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Drosophila telomere capping protein HOAP interacts with DSB sensor proteins Mre11 and Nbs

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Abstract

In eukaryotes, specific DNA-protein structures called telomeres exist at linear chromosome ends. Telomere stability is maintained by a specific capping protein complex. This capping complex is essential for the inhibition of the DNA damage response (DDR) at telomeres and contributes to genome integrity. In *Drosophila* the central factors of telomere capping complex are HOAP and HipHop. Furthermore, a DDR protein complex Mre11-Rad50-Nbs (MRN) is known to be important for the telomere association of HOAP and HipHop. However, whether MRN interacts with HOAP and HipHop, and the telomere recognition mechanisms of HOAP and HipHop are poorly understood. Here, we show that Nbs interacts with Mre11 and transports the Mre11-Rad50 complex from the cytoplasm to the nucleus. In addition, we report that HOAP interacts with both Mre11 and Nbs. The N-terminal region of HOAP is essential for its co-localization with HipHop. Finally, we reveal that Nbs interacts with the N-terminal region of HOAP.

Introduction

Telomeres are nucleoprotein complexes present at the linear chromosome ends. In most organisms, telomeres are elongated by telomerase, a reverse transcriptase which adds short DNA repeats to chromosome ends (Blackburn et al., 2006). In addition, a specific capping protein complex called shelterin in mammal associates with the telomeric sequences to prevent the chromosome ends from being recognized as DNA double-strand breaks (DSBs). Shelterin is also involved in the maintenance of telomere elongation by regulating telomerase recruitment at telomeres (Palm & de Lange, 2008; van Steensel & de Lange, 1997; van Steensel et al., 1998). The lack of shelterin components induces the DDR at telomeric regions and leads to severe

telomere instability (Diotti & Loayza, 2011; Okamoto et al., 2013; Palm & de Lange, 2008). In addition to shelterin, non-shelterin proteins, including DSB repair and replication proteins, are known to be mediators of telomere stability (Drosopoulos et al., 2020; Rai et al., 2017; Zhu et al., 2000). Although these non-shelterin proteins do not recognize telomere sequence directly, they temporarily accumulate at telomeres via an interaction with shelterin components (Diotti & Loayza, 2011). Their accumulation is necessary for the replication and inhibition of DDR at telomeres (Rai et al., 2017; Sarek et al., 2019). Thus, both shelterin and no-shelterin proteins are essential for genome integrity.

Drosophila melanogaster telomeric sequences atypically consist of three kinds of non-long terminal repeat retrotransposons (*HeT-A*, *TART*, *TAHRE*) (Casacuberta, 2017; Mason et al., 2008). In spite of their unique composition, *Drosophila* telomeres are also protected by a capping protein complex, called terminin. Although terminin has no sequence homology with shelterin (except for Ver which contains a conserved OB-fold domain), they are functionally analogous to shelterin. In addition, *Drosophila* orthologs of some non-shelterin proteins are also essential for telomere protection (Bi et al., 2005; Cipressa et al., 2016). These indicate that the *Drosophila* system for telomere protection is similar to that of mammals. Terminin consists of five proteins; HOAP, HipHop, Moi, Ver and Tea (Raffa et al., 2011; Zhang et al., 2016). HOAP and HipHop are known to associate with the telomere duplex regions; Ver shows high affinity to single strand DNAs; Moi and Tea interact with both HOAP and Ver to promote the affinity of Ver to single strand DNAs (Cenci et al., 2003; Cicconi et al., 2017; Gao et al., 2010; Raffa et al., 2010; Raffa et al., 2009; Zhang et al., 2016). These capping proteins are essential for telomere stability in *Drosophila*. However, several studies have shown that *Drosophila* telomere capping is independent of DNA

sequence, and how terminin recognizes telomeres is still unknown (Gao et al., 2010; Kurzhals et al., 2017; Rong, 2008). Recently, Zhang *et al.* reported that HOAP and HipHop are essential for the accumulation of other terminin components to telomeric regions (Zhang et al., 2016). This suggests that telomere association of HOAP and HipHop is the first step of telomere capping. Therefore, dissection of the mechanisms by which HOAP and HipHop recognize telomeres is vital to allow an understanding of the whole telomere protection system in *Drosophila*.

