INTRODUCTION

Ciliates are unique unicellular eukaryotes that contain two functionally and structurally distinct nuclei: a macronucleus (MAC) and a micronucleus (MIC) (Karrer, 2012; Orias, Cervantes, & Hamilton, 2011). The MAC is the larger nucleus and has somatic functions; it contains thousands of fragmented small chromosomes generated by programmed DNA rearrangement, which eliminates MIC-specific internal sequences (Chalker, 2008; Chalker, Meyer, & Mochizuki, 2013). The MAC divides by amitosis, and its chromosomes are transcriptionally active during all stages of the life cycle. In contrast, the MIC is the smaller nucleus bearing a diploid genome, with germline functions, and divides by mitosis.
MIC chromosomes remain transcriptionally inactive during all vegetative stages and are involved in the process of sexual reproduction including conjugation, meiosis and MAC regeneration. These two nuclei undergo DNA replication and nuclear division at different times within the same cell. Thus, the nuclear proteins involved in transcription, DNA replication and nuclear division must be strictly targeted to an appropriate nucleus at an appropriate time of the cell cycle.

The nuclear transport system regulating transport specific to either MAC or MIC is important for binucleated ciliates, however, the mechanisms to distinguish each of the two nuclei are largely unknown. Nuclear transport receptor proteins, importin α and importin β, with varying affinities for the MAC and MIC have been reported (Malone et al., 2008), although their involvement in the nuclear-specific transport is unclear. The nuclear pore complex, the sole gateway for nucleocytoplasmic transport, is known to be involved in the nucleus-specific transport systems are conserved in binucleated ciliates. In this study, we identified MAC-specific NLSs from Tetrahymena linker histones (MAC-specific H1 and MIC-specific Mlh1). Our findings advance the understanding of the nuclear transport systems to distinguish the two functionally distinct nuclei contained in the same cell.

2 | RESULTS AND DISCUSSION

2.1 | MAC-specific NLSs of histone H1

MAC-specific histone H1 of T. thermophila (T1H1) is an ortholog of the metazoan H1 (Hayashi et al., 1987). To understand the MAC-specific targeting domain of T1H1, we examined the subcellular localization of various T1H1 fragments tagged with GFP-GST at the N-terminus in living cells using fluorescence microscopy. Similar to the GFP-GST fusion of full-length T1H1, GFP-GST-T1H11^-87 and -T1H173^-164 were localized in the MAC, whereas GFP-GST alone as a control was not (Figure 1a,b), suggesting that both fragments contained the MAC-targeting NLSs. To find the minimal sequence required for MAC targeting, we tested the subcellular localization of shorter, GFP-tagged fragments of the T1H11^-87 and T1H173^-164 sequences (Figure 1a). Of the two fragments of T1H11^-87, 1^-47 and 41^-87, the fragment containing amino acid residues 1^-47 was localized to the MAC, but the fragment containing amino acids 41^-87 was not, suggesting that the amino acid residues 1^-47 contain the MAC-targeting NLS. To further narrow down the sequence within the 1^-47 fragment required for MAC targeting, we tested the subcellular localization of shorter fragments of T1H11^-47. Among the tested fragments, 1^-28, 20^-47, 15^-33 and 13^-30, we found that fragment 13^-30 was localized to the MAC,
FIGURE 1 Identification of MAC-specific NLS sequences in *Tetrahymena* histone H1. (a) Diagram representing various fragments of H1 tested in this study. The fragments were tagged with GFP-GST at the N-terminal end. Green and white bars represent MAC-localizing and non-MAC-localizing fragments, respectively. Numbers on the bars indicate the first and last amino acid numbers of the fragments. Shaded areas represent putative NLSs predicted by the PSORT II software. Amino acid sequences of MacNLS1 (fragment “13–30”) and MacNLS2 (fragment “101–130”) were the experimentally identified NLSs in this study. (b) Subcellular localization of GFP-GST-fused fragments shown in (a). GFP-GST alone was expressed as a control. White broken lines represent the cell outline. The asterisk indicates fluorescence accumulation in the oral cilia by unknown mechanisms. The cytoplasmic puncta appearing for some non-MAC-localizing constructs (most strikingly in 15–33) were probably caused by the cellular stress generated when the cells were squeezed between coverslips for fluorescence observation. Scale bar, 20 μm. (c) MAC localization of GFP-GST-H113–30 (MacNLS1) carrying a single amino acid substitution at the indicated amino acids. Dotted amino acids represent target amino acids for substitution. (+++) represents fluorescence signals predominantly in the MAC; (+) represents weaker fluorescence signals in the MAC and some signals in the cytoplasm; (−) represents fluorescence signals predominantly in the cytoplasm as shown in the schematic drawings. (d) MAC localization of GFP-GST-H1101–130 (MacNLS2) carrying a single amino acid substitution at the indicated amino acids. The marks are as defined in (c).
but the other fragments were not (Figure 1b). To find the minimal sequence required for MAC targeting within the \( \text{H1}_{73-164} \) fragment in the C-terminal region, we also tested the subcellular localization of shorter fragments of \( \text{H1}_{73-164} \) (Figure 1a). Among the tested fragments, 73–117, 114–164 and 101–130, we found that fragment 101–130 was localized to the MAC, but the others were not (Figure 1b). These results suggest that two independent amino acid sequences in the N-terminal and C-terminal regions of \( \text{H1} \) (residues 13–30 and 101–130, respectively) are responsible for MAC targeting. Our result is consistent with the finding that \( \text{H1} \) in human cells has two independent regions responsible for nuclear targeting (Schwamborn et al., 1998). Thus, we named these N-terminal and C-terminal minimum sequences MacNLS1 and MacNLS2, respectively.

