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RESEARCH ARTICLE

Heterochromatin aggregation during DNA elimination in *Tetrahymena* is facilitated by a prion-like protein

Kensuke Kataoka[1] and Kazufumi Mochizuki[1,2,*]

ABSTRACT

Regulated aggregations of prion and prion-like proteins play physiological roles in various biological processes. However, their structural roles in the nucleus are poorly understood. Here, we show that the prion-like protein Jub6p is involved in the regulation of chromatin structure in the ciliated protozoan *Tetrahymena thermophila*. Jub6p forms sodium dodecyl sulfate (SDS)-resistant aggregates when it is ectopically expressed in vegetative cells and binds to RNA *in vitro*. Jub6p is a heterochromatin component and is important for the formation of heterochromatin bodies during the process of programmed DNA elimination. We suggest that RNA–protein aggregates formed by Jub6p are an essential architectural component for the assembly of heterochromatin bodies.

KEY WORDS: Heterochromatin, Heterochromatin body, Prion, DNA elimination, *Tetrahymena*

INTRODUCTION

In certain cell types, multiple heterochromatic loci are assembled into higher-order structures known as heterochromatin bodies, which include chromocenters (Fransz and de Jong, 2002; Probst and Almouzni, 2011), Barr bodies (Rego et al., 2008) and senescence-associated heterochromatin foci (Narita et al., 2003). In addition to the local compaction of individual heterochromatic loci, the assembly of these into heterochromatin bodies is suggested to be important for regulating chromatin activities. However, the molecular mechanisms regulating the assembly of heterochromatin bodies are poorly understood.

Heterochromatin bodies are formed *de novo* in the macronucleus (MAC) that is newly formed from the germline micronucleus (MIC) during sexual reproduction of the ciliated protozoan *Tetrahymena thermophila* (Chalker, 2008). In the new MAC, an RNA interference (RNAi)-related mechanism induces the accumulation of histone H3 methylated at lysine residues 9 and 27 (H3K9me and H3K27me, respectively) and of the associated heterochromatin components at internal eliminated sequences (IESs), which occupy approximately one-third (50 Mb) of the MIC genome (Chalker and Yao, 2011). The heterochromatized IESs are assembled into heterochromatin bodies and are eventually eliminated. Because sexual reproduction of *Tetrahymena* can be synchronously induced at a large scale, it serves as an ideal model for analyzing the process of heterochromatin body formation.

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Several proteins are known to be required for the formation of heterochromatin bodies during the process of DNA elimination in *Tetrahymena* and can be divided into two groups. The first group consists of proteins required for the formation of heterochromatin at IESs, including the RNAi machinery components Dcl1p, Twi1p, Giw1p and Ema1p, the histone methyltransferase Ezl1p and the HP1-like protein Pdd1p (Aronica et al., 2008; Coyne et al., 1999; Liu et al., 2004, 2007; Malone et al., 2005; Mochizuki and Gorovsky, 2005; Noto et al., 2010). The second group includes proteins that are dispensable for heterochromatin formation but are required for its assembly into heterochromatin bodies: Jub1p, Lia1p, Lia4p, Lia5p, Pdd2p, Tku80p and Tpb2p (Nikiforov et al., 1999; Rexer and Chalker, 2007; Horrell and Chalker, 2014; Shieh and Chalker, 2013; Liu et al., 2007; Cheng et al., 2010; Lin et al., 2012; Vogt and Mochizuki, 2013; Kataoka and Mochizuki, 2015). Although some of the mechanisms responsible for the former RNAi-directed heterochromatin formation process have been elucidated at the biochemical level, the latter heterochromatin body formation process remains elusive.

It has long been known that Pdd1p is highly phosphorylated upon the formation of heterochromatin, and its dephosphorylation coincides with heterochromatin body formation (Madireddi et al., 1994). More recently, it has been shown that Pdd1p dephosphorylation and heterochromatin body formation can be triggered by a DNA damage response, which could be caused by the DNA elimination process (Shieh and Chalker, 2013). In addition, we have shown that Pdd1p dephosphorylation is required for the formation of heterochromatin bodies, possibly by enhancing its RNA-binding activity (Kataoka and Mochizuki, 2015). However, it is still unclear how Pdd1p dephosphorylation eventually results in the formation of heterochromatin bodies and how the other proteins are involved in this process.

Prion-like proteins play important pathological roles in the development of neurodegenerative diseases (Jucker and Walker, 2013), in addition to physiological roles in various biological processes, such as transcriptional regulation for environmental adaptability, translational regulation in the formation of long-term memory and RNA localization in cell cycle regulation (Halfmann et al., 2012; Holmes et al., 2013; Lee et al., 2013; Majumdar et al., 2012; Si et al., 2003). Because heterochromatin bodies are considered nucleoprotein aggregates, we chose to investigate the involvement of a prion-like protein in this process in *Tetrahymena*.

RESULTS

Prediction of prion-like proteins in *Tetrahymena*

To identify prion-like proteins that are involved in heterochromatin body formation, we performed *in silico* analysis of *Tetrahymena* proteins with prion-forming propensities. From 24,559 predicted *Tetrahymena* proteins, we first selected 21,091 proteins with potential disordered region(s) that we identified using the FoldIndex algorithm (Prilusky et al., 2005). Among these

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proteins, 1431 were predicted to exhibit strong prion-forming properties (score >0.1) using the PAPA algorithm, which was designed to predict the prion propensity of glutamine- and asparagine-rich prion-forming domains (Toombs et al., 2012) (Fig. 1A). Because heterochromatin body formation occurs during sexual reproduction, we next selected proteins that are highly and specifically expressed during sexual reproduction based on mRNA expression (Miao et al., 2009). We found that the gene expression of five putative prion-like proteins was dramatically upregulated during conjugation (Fig. S1A). One of these proteins was the MIC-specific nuclear pore protein MicNup98B (Iwamoto et al., 2009; Malone et al., 2008), which is unlikely to be involved in heterochromatin body formation in the new MAC. Therefore, we further analyzed the other four proteins: TTHERM_01415190, TTHERM_00647510, TTHERM_00145310 and TTHERM_00421130 (*Tetrahymena* Genome Database).