The MRN complex is known as a DSB sensor which rapidly accumulates at DSB sites and induces DNA end resection. Subsequently, other DDR proteins carry out error-free repair by homologous recombination (Lamarche et al., 2010; Liu and Huang, 2016). Therefore, MRN acts as a primary activator in the DSB repair pathway. In MRN fly mutants, the accumulation of HOAP and HipHop at telomeres was reduced (Ciapponi et al., 2004; Ciapponi et al., 2006; Gao et al., 2010), which suggests that MRN also plays a critical role in telomere recognition by HOAP and HipHop. However, the function of MRN as non-terminin proteins in telomere capping and whether MRN interacts with HOAP and/or HipHop remain poorly understood.

Here, we used immunofluorescent (IF) staining and immunoprecipitation (IP) to reveal that Nbs transports Mre11-Rad50 complex from the cytoplasm to the nucleus. Baculovirus based expression and IP revealed that HOAP interacted with both Mre11 and Nbs. Moreover, we mapped the Nbs interaction region to the N-terminus of HOAP and showed that this N-terminal region is important for the telomere recognition of HOAP.

Results

The nuclear localization of MRN is dependent on Nbs

To focus on the nuclear transport mechanism of MRN, we expressed tagged or EGFP fused components of the MRN complex in S2 cells and carried out IF to examine their cellular distribution (Fig. 1A-C). Tagged Mre11, Rad50 and Nbs showed obviously different cellular distributions (Fig. 1A), while FLAG, HA and EGFP showed only diffuse signals all over the cells (Fig. S1A). Nbs localized in the nucleus, Mre11 was diffuse throughout the whole cell, and Rad50 formed huge foci in the cytosol (Fig. 1A). Because MRN was known as a nucleoprotein complex (Carney et al., 1998; Desai-Mehta et al., 2001; Zhu et al., 2000), we predicted that Nbs transports Mre11 and Rad50 from the cytoplasm to the nucleus. To confirm our prediction, we carried out double and triple transfections of the MRN components into S2 cells and carried out immunostaining (Fig. 1B, C). We found that co-expression of Nbs and Mre11 altered Mre11 localization to the nucleus, but co-expression of Nbs and Rad50 did not affect the cytoplasmic localization of Rad50 (Fig. 1B). Furthermore, co-expression of Mre11 and Rad50 generated large co-localized signals in the cytosol (Fig. 1B), which suggested that Mre11 interacted with Rad50 and formed a Mre11-Rad50 complex in the cytosol. We confirmed that introduction of a tag onto Nbs and Mre11 did not affect the cellular localizations of the proteins (Fig. S1B). In addition, triple expression of Mre11, Rad50 and Nbs altered the localization of Mre11 and Rad50 from the cytoplasm to the nucleus (Fig. 1C). We also observed a similar nuclear transport system for overexpressed Ku heterodimer and Mei-9-Ercc1 dimer (unpublished data). Thus, our results suggested that nuclear transport and protein interactions were conserved in the overexpressed proteins. To confirm the interaction between Nbs-Mre11 and Mre11-Rad50, we used a baculovirus based expression system and IP (Fig. 1D). We co-expressed Myc-Mre11 with either HA-Nbs or FLAG-Rad50 in Sf21 cells and detected both the Nbs-Mre11 and Mre11-Rad50

interaction (Fig. 1D). These results suggested that Nbs transported the Mre11-Rad50 complex to the nucleus via its interaction with Mre11.