To identify the amino acid residues critical to the MAC-targeting NLS activity in these putative NLS sequences, we examined the subcellular localization of GFP-GST fragments (\( \text{H1}_{13-30} \) and \( \text{H1}_{101-130} \)) carrying a single amino acid substitution at a lysine residue within the NLS sequences because the basic amino acid residues are known to function as critical residues in the various NLSs (reviewed in Dingwall & Laskey, 1991; Imamoto, 2000). Among the mutants at lysine residue 15, 16, 19, 20, 24, 25, 29 or 30 in MacNLS1 (dotted residues in \( ^{13}\text{REKKDHKKAPIKKAAKK}^{30} \)) tested, the mutants at residue 20, 25 or 29 were localized predominantly to the cytoplasm (designated as [−] in “MAC localization” in Figure 1c), the mutants at residue 24 or 30 weakly localized to the MAC and were also localized in the cytoplasm (designated as [+ ] in Figure 1c), and the mutants at residue 15, 16 or 19 were localized predominantly in the MAC (designated as [++] in Figure 1c). Because the first two amino acid residues, RE, are important for MAC-targeting NLS activity (see above), we concluded that RE\(^{13,14} \), K\(^{20} \), K\(^{24} \), K\(^{25} \), K\(^{29} \) and K\(^{30} \) are critical residues in the MacNLS1 sequence (Figure 1c).

In similar manner, we identified the amino acid residues critical to the MAC-targeting NLS activity in MicNLS2. Among the mutants at a lysine residue 101, 102, 108, 109, 115, 118, 119, 122, 123, 126, 129 or 130 in MacNLS2 (dotted residues in \( ^{101}\text{KKAINPGKKAAQP}^{29} \text{KST}^{30} \text{KDEVKKDKNTAKK}^{30} \)) tested, the mutants at residue 108, 109, 115, 118 or 119 were localized predominantly in the cytoplasm (designated as [−] in Figure 1d), the mutants at residue 101, 102, 122, 129 or 130 weakly localized to the MAC (designated as [+] in Figure 1d), and the mutants at residue 123 or 126 were predominantly localized in the MAC (designated as [++] in Figure 1d). Based on these results, we concluded that K\(^{101} \), K\(^{102} \), K\(^{108} \), K\(^{109} \), K\(^{115} \), K\(^{118} \), K\(^{119} \), K\(^{122} \), K\(^{129} \) and K\(^{130} \) are critical residues in the MacNLS2 sequence (Figure 1d).