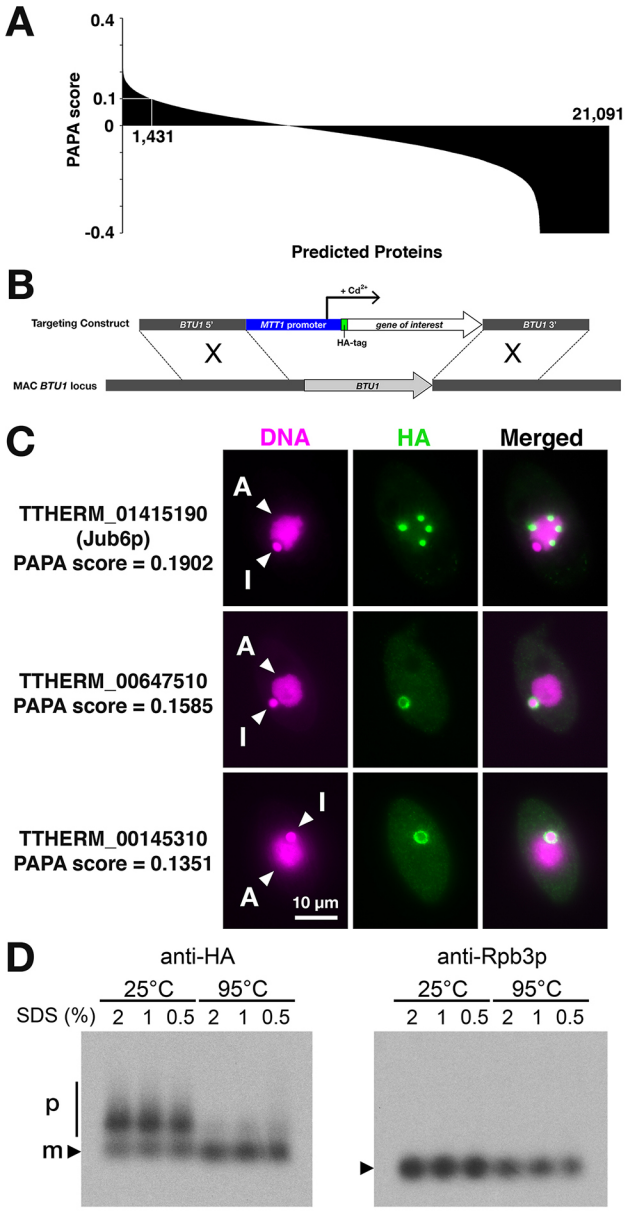

Fig. 1. Identification of proteins possessing prion-forming properties. (A) The PAPA scores of 21,091 predicted *Tetrahymena* proteins with potential disordered region(s) are shown, among which 1431 exhibited strong prion-forming properties (score >0.1). (B) Constructs designed for the ectopic expression of HA-tagged TTHERM_01415190 (Jub6p), TTHERM_00647510 or TTHERM_00145310 from the Cd^{2+}-inducible *MTT1* promoter were introduced into the *BTU1* locus in the MAC. (C) The localization of the proteins in vegetative cells was analyzed using an antibody against HA (green). DNA was counterstained with DAPI (magenta). MAC and MIC are indicated by arrowheads and the letters A and I, respectively. (D) Vegetative cells expressing HA–Jub6p were lysed in buffers containing the indicated concentration of SDS, followed by incubation at room temperature (25°C) or 95°C; the proteins were then separated using SDD-AGE and subjected to western blotting using anti-HA (left) and anti-Rpb3p (right) antibodies. Polymers (p) and monomers (m) are indicated.

Ectopically expressed Jub6p forms foci in the MAC

To determine whether these proteins form prion-like protein aggregates *in vivo*, we expressed the candidate proteins in vegetative *Tetrahymena* cells. The full-length cDNA of each of the four proteins was cloned into an expression vector with a Cd^{2+}-inducible *MTT1* promoter, and each protein of interest was expressed as a hemagglutinin (HA)-tagged protein (Fig. 1B). The expression vectors carrying the cloned genes were introduced into the non-essential *BTU1* locus of wild-type *Tetrahymena* cells. Protein expression was then induced by adding cadmium to the starved cell culture, and HA-tagged proteins were detected through immunofluorescence staining using an antibody against HA. We found that TTHERM_01415190 localized to several foci in the MAC (Fig. 1C, top). In contrast, TTHERM_00647510 and TTHERM_00145310 localized to the periphery of the MIC (Fig. 1C, middle and bottom). We were unable to detect TTHERM_00421130. Because TTHERM_01415190 formed foci reminiscent of prions in the MAC, we chose to characterize this protein further. The TTHERM_01415190 protein was named Junk buster 6 [Jub6p, according to our previously described nomenclature (Kataoka and Mochizuki, 2015)], and the gene encoding this protein was designated *JUB6*.

In *Tetrahymena*, H3K9me and its associated heterochromatin components accumulate only on eliminated chromatin in the new MAC. One known exception to this rule is H3K27me, which accumulates in both the MIC and MAC in vegetative cells, albeit at much lower levels than in the new MAC (Fig. S1B). We found that trimethylated H3K27 (H3K27me3) did not accumulate in the Jub6p-containing foci in vegetative cells (Fig. S1C). Considering these findings together, we conclude that Jub6p has the ability to form foci in the vegetative MAC, and Jub6p foci are likely to form without heterochromatin.

Ectopically expressed Jub6p forms prion-like aggregates

We next investigated whether Jub6p forms prion-like aggregates. Cells ectopically expressing HA–Jub6p were lysed in buffer containing 0.5–2% sodium dodecyl sulfate (SDS) and were incubated at either room temperature or 95°C, followed by separation through semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) and western blotting with an antibody against HA (Fig. 1D, left). We found that although the lysate that had been incubated at 95°C showed a single band in western blots (arrowhead with 'm'), the lysate that had been incubated at room temperature exhibited additional more slowly migrating HA–Jub6p smears, even in 2% SDS (marked with 'p'). In contrast, in the same lysate, Rpb3p (PAPA score=−0.03), a subunit of RNA polymerase II, which was detected using an antibody against Rpb3p, did not show SDS-resistant aggregates in the same blot (Fig. 1D right). Therefore, consistent with its predicted strong prion-forming propensity and foci-forming characteristics, Jub6p

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forms SDS-resistant prion-like multimers *in vivo* when it is ectopically expressed in vegetative cells. It seems the detected Jub6p multimers migrate faster than the previously reported amyloid aggregates (Alberti et al., 2009; Halfmann et al., 2012; Holmes et al., 2013). This suggests that Jub6p aggregates are stable only as oligomers. Alternatively, Jub6p aggregates might form a more compacted SDS-resistant structure.

The predicted prion-forming domain of Jub6p is required for its aggregation

Although there are no detectable Jub6p homologs in non-*Tetrahymena* organisms, there are six genes encoding Jub6p-like proteins (Jsl1p to Jsl6p) in the genome of *Tetrahymena thermophila* (Fig. 2A,B). However, none of the proteins were predicted to exhibit prion-forming properties (score <0.05) according to the PAPA algorithm (Fig. 2B). We chose two of these proteins, Jsl3p and Jsl4p, and tested their aggregation propensity by expressing them using the *MTT1* promoter in vegetative cells, as described above. Consistent with their low predicted prion-forming potential, these proteins did not form any detectable foci but instead were distributed throughout the cytoplasm and the nuclei (Fig. 2C,D).

Then, to analyze the importance of the predicted prion-forming region (PFR; the region showing a PAPA score above 0.05, from amino-acid residues 319 to 427 of Jub6p; indicated in red in Fig. 2A,E) of Jub6p, we replaced it with the corresponding region of Jsl3p. When this chimeric protein (Jub6p-Jsl3pPFR) was expressed in vegetative cells, it was found to be dispersed in the MAC and did not form any detectable foci (Fig. 2F). Additionally, Jsl3p-Jub6pPFR, in which the PFR of Jub6p was used to replace the corresponding region of Jsl3p, also did not form foci (Fig. 2G). These results indicate that the predicted PFR is essential for the focus formation activity of Jub6p in vegetative cells. However, the predicted PFR is not sufficient for the formation of foci, and some additional region(s) of Jub6p are expected to act cooperatively to facilitate the formation of nuclear foci. Alternatively, the predicted PFR might induce the foci formation only in the nucleus.