The nuclear localization of HOAP and HipHop is independent of MRN

Localization of HOAP and HipHop at telomeric regions was dramatically reduced in MRN mutant flies (Ciapponi et al., 2004; Ciapponi et al., 2006; Gao et al., 2010). Therefore, we predicted that MRN mediated the distributions of HOAP and HipHop. First, we expressed HOAP and HipHop in S2 cells, and carried out IF to examine their cellular localization. We found that both HOAP and HipHop distributed to nuclei (Fig. 2A). Next, we co-expressed each component of MRN with HOAP or HipHop to compare how this affected the cellular localization of each individual protein (Fig. 2B, C). Unexpectedly, the expression of MRN components did not affect HOAP and HipHop nuclear localizations (Fig. 2B, C). Moreover, neither HOAP or HipHop altered the cytosolic distributions of Mre11 and Rad50, or the nuclear localization of Nbs (Fig. 2B, C). Thus, these results suggested that MRN, HOAP and HipHop independently localize in the nucleus.

HOAP interacts with Mre11 and Nbs

Next, we examined the protein-protein interaction between HOAP, HipHop and MRN. We quadruply expressed FLAG-Mre11, FLAG-Rad50 and FLAG-Nbs with HA-HOAP or HA-HipHop in S2 cells and carried out IPs with α-FLAG antibody (Fig. 2D). We found that MRN interacted with HOAP, but not with HipHop (Fig. 2D). Therefore, we focused on the HOAP-MRN interaction and examined which MRN components interacted with HOAP. We carried out IPs using Sf21 cells expressing HOAP and each of three MRN components. In the IPs, we found that

HOAP interacted with both Mre11 and Nbs, but not with Rad50 (Fig. 2E). This result suggested the possibility that MRN accumulated at telomere via the interaction with HOAP, and vice versa.

HOAP and HipHop form characteristic telomeric localized dots on detergent treatment

HOAP and HipHop are telomere capping proteins and are detected as dot-signals in nuclei and only localize to telomeres (Cenci et al., 2003; Cicconi et al., 2017; Gao et al., 2010; Raffa et al., 2011; Raffa et al., 2010; Raffa et al., 2009; Zhang et al., 2016). In contrast, our IF experiments showed diffuse-signals of HOAP and HipHop in nuclei when overexpressed (Fig. 2A, 3A). Since excess proteins could mask the dot-signals at telomeric region, to observe the telomeric dot-signals of HOAP and HipHop, we removed interfering nucleoplasmic proteins by detergent treatment (Zhu et al., 2000). Unlike the dispersed IF signals observed in untreated cells, detergent treated cells showed clear HOAP-HipHop co-localized dot-signals (Fig. 3A). To confirm that these dot-signals were telomere associated HOAP and HipHop, we established endogenous HOAP-FLAG (endo-HOAP) expressing cell lines using CRISPR/*Cas9*. Although we could not observe endo-HOAP association with chromosome ends in metaphase cells, the endo-HOAP exhibited clear dot-signals in anaphase cells as previously reported (Fig. 3B). Thus, we used endo-HOAP as a putative telomere marker and found that the putative telomeric signals perfectly merged with exogenous HipHop (Fig. 3B). This indicated that overexpressed HOAP and HipHop also associated with the putative telomeric sites.

To examine whether Nbs localized at telomeres, we carried out detergent treatment on Nbs-HOAP and Nbs-HipHop co-expressed cells. Nbs signals disappeared from nuclei and only HOAP or HipHop telomeric dot-signals were observed (Fig. 3C), although untreated cells showed

Nbs-HOAP or HipHop co-dispersed signals in nuclei (Fig. 2B, C). In addition, expressed Nbs also did not show any signals after detergent treatment in endo-HOAP cells (date not shown). These data suggested that Nbs did not bind tightly to putative telomere regions. However, we could not exclude the possibility that the amount of Nbs at telomeric region was not sufficient to detect by IFs.