To further verify the MAC-targeting NLS activity of the MacNLS1 and MacNLS2 sequences, we introduced substitutions into the putative NLS sequences: Lysine residues 24, 25, 29 and 30 in MacNLS1 (dotted residues in \( ^{13}\text{REKKDHKKAPIKKAAKK}^{30} \)) and lysines 101, 102, 108 and 109 in MacNLS2 (dotted residues in \( ^{101}\text{KKAINPGKKAAQP}^{29} \text{KST}^{30} \text{KDEVKKDKNTAKK}^{30} \)) were mutated to alanine residues (designated as MacNLS1-KA and MacNLS2-KA, respectively) (Figure 2a). GFP-GST-tagged \( \text{H1} \), bearing mutations in either or both NLSs, was expressed in Tetrahymena cells, and its subcellular localization was observed by fluorescence microscopy. GFP-GST-\( \text{H1}_{13-30} \) and GFP-GST-\( \text{H1}_{101-130} \) carrying mutations in either NLS were localized to the MAC, whereas GFP-GST-\( \text{H1}_{13-30} \) and GFP-GST-\( \text{H1}_{101-130} \) carrying mutations in both NLSs were not (Figure 2b). These results indicate that either of MacNLS1 or MacNLS2 of \( \text{H1} \) is sufficient for MAC targeting.

These two experimentally identified NLS sequences in \( \text{H1} \) were different from NLS sequences predicted by the PSORTII software (https://psort.hgc.jp/form2.html) (black-shaded regions in Figure 1a), and they did not match any of the known NLS sequences (NLSdb software; https://rostlab.org/services/nlsdb/), suggesting that MacNLS1 and MacNLS2 do not share a consensus sequence with any known monopartite or bipartite NLSs in other model organisms. In addition, the sequence homology between MacNLS1 and MacNLS2 determined using GENETYX software (GENETYX Corporation, Tokyo, Japan) was limited (Supporting Information Figure S2). Thus, these two NLSs may be recognized by different importins unique
in *Tetrahymena*. Based on these results, we concluded that *Tih*1 has two independent NLS sequences. This finding in *Tetrahymena* histone H1 is consistent with the previous finding that mammalian histone H1 contains multiple NLS-like sequences (Schwamborn et al., 1998).

### 2.2 MIC targeting and cleavage of micronuclear linker histone Mlh1

Mlh1 is a polyprotein that is expressed from the *MLH1* gene as a precursor protein with a MW of 70.6 kDa, which generates three mature proteins by cleavage. The precursor protein is first cleaved into two proteins, α and β, and then, the α protein is further cleaved into two proteins, δ and γ, to generate three mature proteins, δ, γ and β (Figure 3a). None of these proteins resemble other canonical histone H1 orthologs in the primary amino acid sequence (Wu et al., 1994). Endogenous α, β, γ and δ proteins as well as the precursor Mlh1 protein are located in the MIC (Allis et al., 1984; Wu et al., 1994).