Jub6p is a component of heterochromatin bodies in the new MAC

We next raised an antibody against Jub6p in rabbit and used it to analyze the localization of endogenous Jub6p through immunofluorescence staining. In wild-type cells, Jub6p was first detected homogeneously in the new MACs following their differentiation [wild type, 10 hours post mixing (hpm) in Fig. 3A]. Jub6p was then localized into numerous small foci at 12 hpm and into several large foci at 14 hpm (Fig. 3A). These results indicate that some factor(s) in the new MAC either inhibit the aggregation of Jub6p in the early stages of MAC development (until 10–12 hpm) or promote Jub6p aggregation in late stages (after 12 hpm). The localization pattern of Jub6p was reminiscent of those of heterochromatin body components, such as H3K9me and H3K27me3 and the HP1-like protein Pdd1p (Coyne et al., 1999; Liu et al., 2007; Taverna et al., 2002). Indeed, double immunofluorescence staining using antibodies against Pdd1p and Jub6p revealed that Jub6p colocalizes with Pdd1p in heterochromatin bodies at 14 hpm (Fig. 3B). Therefore, we conclude that Jub6p is a heterochromatin body component during the process of DNA elimination.

Because several proteins are known to localize to heterochromatin bodies during DNA elimination in *Tetrahymena*, we next asked whether the foci-forming activity of Jub6p is a common feature of proteins that localize to heterochromatin bodies. For this purpose, we chose six heterochromatin body components: Jub1p, Lia1p, Lia4p, Lia5p, Pdd1p and Pdd2p, all of which are exclusively expressed during conjugation and are required for the formation of heterochromatin bodies (Coyne et al., 1999; Horrell and Chalker, 2014; Kataoka and Mochizuki, 2015; Nikiforov et al., 1999; Rexer and Chalker, 2007; Shieh and Chalker, 2013). We expressed these proteins using the *MTT1* promoter in vegetative cells as HA-tagged proteins and analyzed their localization (Fig. 4). Although five of the six proteins were localized homogeneously either in the MAC or in both the MAC and the cytoplasm, Pdd1p clearly formed foci in the cytoplasm. Therefore, the ability to form foci is not a common feature of heterochromatin body components, but Jub6p and Pdd1p, at the least, exhibit such activity. The relationship between Jub6p and Pdd1p in the process of heterochromatin body formation will be discussed below.

Jub6p is important for DNA elimination

To analyze the function of Jub6p, we produced *JUB6* MIC knockout (MIC-KO) strains, in which the entire *JUB6* protein-coding sequences of both alleles of the *JUB6* gene in the MIC were replaced with a drug resistance gene (Fig. S2). We detected no Jub6p through immunostaining using the Jub6p-specific antibody in mating *JUB6* MIC-KO strains (Fig. 3A, KO). This indicates that *JUB6* is exclusively expressed from zygotic copies of *JUB6* in the new MAC in wild-type cells, as expected based on the expression pattern of *JUB6* mRNA (Fig. S1B), and disruption of the MIC copies of *JUB6* is sufficient to eliminate the expression of *JUB6*.

Previous studies have shown that many heterochromatin components are required for DNA elimination and for the production of viable sexual progeny (Coyne et al., 1999; Mochizuki et al. 2002; Malone et al., 2005; Liu et al., 2007; Cheng et al., 2010). DNA elimination in the exconjugants (progeny) of *JUB6* MIC-KO was analyzed by using DNA fluorescent *in situ* hybridization (FISH) with probes complementary to the *Tlr1* IESs, which show moderate levels of repetition (Wuitschick et al., 2002). At 36 hpm, *Tlr1* IESs were detected in the new MICs, but not in the new MACs, in the exconjugants from wild-type cells (Fig. 5A). In contrast, almost all of the exconjugants from *JUB6* MIC-KO cells showed staining for *Tlr1* IESs in the new MACs (Fig. 5A, *JUB6* KO). The DNA-FISH staining for *Tlr1* IESs in the exconjugants from *JUB6* MIC-KO cells was sparser than in the exconjugants from *PDD1* knockout cells (Fig. 5A, *PDD1* KO), in which DNA elimination of all IESs was completely blocked (Kataoka and Mochizuki, 2015). Therefore, in the absence of Jub6p, DNA elimination does occur but is not completed. *JUB6* MIC-KO cells produced no viable sexual progeny, whereas approximately 70% of the mating pairs of the control wild-type cells produced viable progeny (Fig. 5B), indicating that Jub6p is essential for completing sexual reproduction, similar to most other known heterochromatin components that are important for DNA elimination.

Jub6p is essential for the formation of heterochromatin bodies

We next determined whether Jub6p is required for the formation of heterochromatin bodies, which mostly occurs before DNA elimination in wild-type cells. As previously reported (Chalker, 2008), most of the exconjugants from wild-type cells formed several distinct heterochromatin bodies, which were visualized in the new MACs at 14 hpm by immunofluorescence staining with an antibody against Pdd1p (Fig. 5C; Fig. S3). Following the completion of DNA elimination, Pdd1p-containing bodies were mostly lost by 16 hpm (Fig. S3). In contrast, in the exconjugants from *JUB6* MIC-KO cells, heterochromatin bodies were barely detectable, and Pdd1p instead