HOAP N-terminal region is essential for telomere accumulation

Despite of the important role of HOAP in telomere protection, no functional domains in HOAP have been reported. We aligned the HOAP amino acid (aa) sequences from eight *Drosophila* species and found two conserved sites: an N-terminal region (residues 72 to 145) and three amino acids, STQ (residues 279 to 281) in the C-terminus (Fig. 4A). In addition, a previous study reported that the C-terminal region of HOAP was essential for telomere stability (Cenci et al., 2003). Therefore, we created four tagged HOAP deletion constructs: $HOAP^{113\text{-}337}$, $HOAP^{225\text{-}337}$, $HOAP^{1\text{-}286}$ and $HOAP^{\Delta STQ}$ to determine the essential region for HOAP telomere recognition (Fig. 4E). We co-expressed the truncated HOAP constructs with HipHop in S2 cells and carried out IF. We found that $HOAP^{113\text{-}337}$, $HOAP^{1\text{-}286}$ and $HOAP^{\Delta STQ}$ localized in the nucleus, but $HOAP^{225\text{-}337}$ disrupted the nuclear localization and dispersed the protein in the cytosol (Fig. 4B). These results suggested that nuclear localization signals of HOAP are located in the amino acid region between 113 and 224. The truncated $HOAP^{225\text{-}337}$, which lacked nuclear accumulation, did not alter the nuclear distribution of HipHop (Fig. 4B). This implied that HipHop nuclear distribution was independent of HOAP. Moreover, we used HipHop as a telomere marker and carried out IF on detergent treated cells carrying truncated HOAP proteins (Fig. 4C). We found that $HOAP^{113\text{-}337}$

lacked the nuclear foci, although HOAP$^{\Delta STQ}$ and the other truncated HOAP showed clear co-localization with HipHop (Fig. 4C). Therefore, we concluded that the N-terminal region (1-112 aa) of HOAP, but not the conserved STQ in the C-terminus of HOAP, was essential for its co-localization with HipHop.

The N-terminus of HOAP interacts with Nbs

To analyze whether the conserved N-terminal region or the C-terminal region of HOAP interacted with Nbs, we carried out IPs using Sf21 cells expressing Nbs and each deleted HOAP (Fig. 4D, E). In the IPs, we found that both HOAP$^{\Delta STQ}$ and HOAP$^{1-286}$ retained the interaction with Nbs, but HOAP$^{113-337}$ and HOAP$^{225-337}$ lacked interaction (Fig. 4D). Therefore, we concluded that Nbs interacted with the N-terminal region (1-112 aa) of HOAP.

Discussion

Previous studies reported that MRN is important for the telomere localization of HOAP and telomere stability (Ciapponi et al., 2004; Ciapponi et al., 2006; Gao et al., 2009; Gao et al., 2010). Therefore, MRN is thought to be involved in HOAP telomere recruitment. However, whether MRN interacted with HOAP was not clear.

Here, we reported that the MRN components, Mre11 and Nbs, interact with HOAP. HOAP is known for binding to terminal double-strand DNA (dsDNA) regions of the telomere (Gao et al., 2010) while MRN binds to ends of dsDNA (Myler et al., 2017), therefore both HOAP and MRN tend to associate to ends of dsDNA. Thus, there is a possibility that ends of dsDNA could act as a scaffold to promote HOAP-MRN interaction. We also showed that the nuclear localization of

Mre11-Rad50 is dependent on Nbs, but HOAP does not affect the cytoplasmic localization of Mre11 in spite of their detected interaction by IP. Therefore, we predict that HOAP and Mre11 interact in nucleus after Nbs-Mre11 transport from cytoplasm to nucleus. In addition, we could not observe co-localized foci of Nbs-HOAP after detergent treatment. We suspect it is due to the weak chromatin binding of Nbs. In mammals, the accumulation of NBS1 to a DSB site is dependent on the DNA binding activity of MRE11-RAD50 (Myler et al., 2017). Hence, Nbs could also bind to chromatin tightly via the interaction with the Mre11-Rad50 complex in *Drosophila*. Therefore, we predict that the telomeric foci of Nbs after detergent treatment would be detected in Mre11-Rad50-Nbs overexpressing cells. In mammals, NBS1 interacts with TRF2, a mammalian telomere duplex region binding protein, and maintains telomere specific loop formation (Rai et al., 2017). It is therefore possible that, as for the interactions of HOAP-Mre11 and HOAP-Nbs, MRN also interacts with HOAP to promote loop formation in *Drosophila,* and the interaction of MRN-HOAP is essential for maintenance of telomere structure. In addition to HOAP, the association of HP1a at telomere regions is also reduced in the MRN mutants (Ciapponi et al., 2004; Ciapponi et al., 2006). HP1a is known as a heterochromatin protein and interacts with HOAP (Badugu et al., 2003). Moreover, a recent study reports that Nbs also interacts with HP1a (Bosso et al., 2019). The interaction that we have detected between Nbs and HOAP suggests that both Nbs-HP1a and Nbs-HOAP interactions may contribute to telomere stability.