**FIGURE 3** Identification of MIC-localizing protein fragments in the precursor form of micronuclear linker histone Mlh1. (a) Diagram represents an Mlh1 precursor protein and its three processed mature forms. Green and white colors represent MIC-localizing and non-MIC-localizing proteins, respectively. Shaded areas represent putative NLSs predicted by the PSORT II software. (b) Subcellular localization of mature protein (δ, γ or β) tagged with GFP-GST. Protein expression under the *MTTI* promoter was induced by 0.2 μg/ml CdCl₂ for 1 day. The white arrow indicates the MIC. (c) Subcellular localization of GFP-α-mCherry expressed under the vegetative *rpL29* promoter. GFP and mCherry fluorescence signals, after cleavage of the α protein to generate δ and γ proteins, are represented as GFP-δ or GFP-α (left image panel) and γ-mCherry (middle image panel), respectively. Green and red colors in the merged image represent GFP-δ and γ-mCherry, respectively. The rightmost panel represents the data of the western blotting analysis, performed with anti-mCherry antibody for the nuclear fractions of cells expressing GFP-α-mCherry (“GαC”) and wild-type cells not expressing any exogenous gene as a control (“Ctr”). The positions of molecular size markers are indicated on the left. The red arrow indicates the position of γ-mCherry. The black arrow indicates the predicted position of full-length GFP-α-mCherry. The open triangle indicates probable degradation products of mCherry. The asterisk indicates nonspecific bands. (d) Subcellular localization of GFP-Mlh1-mCherry expressed under the vegetative *rpL29* promoter. GFP and mCherry fluorescence signals, after cleavage of Mlh1 to generate three mature forms (δ, γ and β), are represented as GFP-δ (left panel) and β-mCherry (middle panel), respectively. The rightmost panel represents the merged image of the GFP-δ (green) and γ-mCherry (red) signals; yellow in the merged image indicates complete overlap of the green and red signals. Scale bars, 20 μm.
contrast, the MIC NPC has much smaller exclusion pore size (10–20 kDa) (Iwamoto et al., 2017). Thus, the GFP-γ protein (~49 kDa) did not localize to the MIC when expressed as a GFP-tagged protein (Supporting Information Figure S3). In addition, functionality or stability of the fusion protein might explain the difference in localization. We found that fluorescence signals of GFP-Mlh1 were mostly lost when the cells were fixed with 100% ice-cold methanol, probably because these GFP-tagged proteins were not functional in the MIC and thus not integrated into chromosomes. This idea is supported by the finding that none of HA-tagged-α, HA-tagged-β, HA-tagged-γ and HA-tagged-δ fragments were localized in the MAC after fixation with Schaudinn’s fixative containing 100% ethanol in a study by Qiao et al. (2017). Together, these results indicate that δ and β fragments, but not γ fragment, can be actively transported to the MIC, suggesting the presence of MIC-specific NLS sequences in the δ and β fragments.

To confirm this hypothesis, we tested the subcellular localization of α protein (a precursor of δ and γ proteins) tagged with GFP and mCherry at its N-terminal and C-terminal ends, respectively, in living cells. Fluorescence microscopic observation showed that the GFP signal (corresponding to GFP-δ) and the mCherry signal (corresponding to γ-mCherry) were both inside the MIC (Figure 3c), suggesting that α protein was first transported into the MIC and cleaved to generate GFP-δ and γ-mCherry proteins. This result is different from the previous finding that HA-tagged α protein does not localize to the MIC (Qiao et al., 2017), probably reflecting the different experimental conditions between our and their experiments (GFP-GST-tag vs. HA-tag and live vs. fixed cells). We used western blotting to confirm whether GFP-α-mCherry protein was indeed cleaved. Western blotting analysis of cells expressing GFP-α-mCherry using anti-mCherry antibody revealed a protein band with an apparent MW of 50 kDa, corresponding to γ-mCherry (~50 kDa); however, no protein band corresponding to the full-length GFP-α-mCherry protein (MW, ~100 kDa) was detected (black arrow in Figure 3c). By bad luck, western blotting of the same specimens using anti-GFP antibody failed to detect any signal; this is probably owing to the lower titer of the antibody used or unknown reasons associated with protein stability. These results suggest that GFP-α-mCherry is cleaved to generate GFP-δ and γ-mCherry proteins within the MIC and support the finding that the α protein is transported by the NLS of the δ protein and cleaved to release the γ protein in the MIC. This finding is consistent with previous reports that endogenous α protein is cleaved to generate δ and γ proteins (Allis et al., 1984; Wu et al., 1994).

We further tested full-length Mlh1 protein, tagged with GFP and mCherry at its N-terminal and C-terminal ends, respectively, to determine whether the whole precursor form of the Mlh1 protein is required for proper MIC targeting of the mature proteins. The fluorescence signals of both GFP and mCherry were predominantly localized to the MIC (Figure 3d). In an interesting manner, MIC localization of GFP-δ signal was much more evident when introduced as GFP-Mlh1-mCherry than when introduced as GFP-α-mCherry (Figure 3c) or GFP-GST-δ (Figure 3b). In addition, no mistargeted signals of both GFP and mCherry in the MAC were observed for GFP-Mlh1-mCherry (Figure 3d). These results suggest that the precursor form ensures targeting and/or retention of the MIC-specific linker histone proteins in the MIC. Taken together, our results indicate that the precursor Mlh1 is transported to the MIC through NLS sequences located in the δ and β portions and is cleaved to generate the mature δ, γ, and β proteins.