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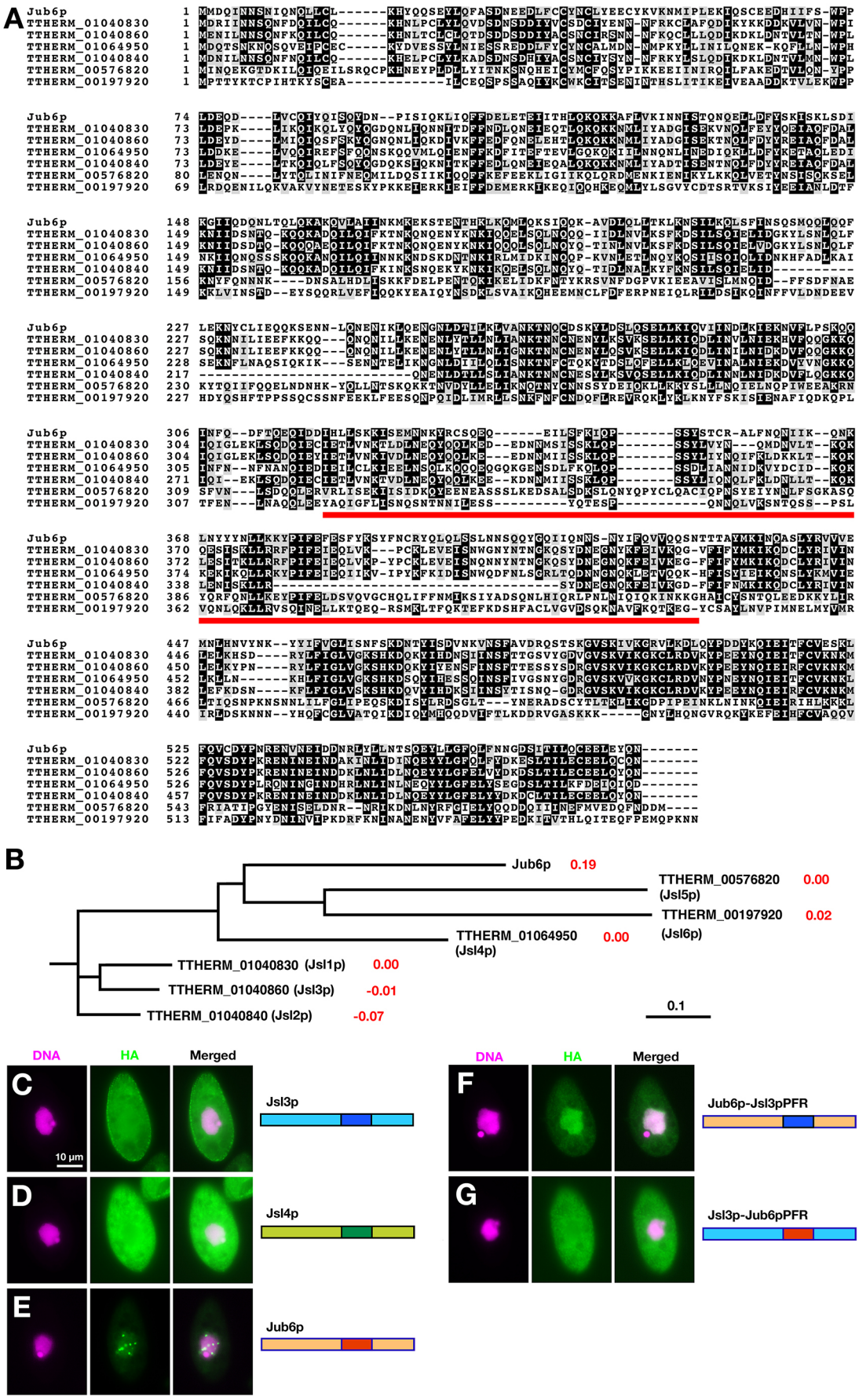

Fig. 2. See next page for legend.

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Fig. 2. Jub6p-like proteins in *Tetrahymena thermophila*. (A,B) The Jub6p and Jub6p-like proteins identified in the *Tetrahymena* MAC genome were aligned using ClustalW (A), and their relationships are shown in trees generated using the neighbor-joining method (B). The region predicted to exhibit prion-forming properties [amino acids 359 to 387 (±40 amino acids) of Jub6p] is underlined in red in A. The PAPA scores of each protein are shown in red in B. The scale (bottom right) represents 10% differences between two sequences. (C–G) The proteins schematically represented on the right were expressed in vegetative cells as fusion proteins with an HA-tag and located using an HA-specific antibody (green). DNA was counterstained with DAPI (magenta). C–G share the scale bar shown in C.

localized to a few larger bodies, which remained in the new MAC, even at 16 hpm (Fig. 5C; Fig. S3).

Trimethylated H3K9 (H3K9me3), which is known to accumulate specifically at IESs in the new MACs (Kataoka and Mochizuki, 2015; Taverna et al., 2002), colocalized with Pdd1p in the heterochromatin bodies of wild-type exconjugants (Fig. 5C, WT 14 hpm) and in the large Pdd1p-containing bodies of *JUB6* MIC-KO exconjugants (Fig. 5C, KO 14 hpm, 16 hpm). These results indicate that the Pdd1p-containing bodies that form in the absence of Jub6p are still associated with heterochromatin and, thus, probably represent an abnormal type of heterochromatin body caused by disturbance of the subnuclear localization of heterochromatin within the new MAC. This abnormal heterochromatin body formation is unlikely to be due to blockage of DNA elimination, as such abnormal heterochromatin bodies have not been detected in our previous studies of other DNA-elimination-defective mutants by the same immunofluorescent staining procedures (Kataoka and Mochizuki, 2015; Kataoka et al., 2016; Vogt and Mochizuki, 2013).

We then analyzed the accumulation of H3K27me3, which is also known to accumulate specifically at IESs in the new MACs (Kataoka and Mochizuki, 2015; Liu et al., 2007). We found that H3K27me3 accumulated in a similar pattern in the new MACs of wild-type and in *JUB6* MIC-KO cells, although H3K27me3 was more homogeneously distributed in the new MACs from *JUB6* MIC-KO cells at 14 hpm when H3K27me3 had accumulated in heterochromatin bodies in wild-type cells (Fig. S3 bottom). Taken together, the above results indicate that Jub6p is not required for the formation of heterochromatin itself but plays an important role in properly assembling individual heterochromatin sequences into heterochromatin bodies.

Jub6p is not required for the phosphorylation–dephosphorylation cycle of Pdd1p

Our previous study suggests that heterochromatin body assembly during new MAC development requires the phosphorylation (Kataoka et al., 2016) and subsequent dephosphorylation (Kataoka and Mochizuki, 2015) of Pdd1p. Pdd1p is phosphorylated at multiple serine and threonine residues, and phosphorylated Pdd1p migrates more slowly than unphosphorylated Pdd1p in standard SDS-PAGE