We showed that Nbs is essential for the nuclear transport of Mre11-Rad50. In addition, several previous studies showed that Rad50 formed nuclear foci in WT flies, but not in *mre11* and *nbs* mutants (Ciapponi et al., 2004; Ciapponi et al., 2006; Gao et al., 2009). This strongly supports our IF results that both Mre11 and Nbs are essential for nuclear localization of Rad50. We

detected overexpressed Rad50 as huge foci in the cytoplasm. Rad50 is known to have long coiled-coil domains and form a homodimer or a heterotetramer via Zn^{+} (He et al., 2012). In addition in *Drosophila*, it was reported that endogenous Rad50 formed large nuclear foci in embryos (Gao et al., 2009). Therefore it seems that Rad50 tends to aggregate, suggesting that overexpressed Rad50 also forms dimers and/or tetramers, which associate as huge blobs in the cytoplasm. Although we have not verified the DSB repair function of overexpressed MRN components, our results suggested that nuclear transport system and protein-protein interactions are conserved even in the overexpression experiments. Taken together, we conclude that Nbs is essential for the nuclear localization of MRN in *Drosophila*. In addition, mammalian MRN uses a similar transport mechanism as *Drosophila* (Carney et al., 1998; Desai-Mehta et al., 2001), suggesting that nuclear localization system of MRN is conserved between mammals and *Drosophila*.

We report that the N-terminal region of HOAP is essential for its co-localization with HipHop. Although HOAP has no functional domains, several studies reported that the N-terminus of HOAP has sequence similarity with the high mobility group (HMG) box domains (Cenci et al., 2003; Vedelek et al., 2015). The HMG-box domain is found in both sequence-dependent and -independent DNA binding domains (Stros et al., 2007). Therefore, the N-terminal region of HOAP is expected to be important in telomeric DNA binding. Here, we showed the first evidence that the N-terminal region of HOAP is necessary for its localization at putative telomeres. Moreover, we mapped the Nbs interacting site to the N-terminus of HOAP, suggesting that Nbs might regulate the DNA binding activity of HOAP.

The C-terminal truncated $HOAP^{1-286}$ showed clear telomere dot-signals with HipHop.

However, the C-terminal region of HOAP was essential for its telomere binding (Cenci et al., 2003). This contradiction could be explained by differences in the deletion sites used in the two studies. In Cenci *et al*., the C-terminal truncated HOAP altered the aa sequence from residue 261 and caused a stop codon at aa 281 (Cenci et al., 2003). This truncated HOAP is shorter than $HOAP^{1-286}$ we used in this study. Therefore, the C-terminal 261-281 aa region is likely to be essential for the telomere binding function of HOAP. We also showed that the conserved STQ (279-281) in the C-terminus of HOAP was not necessary for its telomere localization. As TRF2 is phosphorylated by ATM and ERK1/2 in mammals (Huda et al., 2009; Picco et al., 2016; Tanaka et al., 2005), the conserved STQ may be a target for serine/threonine kinases in the DDR pathway or MAPK signaling pathway.

In mammals, TRF2 binds telomeric DNA in a sequence dependent manner (Diotti & Loayza, 2011; Palm & de Lange, 2008). However, several studies recently reported that TRF2 also possesses DNA sequence-independent binding for its accumulation at DSB sites (Badugu et al., 2003; Huda et al., 2012). In addition, Kong *et al.* reported that the accumulation of TRF2 at DSB sites can be stabilized by the interaction of MRE11 and NBS1 (Kong et al., 2018). Several studies have presented evidence indicating that HOAP binds to telomeres in a DNA sequence independent manner (Cenci et al., 2003; Gao et al., 2010; Raffa et al., 2011), but no evidence has shown that HOAP is involved in the DSB repair pathway. Our present finding of the interactions of HOAP-Mre11 and HOAP-Nbs suggests functional similarities between TRF2 and HOAP. They also provide new approaches to study the DNA sequence-independent binding mechanism of telomere capping proteins, and suggest that *Drosophila* is a useful model for such studies.