### 2.3 MIC-specific NLSs of micronuclear linker histone Mlh1

We also examined the minimum sequence bearing MIC-targeting activity within the δ and β proteins in sequential deletion experiments. First, we prepared several fragments of the δ protein as shown in Figure 4a and expressed each fragment tagged with GFP-GST at its N-terminal end in the cell. Observation of living cells by fluorescence microscopy showed that amino acid fragments 86–199 and 90–189 were localized to the MIC, whereas the other fragments (δ1–116, δ90–186, δ92–199 and δ90–114/145–189) showed slight or no MIC localization (Figure 4b). This result suggests that the amino acid fragment 90–189 is the minimum MIC-targeting sequence in the δ protein. In an interesting manner, this minimum fragment contains a whole high-mobility group (HMG)-box motif at 96–161 as annotated in accession number P40631 in NCBI database (designated as δHMG-box in Figure 4a; underlined region in Figure 5a); in mammalian cells, the HMG-box contains two independent NLS-like sequences at its N-terminal and C-terminal regions (Harley et al., 2003; Smith & Koopman, 2004). The δHMG-box motif of Mlh1 protein also overlaps with two putative NLS-like sequences (a monopartite type at 93–99 and bipartite type at 161–177) at the ends as predicted by the PSORTII software, which is similar to other HMG-boxes. However, a loss of any one of the first two amino acid residues (V90 or K91), the middle 30 residues (115–144) or the last three residues (R187, S188 and S189) of the putative MIC-targeting sequence reduced its NLS activity (Figure 4a,b), suggesting that the entire sequence δ90–189 is required for NLS activity in Tetrahymena. Based on these findings, the amino acid fragment 90–189 is the minimum MIC-targeting NLS sequence in δ protein; this fragment was named MicNLS1 (Figure 4a).

In the β protein, we also examined several fragments, as shown in Figure 4c. Fluorescence microscopy of living cells showed that amino acid fragments β108–234, β213–234 were first transported into the MIC and cleaved to generate GFP-β and γ-mCherry proteins. This result is different from the previous finding that HA-tagged α protein does not localize to the MIC (Qiao et al., 2017), probably reflecting the different experimental conditions between our and their experiments (GFP-GST-tag vs. HA-tag and live vs. fixed cells). We used western blotting to confirm whether GFP-α-mCherry protein was indeed cleaved. Western blotting analysis of cells expressing GFP-α-mCherry using anti-mCherry antibody revealed a protein band with an apparent MW of 50 kDa, corresponding to γ-mCherry (~50 kDa); however, no protein band corresponding to the full-length GFP-α-mCherry protein (MW, ~100 kDa) was detected (black arrow in Figure 3c). By bad luck, western blotting of the same specimens using anti-GFP antibody failed to detect any signal; this is probably owing to the lower titer of the antibody used or unknown reasons associated with protein stability. These results suggest that GFP-α-mCherry is cleaved to generate GFP-δ and γ-mCherry proteins within the MIC and support the finding that the α protein is transported by the NLS of the δ protein and cleaved to release the γ protein in the MIC. This finding is consistent with previous reports that endogenous α protein is cleaved to generate δ and γ proteins (Allis et al., 1984; Wu et al., 1994).

We further tested full-length Mlh1 protein, tagged with GFP and mCherry at its N-terminal and C-terminal ends, respectively, to determine whether the whole precursor form of the Mlh1 protein is required for proper MIC targeting of
(a) δ (199 aa, 23.4 kDa)

(b) β (234 aa, 25.5 kDa)

(c) Critical residues

(e) MicNLS2 localization

Critical residues

KGGKKSKEGKTGAYGKKAN231
and β<sup>213–231</sup> were localized to the MIC, whereas the other fragments (β<sup>1–135</sup>, β<sup>108–178</sup>, β<sup>164–224</sup>, β<sup>215–234</sup> and β<sup>213–229</sup>) were not (Figure 4d). In an interesting manner, a loss of either one of the first two amino acid residues (K or G) and the last two residues (A and N) in the β<sup>213–231</sup> fragment resulted in a loss of its MIC-targeting NLS activity (Figure 4c,d). This suggests that fragment 213–231 (KGKKKSKEKGTGAYGKKAN) is the minimum MIC-targeting sequence in the β protein, which was named MicNLS2 (Figure 4c,d).