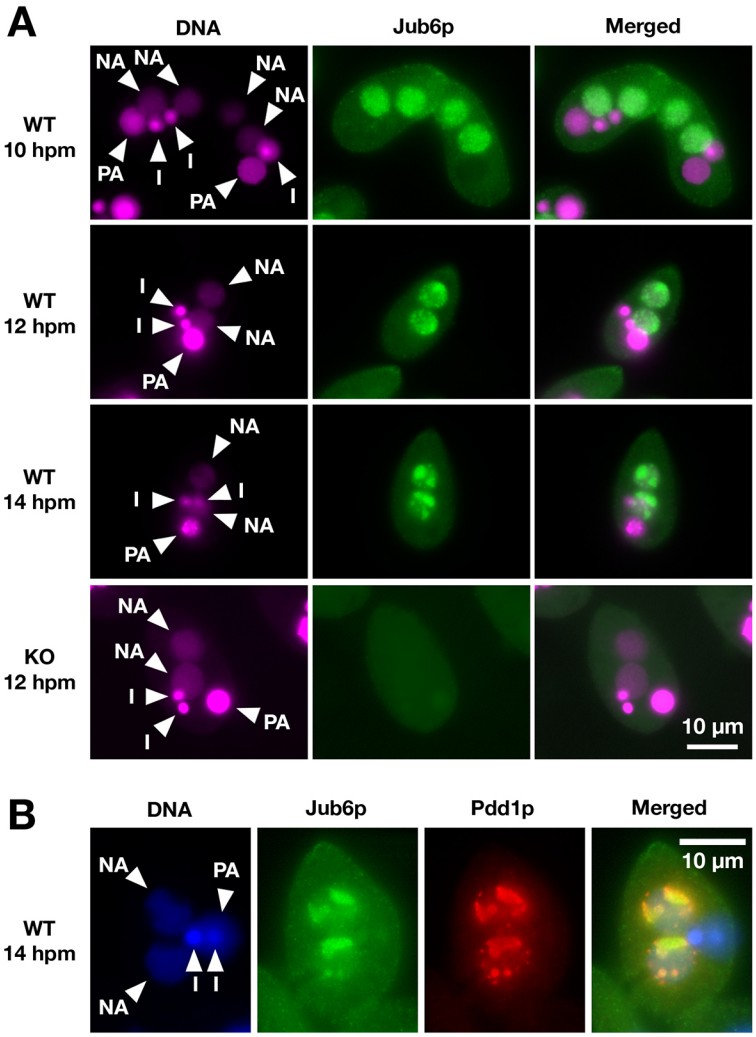

Fig. 3. Localization of endogenously expressed Jub6p. (A) The localization of Jub6p in wild-type (WT) or *JUB6* MIC-KO (KO) cells was analyzed using an antibody against Jub6p (green) at the indicated time points of sexual reproduction. DNA was counterstained with DAPI (magenta). (B) The colocalization of Jub6p and Pdd1p in wild-type (WT) cells at 14 hpm was analyzed using antibodies against Jub6p (green) and Pdd1p (red), respectively. DNA was counterstained with DAPI (blue). I, MIC; NA, new MAC; PA, parental MAC.

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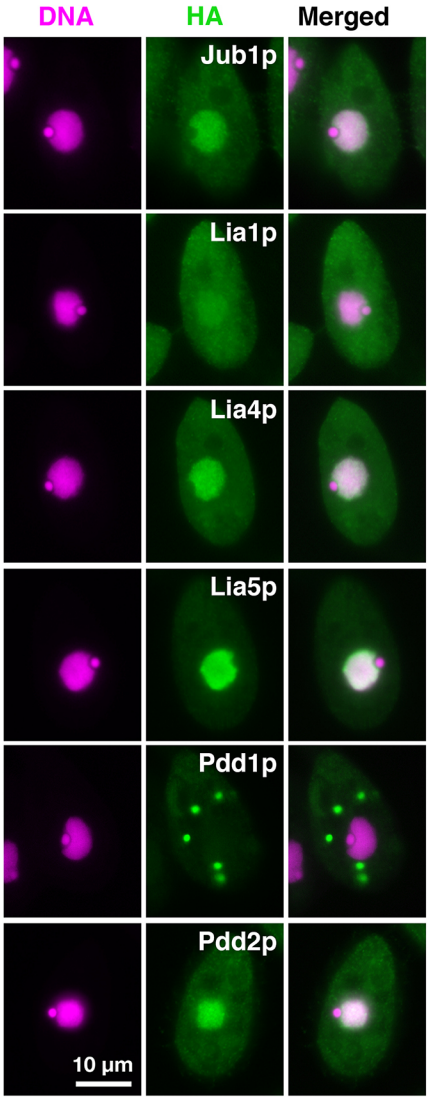

Fig. 4. Localization of heterochromatin body components ectopically expressed in vegetative cells. The indicated heterochromatin body components were expressed as HA-tagged fusion proteins in vegetative cells and analyzed using an HA-specific antibody (green). DNA was counterstained with DAPI (magenta).

gels (Kataoka and Mochizuki, 2015; Madireddi et al., 1996). In wild-type and *JUB6* MIC-KO cells, similar slower-migrating Pdd1p was detected in the early stages of the new MAC development (8 hpm) by western blotting using an antibody against Pdd1p (Fig. 5D). These slower-migrating bands were lost at later stages (10–12 hpm) in both wild-type and *JUB6* MIC-KO cells (Fig. 5D). These results indicate that the timing and the levels of Pdd1p phosphorylation and subsequent dephosphorylation are unaffected by the absence of Jub6p. Therefore, we conclude that the requirement of Jub6p for proper heterochromatin body formation is independent of the phosphorylation cycle of Pdd1p.

Jub6p binds to RNA

Because Jub6p has no detectable chromatin-binding domain, we investigated whether RNA links Jub6p and Pdd1p, which binds to both H3K9me and/or H3K27me and RNA, to facilitate heterochromatin body formation. An electrophoretic mobility shift assay (EMSA) was performed using recombinant maltose-binding protein (MBP)-tagged full-length (FL) Jub6p (Fig. 6A; Fig. S4) and a 1305-nucleotide single-stranded (ss)RNA that was complementary to Cal-IES of *Tetrahymena* (Cal ssRNA) or a 723-nucleotide ssRNA that was complementary to EGFP (EGFP ssRNA). As shown in Fig. 6B,C, MBP-tagged Jub6p, but not MBP that had been purified in a similar way, caused mobility shifts of both RNAs. The binding affinity of Jub6p to RNA was estimated as K_d=~60 nM. Then, to determine whether Jub6p also binds to DNA, similar EMSA experiments were performed using a 50-nucleotide ssDNA and a 50-nucleotide ssRNA, which share corresponding base sequences. We found that the binding affinity of Jub6p to DNA is at least one order lower than that to RNA (Fig. 6D,E). Therefore, Jub6p interacts preferentially with ssRNA in an RNA-sequence-independent manner and could interact with heterochromatin through RNA and Pdd1p.

We next attempted to determine the region of Jub6p to which RNA binds. We prepared N- and C-terminal halves of Jub6p that were fused to MBP (MBP–N and MBP–C, respectively; Fig. 6A; Fig. S4) for EMSA experiments and found that the RNA-binding activity of Jub6p mainly resides in its C-terminal half where the predicted PFR also occurs (Fig. 6F). Then, we produced an MBP–C construct that lacked the predicted PFR (MBP–C-ΔPFR; Fig. 6A; Fig. S4) and found that the binding affinity of this protein to RNA was weaker than that of MBP–C (Fig. 6G). Taken together, these results indicate that the predicted PFR and the region responsible for RNA binding in Jub6p largely overlap.

DISCUSSION

The results of this study indicate that the prion-like RNA-binding protein Jub6p is required for the formation of proper heterochromatin bodies. Although several heterochromatin body components are known to be essential for the formation of heterochromatin bodies, we found that, among the heterochromatin body components we tested, only Jub6p and Pdd1p had the ability to form foci when they were expressed in vegetative cells. As a large mass of abnormal heterochromatin was formed in the absence of Jub6p (Fig. 5C), Pdd1p alone might be able to induce some level of heterochromatin aggregation, but both Pdd1p and Jub6p were necessary for the proper formation of heterochromatin bodies to support DNA elimination. Jub6p might act as a 'cloud seed' for proper assembly of heterochromatin bodies (Fig. 6H). The roles of the other heterochromatin body components remain elusive. They might be required for the formation of a chromatin environment that is a prerequisite for proper arrangement of Pdd1p and Jub6p on heterochromatinized IESs, although we do not exclude the possibility that they play direct roles in the aggregation process of heterochromatin body assembly. Two proteins, Nowa1p and Nowa2p, which are required for small-RNA-dependent IES elimination in the other ciliate *Paramecium tetraurelia*, are similar to the RNA-binding prion protein Prp27 (Gabus et al., 2001; Nowacki et al., 2005). Therefore, although Jub6p and Nowa1p and Nowa2p share no obvious homology at their amino-acid sequence level, prion-like proteins could play essential roles in one or more processes of DNA elimination in ciliates.