Experimental procedures

Plasmid constructs

Full-length cDNA of *cav* (HOAP) and *HipHop* were obtained by RT-PCR using NucleoSpin RNA Plus (MACHEREY-NAGEL, Düren, Nordrhein-Westfalen, Germany) and SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Waltham, MA). Full-length cDNAs of *mre11*, *rad50* and cDNA of *nbs* were obtained from the *Drosophila* Genomics Resource Center (DGRC) (LD08638, LD44438, LP09268 respectively). For full-length *nbs* cDNA, two nucleotides were added using by PrimeSTAR Mutagenesis Basal Kit (TaKaRa, Shiga, Japan). All cDNAs were amplified by PCR and cloned in pAc5.1/V5-His A (Thermo Fisher Scientific) and pFastBac1 (Thermo Fisher Scientific) with tag sequences (FLAG, HA, Myc) or EGFP protein sequences. The truncated cDNAs were generated by cloning the corresponding PCR-generated sequences into pAc5.1/V5-His A. All constructs were confirmed by sequencing.

Transfection

S2 cells (Thermo Fisher Scientific) and S2R+ cells (DGRC) were grown in Shields and Sang M3 insect media (Sigma, St. Louis, MI) containing 10% heat-inactivated fetal bovine serum (Biosera, Nuaille, France) at 28°C. For each immunofluorescence and immunoprecipitation experiment, 0.5×10^6 and 6.0×10^6 cells were seeded and transfected with Fugene HD (Promega, Madison, WI) or siLentFect (Bio-Rad, Hercules, CA). Cells were harvested 24 h or 72 h after transfection for immunofluorescence staining and immunoprecipitation respectively.

Immunofluorescence staining

Cells were incubated for 30 min on poly-lysine coated coverslips, washed with PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) then either fixed for 10 min with 4% paraformaldehyde immediately or incubated in TritonX-100 buffer (20 mM HEPES-KOH [pH7.9], 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, 0.5% TritonX-100) at RT for 3 min before fixation (Zhu et al., 2000). After fixation, cells were permeabilized in PBS containing 0.2% Triton X-100 for 30 min, blocked in 1% milk powder for 10 min, and incubated with the following primary antibodies, mouse anti-DDDDK (1E6) (1:1,000; MBL, Aichi, Japan), mouse anti-HA (TINA2) (1:1,000; cat# M183-3, MBL), rabbit anti-DDDDK (1:1,000; cat# PM020, MBL) and rabbit anti-HA (1:1,000; cat# 561, MBL) for 1 h. After incubation, cells were washed in PBS containing 0.1％ NP-40 and incubated with the following secondary antibodies, Alexa Fluor 594 donkey Anti-Mouse IgG (1:500; cat# 715-586-151, Jackson Immuno Research, West Grove, PA), Alexa Fluor 488 donkey Anti-Rabbit IgG (1:500; cat# 711-546-152, Jackson Immuno Research), Alexa Fluor 647 donkey Anti-Rabbit IgG (1:200; cat# 711-605-152, Jackson Immuno Research), Rhodamine Red donkey Anti-Mouse IgG (1:200; cat# 715-295-151, Jackson Immuno Research) antibodies and 1.5 μg /ml DAPI for 30 min. After incubation, cells were washed in PBS containing 0.1％ NP-40 and mounted in VECTASHIELD Mounting Medium (Vector Lab, Burlingame, CA). Cells were analyzed using a confocal fluorescence microscope (FLUOVIEW FV10i, Olympus, Tokyo, Japan) and pictures were edited by ImageJ (National Institutes of Health, Bethesda, MD).