To identify the amino acid residues critical to the MIC-targeting NLS activity in the MicNLS2 sequences of the β protein, we examined the subcellular localization of the GFP-GST-β<sup>213–231</sup> fragment carrying a single substitution at a lysine residue within the NLS sequences (Figure 4e). Among mutants at lysine or asparagine residue 215, 216, 217, 219, 222, 228, 229 or 231 in MicNLS2 (dotted residues of 213KGKKKSKEKGTGAYGKKAN<sup>231</sup>) tested, the mutants at residue 215, 216, 217, 219 or 229 completely lost MIC targeting (designated as [−] in Figure 4e) and mutants at residue...
228 or 231 partially lost MIC targeting (designated as [+]1), while the mutant at residue 222 retained its MIC localization (designated as [+++]1). In addition, we examined amino acid substitutions at the last two amino acids (AN) to glycine residues (GG), and the result showed that these substitutions resulted in complete loss of MIC localization (Figure 4e).

Because the first two residues are required for NLS activity and the result showed that these substitutions resulted in complete loss of MIC localization (Figure 4e), we concluded that KG213,214, K215, K216, K217, K219, K228, K229, and AN230,231 are critical residues in 213KGKKKSKEGKTGAYGKKAN231) were mutated to alanine residues as indicated in Figure 5a (designated MicNLS1-KR-A and MicNLS2-KA, respectively). GFP-Mlh1 in MicNLS1-KR-A and GFP-Mlh1 in MicNLS2-KA mCherry proteins carrying mutations in either of the two NLSs or GFP-Mlh1 in MicNLS1-KR-A and MicNLS2-KA carrying mutations in both NLSs were expressed in Tetrahymena cells, and their subcellular localization was observed in live cells by fluorescence microscopy. The GFP-Mlh1 in MicNLS1-KR-A and GFP-Mlh1 in MicNLS2-KA-mCherry mutants carrying mutations in any of the NLSs were localized to the MIC, whereas the GFP-Mlh1 in MicNLS1-KR-A and MicNLS2-KA mutant carrying mutations in both NLSs was not (Figure 5b). These results indicate that the two MIC-targeting NLS sequences function independently of each other.

These two experimentally identified MIC-targeting NLS sequences in 7mMlh1 were compared with other NLS sequences using NLSdb software (https://rostlab.org/services/nlsdb/), but they did not match any of the known NLS sequences, suggesting that both MicNLS1 and MicNLS2 are novel and show no resemblance to classical monopartite or bipartite NLSs. In fact, both classical SV40 monopartite NLS and Xenopus nucleoplasmic bipartite NLS were not localized in the MIC when expressed in Tetrahymena cells (Supporting Information Figure S1c), suggesting that MicNLS1 and MicNLS2 are different from the classical NLSs.

In this study, we identified MAC-specific and MIC-specific NLSs from among the native proteins in Tetrahymena. In particular, MIC-specific NLSs identified from Mlh1 were the first experimentally defined MIC-specific NLSs in ciliates and thus are useful for identifying nucleus-specific nuclear transport systems to distinguish nuclei with different functions. The importin βs responsible for this nucleus-specific targeting are important for understanding the mechanisms of nucleus-specific nuclear transport in ciliates. Our findings contribute to the understanding of how nucleus-specific nuclear targeting is achieved in binucleated ciliates.

3 EXPERIMENTAL PROCEDURES

3.1 Tetrahymena cell strains

Tetrahymena thermophila inbred B strains, CU427.4 (chxl-1/chxl-1 [CHX1; cy-s, VI]) and CU428.2 (mpr1-1/mpr1-1 [MPR1; mp-s, VII]), were used for mRNA preparation and DNA plasmid transfection. The cells were cultured in culture medium (1.5% proteose peptone, 0.5% yeast extract, 0.5% d-glucose), and conjugation was induced in conjugation medium (10 mM Tris-HCl, pH 7.5, 40 mM CaCl2), as described previously (Iwamoto et al., 2015, 2017).