RNA-binding proteins that contain disordered regions are involved in the formation of membraneless RNA–protein (RNP) bodies such as stress granules (Gilks et al., 2004) and P-bodies (Decker et al., 2007). The heterochromatin bodies found in *Tetrahymena* are also membraneless electron-dense granules (Madireddi et al., 1996). Therefore, RNA might be involved not only in the recruitment of Jub6p to heterochromatin but also in the formation of Jub6p-containing RNP granules to trigger the assembly of the

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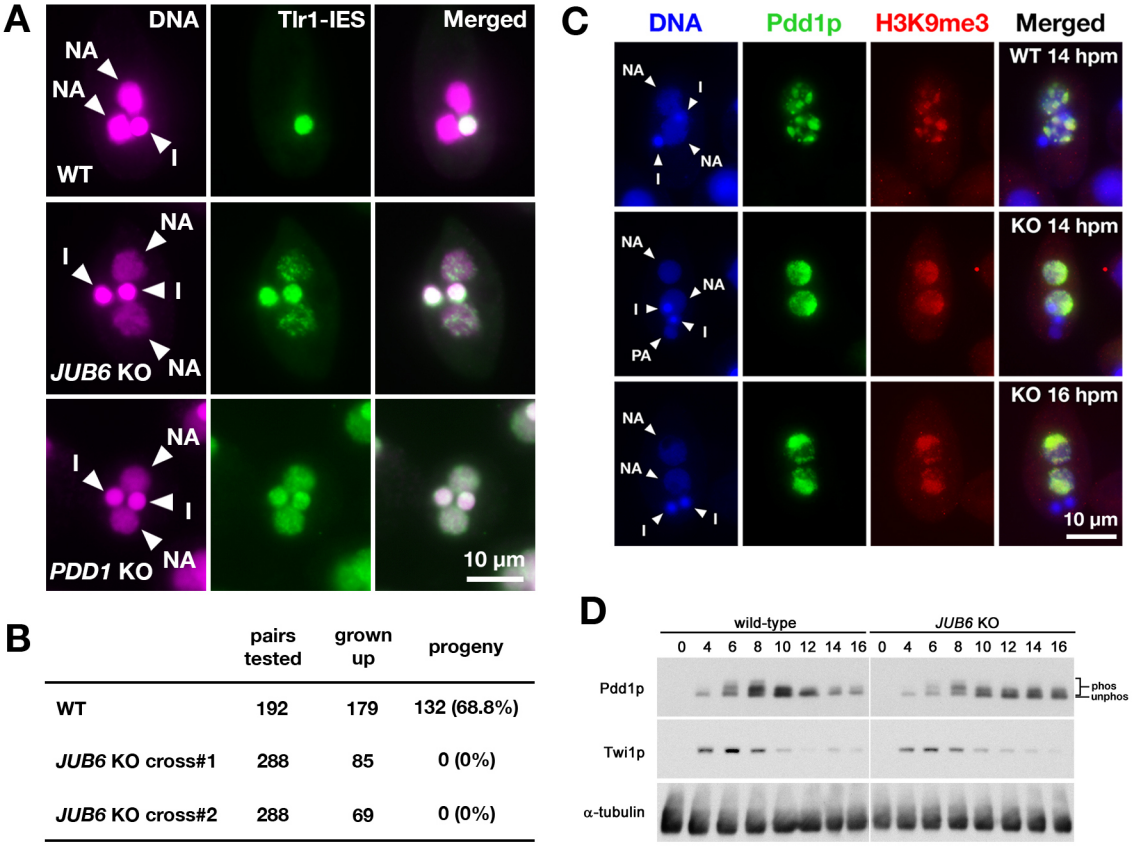

B

	pairs tested	grown up	progeny
WT	192	179	132 (68.8%)
JUB6 KO cross#1	288	85	0 (0%)
JUB6 KO cross#2	288	69	0 (0%)

Fig. 5. DNA elimination, progeny production and heterochromatin body formation in the absence of Jub6p. (A) Exconjugants from the wild-type (WT), *JUB6*-KO and *PDD1*-KO strains at 36 hpm were hybridized with probes complementary to the *Tlr1* IES (green). DNA was counterstained with DAPI (magenta). I, MIC; NA, new MAC; PA, parental MAC. (B) Viability tests for the sexual progeny of *JUB6* KO cells. The numbers of isolated mating pairs that gave rise to growing cells and progeny are shown. The growing cells were counted as progeny or parental cells that aborted conjugation after isolation. The completion of conjugation of the growing clones was tested by checking for the expression of drug resistance markers that existed only in the MICs of the parental strains. (C) Pdd1p and H3K9me3 were localized by immunostaining using an antibodies against Pdd1p (green) and H3K9me3 (red) in wild-type or *JUB6* KO cells at the indicated time points of sexual reproduction. DNA was counterstained with DAPI (blue). (D) Proteins from wild-type and *JUB6*-KO cells during conjugation at the indicated time points (above blots) of sexual reproduction (0–16 hpm) were analyzed by western blotting with an antibody against Pdd1p. Twi1p and α-tubulin were analyzed as controls for the progression of sexual reproduction and loading, respectively. Phosphorylated (phos) and unphosphorylated (unphos) Pdd1p is indicated.

heterochromatin bodies. Because heterochromatin bodies are widely observed in eukaryotes, it would be of interest to determine whether similar prion-like proteins are involved in heterochromatin body formation in other eukaryotes. It is important to note that some known heterochromatin-related proteins, such as methyl-CpG-binding domain protein 5 in mammals (PAPA score=0.05), polyhomeotic in *Drosophila* (PAPA score=0.10) and polyhomeotic-like protein 1 in mammals (PAPA score=0.07) are predicted to exhibit a propensity for prion formation (a PAPA score >0.05).

MATERIALS AND METHODS

Prediction of the prion-forming propensity of proteins

Predicted protein sequences from the *Tetrahymena thermophila* macronuclear genome (version released in 2008) were analyzed using the PAPA algorithm (Toombs et al., 2012). Proteins with scores above 0.1 were selected. Proteins that are highly and specifically expressed during conjugation were then selected using gene expression data that has been previously published (Miao et al., 2009). The upregulation of genes during conjugation was scored by dividing the maximum mRNA expression value for a gene during conjugation by that for the same gene during the vegetative stages (c/v score). Proteins with a c/v score above 20 and a maximum expression value above 7500 were selected as being highly and specifically expressed proteins during conjugation.