Immunoprecipitation and Western blotting

Infected Sf21 cells and transfected S2 cells were washed in ice-cold PBS and incubated in lysis buffer (20 mM Tris-HCl [pH7.5], 350 mM NaCl, 10% glycerol, 0.1% TritonX-100) containing

protease inhibitor cocktail (Fujifilm Wako, Osaka, Japan) at 4°C for 1 h. Cell extracts were spun at 14,000 x g for 15 min at 4°C. For IP samples, 1 mg cell extracts were incubated with 40 µl of Anti-DYKDDDDK tag antibody beads (1E6) (cat# 018-22783, Fujifilm Wako) or Anti c-Myc antibody beads (10D11) (cat# 106-26503, Fujifilm Wako) for 4 h at 4°C. The beads were washed three times in lysis buffer for 10 min at 4°C, and proteins were eluted with 40 µl of sample buffer (125 mM Tris, 0.1% SDS, 5% 2-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue) for 5 min at 95°C. For immunoblotting, protein samples were run into SDS-polyacrylamide gels and electro-blotted on polyvinylidene fluoride microporous membranes (Merck Millipore, Burlington, MA). Membranes were blocked in 5% milk powder for 1 h and then probed with Anti-DDDDK-tag mAb-HRP-DirectT (FLA-1) (1:10,000; cat# M185-7, MBL), Anti-HA-tag mAb-HRP-DirectT (TINA2) (1:10,000; cat# M180-7, MBL), Anti-Myc-tag mAb-HRP-DirectT (My3) (1:10,000; cat# M192-7, MBL) antibodies for 1 h. For detecting tagged-proteins, ImmunoStar Zeta (Fujifilm Wako) and ImageQuant LAS 500 (Thermo Fisher Scientific) were used.

Baculovirus based expression system

Spodoptera frugiperda Sf21 insect cells were cultured in Grace's Insect Cell Culture Medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (Biosera) at 28°C. For producing recombinant baculovirus, the Bac-to-Bac Baculovirus Expression System (Thermo Fisher Scientific) was used following the manufacture's-user manual. For IP, 2.0×10^6 cells were infected or co-infected by the appropriate recombinant baculovirus (multiplicity of infection ≈6) and harvested 60 h after infection.

CRISPR/*Cas9*

For genome editing, S2R+ cells were used. pAc-sgRNA-*cas9*, pMH4 plasmids were obtained from Addgene (Watertown, MA) (#49330, #52529). The primer sequences for *cav*-sgRNA and *cav*-targeted HR-mediated template were listed below. All experiments were performed as described previously (Bassett et al., 2014; Bottcher et al., 2014). Clonal selection was carried out after confirmation of template recombination by PCR. Integration of FLAG sequences were verified by sequencing, and HOAP-FLAG expression were confirmed by immunoblotting.

The following oligonucleotides were used in CRISPR/*Cas9*: *cav*-sgRNA: 5′-ATATGGACATCAGGCTATTG, *cav*-targeted up: 5′-TGGACCGTCAATTGATTCGGAAGGAACTATCACTTTTCAAAATACAGAATCTGAGCCGATCGACGTCACCTCAATAGCCTTGGACCGTCAATTTTCGGAAGGAACTATCACTTTTCAAAATACAGAATCTGAGCCGATCGACGTCACTCAATAGCC, *cav*-targeted down: 5′-TTTTTAATTAATTAACACCAATAAATGAAGAACTTTATATAGTTTAATATATGGACATCAGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCATATG.

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Competing interests

The authors declare no competing or financial interests.

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Figure legends

Fig. 1 Nbs interacts with Mre11 and transports the Mre11-Rad50 complex to nuclei. (A-C) Nbs was essential for MRN nuclear localization. Mre11, Rad50 and Nbs were transfected into S2 cells. DNA was visualized with DAPI (cyan). Scale bars: 10 μm. (A) Most Mre11 and Rad50 localized to the cytosol, but Nbs accumulated in the nucleus. HA-Mre11, HA-Rad50 and HA-Nbs were transfected individually and visualized by α-HA antibody (red). (B) Nbs transported Mre11 to the nucleus, but not Rad50. EGFP-Nbs was co-transfected with HA-Mre11 or HA-Rad50, and EGFP-Mre11 was co-transfected with HA-Rad50. Proteins were visualized by α-HA antibody