3.2 Construction of DNA plasmids and transfection

A DNA fragment encoding GST was amplified from the pGEX vector (GE Healthcare, Tokyo, Japan) and inserted at the Xhol site into the 3’ end of the GFP-coding sequence of the pIGF1 vector (Malone, Anderson, Motl, Rexer, & Chalker, 2005) to generate the pGF1-GST vector (Supporting Information Figure S4a). A DNA fragment encoding mCherry, codon-optimized for T. thermophila (Kataoka, Schoeberl, & Mochizuki, 2010), was amplified using a forward primer containing the DNA sequence coding a Xhol-KpnI site and a reverse primer containing the DNA sequence coding a BstXI site and was inserted at the Xhol and Apal sites in the pVGIF1 vector (Wiley, Ohba, Yao, & Allis, 2000) to generate the pVGIF1-mCherry vector (Supporting Information Figure S4b).

mRNAs of H1 and Mlh1 were prepared from growing cells in the mid-logarithmic phase. Total RNA was isolated and purified using TRIZol reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer’s protocol. First-strand cDNA was prepared by RT-PCR with SuperScript III reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the open reading frames were amplified by PCR using PrimeSTAR reagent (Takara, Shiga, Japan). The reaction was carried out for 35–40 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 15 s and extension at 72°C for 10 s to amplify the 1.0-kb sequence. The amplified fragment encoding the DNA sequence of interest was digested with Xhol and Apal, and then ligated into the Xhol and Apal sites of the pGF1-GST vector. To tag both the N- and C-termini with GFP and mCherry, respectively, the amplified fragment encoding the DNA sequence of interest was digested with Xhol and KpnI, and the resulting fragment was inserted into the Xhol and KpnI sites of the pVGIF1-mCherry vector.

To introduce a single amino acid substitution in the GFP-GST-tagged H1 or Mlh1 fragments, the coding region of MacNLS1, MacNLS2 or MicNLS2 cloned into the pGF1-GST vector was amplified by PCR, and the amplified DNA fragment was inserted into the pBluescript SK vector. Then,
using a primer carrying each respective mutation, the coding region was amplified by PCR using up to 18 cycles. The PCR products were treated with DpnI to digest and remove template DNAs. The resulting DNA, which contained the coding region with a mutation, was amplified in DH5α Escherichia coli cells. The amplified DNA was digested with XhoI and Apal and inserted into the XhoI and Apal sites of the pIGF1-GST vector.

To introduce multiple amino acid substitutions in the GFP-GST-tagged full-length H1 or Mlh1, the coding region of full-length H1 or Mlh1 was amplified by PCR and the amplified DNA fragment was inserted into the pBluescript SK vector to generate pBluescript-H1 and pBluescript-Mlh1 vectors. Then, a single amino acid substitution was first introduced into the vectors using the method described above. A second substitution was introduced into the vector carrying the first mutation by repeating the procedure. After introducing all mutations of interest, the coding region of full-length Mlh1 carrying the mutations was digested from the pBluescript-H1 or pBluescript-Mlh1 vector and inserted into the XhoI and Apal sites of the pIGF-GST vector and into the XhoI and KpnI sites of the pVGF1-mCherry vector, respectively.

The nucleotide sequences of all of the plasmids were verified using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, CA, USA). The DNA plasmids were introduced into conjugating pairs by electroporation, as described previously (Iwamoto et al., 2015, 2017).

### 3.3 Fluorescence observation

For microscopic observation, the DeltaVision system (GE Healthcare, Little Chalfont, UK), which is based on the inverted fluorescence microscope IX-70 (Olympus, Tokyo, Japan), was used. Live cells were observed in all experiments except for Supporting Information Figure S1a,b. A small aliquot of cells in culture medium was placed between two coverslips to immobilize and flatten the cells. 3D images were acquired using the DeltaVision microscope system, through an Olympus oil-immersion objective lens UApo40 (NA = 1.35) along the z-axis at 0.3-μm intervals, and processed for deconvolution using the SoftWorx software installed on the DeltaVision system.