Strains and culture conditions

The wild-type *Tetrahymena thermophila* strains B2086 and CU428 were provided by Dr Peter J. Bruns (Cornell University, Ithaca, NY). The *JUB6* MIC-KO strains are described below. Cells were grown in SPP medium (Gorovsky et al., 1975) containing 2% proteose peptone at 30°C overnight. To induce conjugation, exponentially growing cells (~5×10^5/ml) of two different mating types were starved in 10 mM Tris-HCl (pH 7.5) at 30°C overnight and were mixed to allow conjugation (5×10^5–7×10^5/ml) at 30°C.

Ectopic expression of proteins in vegetative cells

The open reading frames of *TTHERM_01415190* (*JUB6*), *TTHERM_00647510*, *TTHERM_00145310*, *TTHERM_00421130*, *JUB1*, *LIA1*, *LIA4*, *LIA5*, *PDD1* and *PDD2* were PCR amplified from *Tetrahymena* cDNA and cloned into pBNMB1-HA (Woehrer et al., 2015). The DNA oligonucleotides used to generate the constructs are listed in Table S1. All constructs were digested with XhoI and introduced into the macronucleus of B2086 cells using a biolistic gun (see Fig. S2). Phenotypic assortment was performed until cells could grow in ~20 mg/ml paromomycin. The cells were then starved in 10 mM Tris-HCl pH 7.5 overnight, and the expression of the proteins was induced by incubating the cells in 0.1 µg/ml $CdCl_2$ for 5 h.

Semi-denaturing detergent agarose gel electrophoresis

SDD-AGE was performed as described previously (Alberti et al., 2010) with slight modifications. Approximately 7.5×10^7 cells expressing HA–Jub6p were

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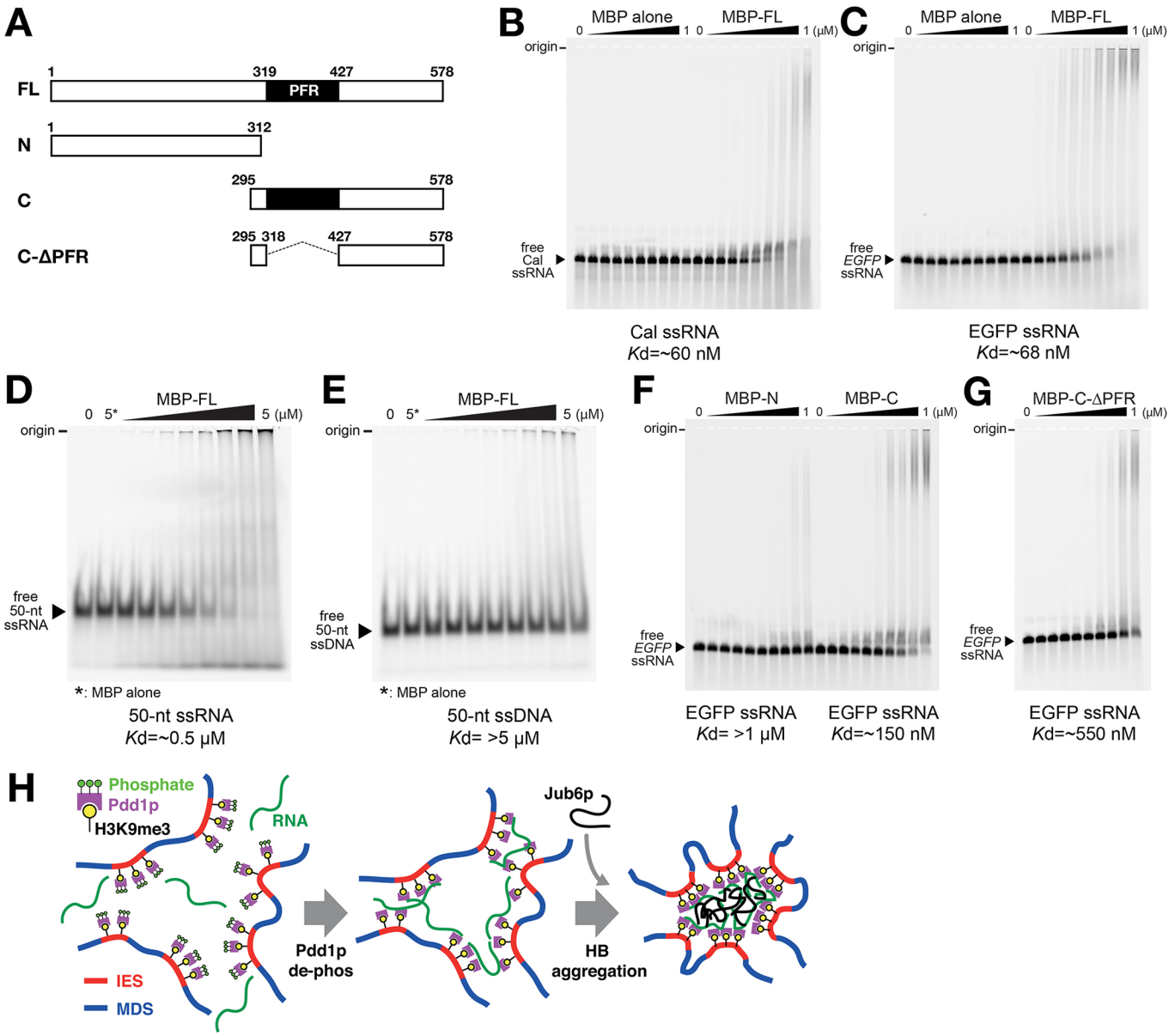

Fig. 6. RNA-binding assays of Jub6p. (A) Schematic representation of the Jub6p proteins used for EMSA experiments. All proteins were tagged with MBP at their N-terminus. (B,C) Fluorescently labeled Cal (B) or EGFP (C) ssRNAs (10.4 nM) were incubated with a 0–1 μM concentration of the indicated proteins, followed by analysis using agarose gel electrophoresis. (D,E) Fluorescently-labeled 50 nM of 50-nucleotide ssDNA (D) or ssRNA (E) oligonucleotide was incubated with 5 μM of MBP alone or 0–5 μM (0, 0.04, 0.1, 0.2, 0.3, 0.6, 1.3, 2.5 and 5 μM) of MBP-tagged full-length Jub6p (MBP–FL) and analyzed by native polyacrylamide gel electrophoresis. (F,G) Fluorescently labeled EGFP ssRNA (10.4 nM) was incubated with 0–1 μM of MBP–N (F, left), MBP–C (F, right) or MBP–C-ΔPFR (G) followed by analysis with agarose gel electrophoresis. (H) 'Cloud seed' model for the role of Jub6p in heterochromatin body formation. Left, Pdd1p is phosphorylated at the onset of heterochromatin formation by IESs (red lines), and this phosphorylation prevents the interaction of RNA and Pdd1p. Middle, Pdd1p is dephosphorylated (de-phos) after heterochromatin formation, which allows RNA to interact with Pdd1p. Right, Jub6p is recruited to heterochromatin through RNA and facilitates the formation of heterochromatin bodies (HBs).

