in individual nuclei were quantified. For images of H4K20me1-mintbody, the ROI areas of the nuclei and cytoplasm were manually defined. The net intensity was obtained by background correction. The average cell cycle fractions (with the standard deviations) were obtained from three independent experiments.

**(Immunofluorescence)**

Cells were fixed with 4% formaldehyde (Electron Microscopy Sciences) in 250 mM Hepes–NaOH (pH 7.4; Wako Purechemicals) containing 0.1% Triton X-100 for 5 min at room temperature, permeabilized with 1% Triton X-100 in PBS for 20 min at room temperature. After blocking with Blocking One-P (Nacalai Tesque) for 20 min at room temperature, cells were incubated with 2 μg/ml fluorescence dye (Alexa Fluor 488, Cy3, or Cy5)-conjugated mouse monoclonal antibody for 2 h at room temperature. After washing with PBS three times, cells were incubated with 0.25–1 μg/ml Hoechst33342 for 20 min. After washing with PBS three times, fluorescence images were collected. For Supplementary Fig. S5a, a glass bottom dish with a grid (Matsunami) was used to match cells in time-lapse and immunofluorescence images. For Fig. 2b–d and Supplementary Fig. S3, 24 h after transfection with HaloTag expression vectors, cells were stained with 2–4 μg/ml antibodies, 0.1 μM HaloTag tetramethylrhodamine ligand (Promega), and 0.25 μg/ml Hoechst33342 for 2 h at room temperature.

**(Metaphase chromosome spreads)**

MC12 cells stably expressing the H4K20me1-mintbody were incubated with colcemid (50 ng/ml; Nacalai Tesque) for 1.5 h, and mitotic cells were collected by pipetting and brief centrifugation. Mitotic cells were swelled using hypotonic buffer (75 mM KCl) for 10 min at room temperature. Hepes–NaOH (Wako Purechemicals; 250 mM final concentration, pH 7.4) and formaldehyde (Electron Microscopy Sciences; 0.4% final concentration) were added just before the cytospin (WESCO; 1000 rpm, 2 min). Chromosome spreads were fixed for 5 min at room temperature, and then stained with 20 ng/ml Hoechst33342 in PBS for 10 min at room temperature.

**(Microscopy for mammalian cells)**

Fluorescence images were collected using confocal microscopes: an FV1000 (Olympus; operated by the built-in FV1000 software FLUOVIEW ver.4.2), a Ti-E (Nikon; operated by NIS-elements software) with a spinning disk (CSU-W1; Yokogawa) and an EM-CCD camera (iXON3 DU888 X-8465, Andor), or an IX-71 (Olympus) equipped with a spinning disk (CSU-X1; Yokogawa) and an EM-CCD camera (iXON3 DU897E-CS0-#BV-Y, Andor). Live-cell imaging was performed with a heated stage (Tokai Hit) and a CO2-control system (Tokken). For Figs. 1b and 2e, and Supplementary Figs. S5b and S7b, 1 μg/ml Hoechst33342 was added to cells 1 h before imaging. Acquisition parameters are shown in Supplementary Table II. Quantification of fluorescence intensities was performed using NIS-elements analysis software (Nikon). For immunofluorescence images, nuclei were automatically defined by thresholding and fluorescence intensities in individual nuclei were quantified. For images of H4K20me1-mintbody, the ROI areas of the nuclei and cytoplasm were manually defined and the fluorescence intensities were quantified. The net intensity was obtained by background...
Live-Cell Imaging of H4K20 Monomethylation

subtraction. To compare the nuclear enrichment of H4K20me1-mintbody in different cells, the nuclear/cytoplasm ratio was frequently used. The nuclear/cytoplasm ratio could be over-influenced by a cytoplasmic intensity that is too low and variable, but the actual intensity counts a few hundred after background subtraction (in a 14-bit scale) and the deviation is comparable to or lower than the nuclear intensity. The statistics of the nuclear and cytoplasmic intensities used for Fig. 3e are shown in Supplementary Fig. S14, as an example. For Supplementary Fig. S4, co-localization analysis was performed using NIS-elements analysis software (Nikon).

Fission yeast experiments

An S. pombe strain (HA1039-3D, h− lys1–131) was used as wild type. For set9+ gene disruption, a gene disruption fragment was amplified by PCR using a G418-resistant gene cassette, and used for transformation of the wild type strain [53]. An H4K20A mutant strain was constructed according to a previous study with some modifications [54]. Briefly, a pair of histone H4.2/H3.2 genes (hht2′−hht2′) was cloned into a plasmid DNA, and an H4.2 K20A mutation was introduced by PCR-mediated site-directed mutagenesis to convert the 20th codon AAG to GCT. The genomic hht2′−hht2′ gene was disrupted by replacement with the ura4+ marker gene, and the ura4+ marker gene was further replaced with the mutant gene. H4.2 K20A mutant cells were selected in a medium containing 5-fluoroorotic acid, which is toxic for ura4+ cells. To disrupt the other two histone H4/H3 genes, H4.1/H3.1 and H4.3/H3.3 were replaced with G418-resistant and nourseothricin-resistant genes [55]. YES and EMM2 were used as complete and minimal media, respectively [56]. To express GFP-fused 15F11-scFv in S. pombe cells, the scFv-coding sequence was inserted downstream of the β-tubulin (nda3+) gene promoter and fused to GFPs65t. The resulting fusion gene was then integrated into the genomic lys1 locus. For Western blot analyses, whole cell extracts were prepared according to the following procedure. S. pombe cells were grown until they reached the log phase and further incubated in the presence of 1 mM PMSF for 10 min. 5 × 10^7 cells were harvested and resuspended in 800 μl of ice-cold water. The cell suspension was mixed with 150 μl of 2 M NaOH, incubated for 10 min on ice, added with 150 μl of 55% (w/v) trichloroacetic acid, mixed, and incubated for a further 10 min on ice. After centrifugation at 10,000 rpm for 2 min at 4 °C using a microcentrifuge, the pellet was washed with cold acetone and then resuspended in SDS-polyacrylamide gel sample buffer (60 mM Tris–HCl [pH 6.8], 2% SDS, 100 mM DTT, 10% glycerol, and bromophenol blue). A volume equivalent to 2 × 10^6 cells from each sample was separated on 15% SDS-polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes in 100 mM Tris–HCl, 192 mM glycerol, and 5% methanol, using a semidy transfer system. Membranes were incubated in H4K20me1-specific (15F11/CMA421; 1 μg/ml) and pan-H4 (CMA400; 0.1 μg/ml) mouse monoclonal antibodies [22], and a GFP-specific rabbit polyclonal antibody (Rockland Immunochemicals Inc.; 0.25 μg/ml), and then in HRP-conjugated goat anti-mouse or anti-rabbit IgG (GE Healthcare). For immunoblot experiments with the 15F11-scFv, membranes were incubated in 20 μg/ml scFv overnight at 4 °C, and then in HRP-conjugated goat anti-mouse IgG.