### 3.4 Western blot analysis

For western blotting analysis, the nuclear fraction was used to detect Mlh1 fragment proteins (GFP-α-mCherry); this is because the amount of MIC-targeting proteins, such as Mlh1 and its fragments, was too low to be detected in whole-cell extract. Therefore, they needed to be concentrated by fractionation. The method for the nuclear fractionation was modified from that established by Gorovsky (1970) as follows: For a short time, logarithmically growing Tetrahymena cells (WT: CU427 and GFP-α-mCherry) were collected by centrifugation at ~700 × g for 1 min and were washed once with ice-cold homogenizing buffer (6.0% arabic gum [Wako, Osaka, Japan], 3.4% sucrose, 0.125% spermidine [Wako], 10 mM Tris, 2.0 mM MgCl₂ and 2.0 mM phenylmethylsulfonyl fluoride [Wako], and adjusted to pH 6.8 with HCl). Thereafter, all steps were performed on ice or at 4°C. About 5 × 10⁶ cells were resuspended in 10 ml of the homogenizing buffer in a 15-ml conical tube. Sixty-four microliters of octanol was added to the cell suspension, and immediately, the tube was vigorously shaken ~60 times by hand. After centrifugation at 1,000 × g for 10 min with a swing rotor (TS-7C; Tomy, Tokyo, Japan), the pellet was collected as a crude nuclear fraction. To collect the remaining nuclei in the other fractions, the cloudy upper layer, including a thick flesh-colored material appearing near the surface, was transferred to a new tube, vigorously shaken several times and centrifuged. This step was repeated three times. The pellets obtained from each of the steps were pooled and washed twice with the homogenizing buffer and once with 10 mM Tris-HCl (pH 6.8) containing 2 mM MgCl₂. The nuclear fraction pellet contained both MICs and MACs because the majority of MICs (~80%) are physically bound to MACs.

The nuclear fraction was resuspended in sonication buffer (1% Triton X-100, 5 units of DNase I [Takara, Kyoto, Japan], ~1% RNase A [R6148; Sigma-Aldrich, St. Louis, MO] and 10 mM Tris-HCl [pH 6.8]), and sonicated. Excess amount (5.7 times) of 2% SDS dissolved in 10 mM Tris-HCl (pH 6.8) was then added and mixed with a vortex mixer until the pellet was completely dissolved. Next, ice-cold trichloroacetic acid was added to the mixture at a final concentration of 10% and the mixture was incubated on ice for 10 min. The mixture was then centrifuged at 15,000 rpm (18,800 x g) for 5 min in a microcentrifuge (T15AP21 rotor; Hitachi, Tokyo, Japan), and the pellet was washed with distilled water three times; a few microliters of 1 M Tris was used to adjust the pH. The pellet (the nuclear protein fraction) was then lysed in 20 times the amount of the SDS sample buffer by sonication.

Volume equivalents for nuclear number were calculated to be ~1 × 10⁵ micronuclei/μl. The samples were incubated at 70°C for 10 min, and 12 μl of each sample, corresponding to 1.2 × 10⁶ micronuclei/lane, was separated by 10% SDS-PAGE. Proteins were then transferred to a polyvinylidene fluoride membrane in transfer buffer (48 mM Tris, 39 mM glycine, pH 9.2 and 20% methanol) using a semi-dry transfer system (at a voltage of 0.8 mA/cm² for 2 hr). The membrane was blocked with 1% skim milk for 1 hr at room temperature (~25°C) and incubated with a rabbit polyclonal anti-mCherry (Abcam, Cambridge, UK) diluted to 1:1,000 for 2 hr at room temperature. The membrane was washed with PBS containing 0.1% Tween-20 three times and then incubated with secondary antibodies (1:5,000) for 2 hr at room temperature. Horseradish peroxidase-conjugated...
Goat anti-rabbit IgG (MP Biomedicals, Santa Ana, CA, USA) was used as the secondary antibody. Protein bands were detected by chemiluminescence using ImmunoStar LD (Wako).

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