lysed in TMSN buffer [0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 3 mM $CaCl_2$, 0.016% NP-40, 1 mM PMSF, 1× complete protease inhibitor (cpi) cocktail (Roche), 0.1 μg/ml DAPI] using a Dounce homogenizer (15 strokes), and the nuclei were collected by centrifuging at 4500 ***g*** and 4°C for 5 min. The nuclei were washed three times and suspended in 11 ml of TMSN buffer. Three volumes of the suspension were mixed with 1 volume of 4× sample buffer (2× TAE; 20% glycerol; 2, 4or 8% SDS; 20× cpi cocktail; 4 mM PMSF), followed by incubation at either 95°C (denatured) or room temperature (semi-denatured) for 10 min. The lysate was separated on a 1.5% agarose gel in 1× TAE and 0.1% SDS at room temperature at 40 V for 4 h. The proteins were blotted onto a PVDF membrane (Immobilion-PSQ, 0.2 μm pore, Millipore) through capillary transfer with 1× TBS (150 mM NaCl, 100 mM Tris-HCl, pH 7.5), and HA–Jub6p was detected with the antibody against HA.11, clone 16B12 (Covance; 1:5000). Then, the antibodies were stripped, and endogenous Rpb3p was detected with an antibody that had been raised against the peptide (LRDEYGNKREVGC) corresponding to amino acids 137–148 of *Tetrahymena* Rpb3p (underlined, the last cysteine residue is for KLH conjugation) (1:1000).

Immunofluorescence staining and DNA-FISH

To produce the antibody against Jub6p, a rabbit was immunized with a peptide (EQQKSENNLQNENIKLQENGNLC) corresponding to amino acids 235–256 of Jub6p (underlined, the last cysteine residue is for KLH conjugation). The rabbit antibodies against Twi1p (Aronica et al., 2008) and guinea pig anti-

Pdd1p antibody (Kataoka and Mochizuki, 2015) used in this study have been described previously. Cells were fixed and processed as previously described (Kataoka and Mochizuki, 2015). For immunofluorescence staining, the fixed cells were incubated with a primary mouse anti-HA antibody (1:2000, HA.11 clone 16B12), rabbit antibodies against Ju6p (1:1000), H3K9me3 (07-442, Merck Millipore; 1:500), H3K27me3 (07-449, Merck Millipore; 1:500) or Pdd1p (ab5338, Abcam; 1:1000), or a guinea pig antibody against Pdd1p (1:1000) at 4°C overnight and then incubated with secondary antibodies [1:1000–1:2000 anti-mouse, anti-rabbit or anti-guinea-pig IgG conjugated with Alexa-Fluor-488, Alexa-Fluor-568 or Alexa-Fluor-647 (Invitrogen)] at room temperature for 2 h. They were counterstained with 40 ng/ml DAPI and analyzed using an epifluorescence microscope. DNA-FISH was performed as previously described (Loidl and Scherthan, 2004; Noto et al., 2010).

Construction of *JUB6* MIC-KO strains

To generate the *JUB6* KO construct (Fig. S3A), the 5′ and 3′ flanking sequences of *JUB6* were first amplified using PCR with the primers *PRN1*-KO-5FWXba+*PRN1*-KO-5RVBSS (Product-5′) and the primers *PRN1*-KO-3FWBSS+*PRN1*-KO-3RVXho (Product-3′). Then, Product-5′ and Product-3′ were connected through overlapping PCR amplification (Product-5′+3′), which created a SmaI site in the middle of the product. Product-5′+3′ was digested with XbaI and XhoI, and cloned into the pBlueScriptSK(+) vector. Next, a *neo4* cassette was inserted into the SmaI site. The *JUB6* KO construct was obtained from the vector through digestion with XbaI and XhoI, and was introduced into conjugating UMPS strains (Vogt and Mochizuki, 2013) through biolistic transformation. Paromomycin- and 5-fluoroorotic acid (5-FOA)-resistant progeny cells were selected, and cells with the *JUB6*-KO locus in the MIC were selected following genomic PCR with PRN1-gKO_cFW2 and PRN1-gKO_cRV2 and EcoRI digestion (Fig. S3B). *JUB6*-KO heterozygous strains were then mated with the 'star strain' B*6 (Fig. S3B) to obtain *JUB6*-KO homozygous (*JUB6* MIC-KO) strains (Fig. S3D). Finally, *JUB6* KO loci in the MAC were removed through phenotypic assortment (Fig. S3C). The DNA oligonucleotides used to generate the targeting and transgenic constructs are listed in Table S1.

Viability testing

Single mating pairs at 6–8 h post mixing were isolated in drops of SPP medium. After ∼60 h of incubation at 30°C, the drops were examined for cell growth. To assess the completion of conjugation after mating, cells were incubated in SPP with 15 µg/ml 6-methylpurine (6-mp, Sigma) for wild-type cells or with 120 µg/ml paromomycin for *JUB6* MIC-KO cells and were then scored for resistance.

EMSA

To express MBP–Jub6p, the *JUB6* gene sequence that had been codon-optimized for expression in *Escherichia coli* was cloned into pMAL-c2X (NEB). MBP–Jub6p and MBP were expressed in the *E. coli* strain BL21 (DE3) and purified as described previously (Kataoka and Mochizuki, 2015). The proteins were then dialyzed against interaction buffer (20 mM HEPES-NaOH pH 7.5, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol) at 4°C overnight. MBP–Jub6p was further concentrated using Vivaspin concentrators (either 50- or 100-kDa cutoff, Sartorius). Fluorescein-12 UTP-labeled EGFP ssRNA (723 nucleotides) and Cal IES ssRNA (1305 nucleotides) were produced as described previously (Kataoka and Mochizuki, 2015). Two microliters of protein (0.125–12.5 pmol) in interaction buffer was incubated with 8 µl of EMSA buffer (20 mM HEPES-NaOH pH 7.5, 100 mM KCl, 0.05% NP-40) at room temperature for 10 min. Two microliters of EGFP ssRNA or Cal IES ssRNA (125 fmol) was subsequently added and incubated at room temperature for 30 min. For EMSA using 50-nucleotide oligonucleotides, ATTO647N-labeled 50-nucleotide DNA and RNA oligonucleotides encoding a part of EGFP (Opt.EGFP_DNA_50_S_647N and Opt.EGFP_RNA_50_S_647N) were obtained from Integrated DNA Technologies. The oligonucleotide sequences can be found below. Two microliters of the oligonucleotide (0.5 pmol) was incubated with 8 µl of protein (0.4– 50 pmol) in interaction buffer at room temperature for 30 min. The reactions with the long ssRNA and with 50-nuncleotide oligonucleotides were separated in a 0.5% agarose gel with 0.5× TBE and in a 5% native polyacrylamide gel with 0.5× TBE, respectively. The labeled nucleotides were detected using a Typhoon Trio imaging system and quantified with ImageQuant TL (GE Healthcare) or ImageJ software.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.M.; Methodology: K.K. and K.M.; Investigation: K.K. and K.M.; Writing – Original Draft: K.K. and K.M.; Writing – Review & Editing: K.K. and K.M.; Funding Acquisition: K.K. and K.M.; Supervision: K.M.

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Supplementary information
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