Live-cell imaging was performed at 26 °C using a DeltaVision fluorescence microscopy system (Applied Precision), which is built on a wide-field fluorescence microscope IX71 (Olympus), equipped with a 60× PlanApoN (NA 1.40) oil-immersion lens and a CoolSNAP HQ2 CCD (Photometrics). Cells were immobilized as described previously [57]. Images were deconvolved using SoftWoRx software.

Fluorescence microscopy using anti-H4K20me1 antibody (CMA421) and 15F11-scFv was done as follows. Cells grown in complete medium were harvested and fixed using 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2) for 10 min at room temperature. After cell wall digestion by 0.6 mg/ml zymolyase 100T (Nacalai tesque) in PEMS buffer (100 mM Pipes, 1 mM EGTA, 1 mM magnesium chloride and 1.2 M sorbitol) for 70 min at 36 °C, cells were permeabilized by 1% Triton X-100 in PEMS for 2 min at room temperature. Blocking was done with 1% bovine serum albumin for 30 min at room temperature. For staining with CMA421, cells were incubated with 1 μg/ml antibody for 1 h at room temperature. For staining with 15F11-scFv, cells were incubated with 100 μg/ml scFv overnight at 4 °C, although the same result was obtained by incubation with 20 μg/ml scFv. An Alexa488-conjugated goat anti-mouse antibody (GE Healthcare) was used as a secondary antibody. Chromatin was stained with a 0.2 μg/ml 4′,6-diamidino-2-phenylindole. Cells were observed using DeltaVision fluorescence microscopy system (Applied Precision).

Expression and imaging of the H4K20me1-mintbody in the C. elegans embryos

The DNA sequence of 15F11-scFv was synthesized, taking into consideration codon usage in C. elegans (GeneArt Gene Synthesis; Thermo Fisher Scientific). To generate an expression vector compatible with Mos1-mediated single copy insertion (MosSCI), the following four segments were joined using a Gateway Compatible Plasmid Toolkit and Multisite Gateway Technology (Thermo Fisher Scientific), as previously described [58]. The segments joined were as follows: (i) mex-5′ untranslated region, (ii) codon-optimized 15F11-scFv sequence, (iii) mex-5′ 3′ untranslated region following to gfp F64LS65T, and (iv) MosSCI
integration site followed by unc-119* rescue marker. Using the MosSCI technology, the resulting vector was integrated into the unc-119 (ed3) mutant genome. The integrated lines were screened according to previously described protocols [59]. Strains were maintained at 22 °C or 26 °C. For microscopic imaging, adult hermaphrodites were dissected in M9 buffer and released embryos were transferred onto a 2% agarose pad mounted on a glass slide and covered with a coverslip. Fluorescence signals of H4K20me1-mintbody were collected at room temperature using a microlens-enhanced spinning-disk confocal unit (CSU-X1; Yokogawa) mounted on an inverted microscope (IX71; Olympus) equipped with a 100× UPlanSApo (NA 1.40) lens and an EM-CCD camera (iXon; Andor), and controlled by MetaMorph imaging software (ver 7.7.10.0; Molecular Devices). z-Stacks of 12 planes with 2-μm intervals were collected using a 488-nm laser line (100-ms exposure). Stacked images, which were composed by maximum projection of seven to nine serial z-planes, and extracted single section images (Fig. 5) were generated using MetaMorph imaging software (ver 7.7.10.0).

DNA and Protein Data Bank accession numbers

LC129890, LC129891, LC129892, PDB ID: 5B3NPDB.

Author Contributions

Y.S. and H. Kimura conceived the study, designed the experiments, and wrote the manuscript. Y.S. performed most experiments and analyzed data. T.K. and N.H. performed structural and biochemical analyses under the supervision of H. Kurumizaka. R.A. performed nematode experiments under the supervision of A.K. H.A. and C.O. performed yeast experiments under the supervision of T.H. and Y.H. K.Y. and J.U. collected some images using a spinning disk confocal microscope. T.N. provided HaloTag expression vectors.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2016.08.010.

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http://www.pymol.org

Abbreviations used:
H4K20me1, histone H4 Lys20 monomethylation; Mintbody, modification-specific intracellular antibody; scFv, single-chain variable fragment; PCNA, proliferating cell nuclear antigen; FR, framework region; Xi, inactive X chromosome.

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