(red) or EGFP (green). (C) Rad50 localized in the nucleus when Mre11 and Nbs were expressed. FLAG-Mre11, HA-Rad50 and EGFP-Nbs were triple-transfected into S2 cells. Proteins were visualized by α-HA antibody (red), α-FLAG antibody (blue) or EGFP (green). (D) Nbs interacted with Mre11, and Mre11 interacted with Rad50. Immunoprecipitations were performed with α-Myc antibody using Sf21 cell extracts infected with the indicated virus, and then tagged proteins were immunoblotted with α-Myc, α-FLAG and α-HA antibodies.

Fig. 2 HOAP interacts with Nbs and Mre11. (A-C) HOAP, HipHop and MRN were transfected into S2 cells. DNA was visualized with DAPI (cyan). Scale bars: 10 μm. (A) HOAP and HipHop localized in nuclei, individually. HA-HOAP, HA-HipHop were transfected into S2 cells and visualized by α-HA antibody (red). (B) HOAP did not transport Mre11 or Rad50 to the nucleus. EGFP-HOAP was co-transfected with HA-Mre11, HA-Rad50 or HA-Nbs, respectively. Proteins were visualized by α-HA antibody (red) and EGFP (green). (C) HipHop did not transport Mre11 and Rad50 to the nucleus. EGFP-HipHop was co-transfected with HA-Mre11, HA-Rad50 or HA-Nbs, respectively. Proteins were visualized using α-HA antibody (red) and EGFP (green). (D) MRN interacted with HOAP. Immunoprecipitations were performed with anti-FLAG antibody using quadruple-transfected S2 cell extracts; FLAG-Mre11, FLAG-Rad50, FLAG-Nbs and HA-HOAP or HA-HipHop, and then immunoblotted with α-FLAG and α-HA antibodies. Arrowheads indicate weak Mre11 and Nbs signals. (E) HOAP interacted with Mre11 and Nbs. Immunoprecipitations were performed with α-FLAG or α-Myc antibodies using Sf21 cell extracts infected with the indicated constructs, and then tagged proteins were immunoblotted with α-FLAG and α-HA, α-Myc antibodies.

Fig. 3 No Nbs-HOAP co-localized signals are detected at telomeres. (A) Overexpressed HOAP and HipHop co-localized in S2 cells. EGFP-HOAP were co-transfected with HA-HipHop (upper panels), and cells were treated with detergent before fixation (lower panels). Proteins were visualized by α-HA antibody (red) and EGFP (green). Arrowheads indicate co-localization signals of HOAP and HipHop. (B) Overexpressed HipHop co-localized with endo-HOAP. Non-transfected endogenous HOAP-FLAG (endo-HOAP) expressing cells (upper panels) and EGFP-HipHop were transfected into endo-HOAP expressing cells (lower panels). All cells were treated with detergent before fixation. Proteins were visualized by α-FLAG antibody (red) and EGFP (green). Arrowheads indicate co-localization of HOAP and HipHop. (C) Nbs did not co-localize with HOAP and HipHop in S2 cells. EGFP-HOAP or EGFP-HipHop was co-transfected with HA-Nbs, and expressed proteins were visualized by α-HA antibody (red) and EGFP (green). All cells were treated with detergent before fixation. Arrows indicate HOAP or HipHop dot-signals. DNA was visualized with DAPI (cyan). Scale bars: 10 μm.

Fig. 4 HOAP N-terminal region is necessary for the HOAP telomere localization. (A) HOAP N-terminal region and STQ in the C-terminus were highly conserved among *Drosophila* species. Multiple HOAP amino acid sequences among eight *Drosophila* species were aligned by COBALT, NCBI. The general evolutionary relationship among *Drosophila* species is shown on the left. Highly and moderately conserved aa are indicated in red and blue, respectively. D. mel, *D. melanogaster*; D. sim, *D. simulans*; D. yak, *D. yakuba*; D. ana, *D. ananassae*; D. pse, *D. pseudoobscura*; D. wil, *D. willistoni*; D. moj, *D. mojavensis*; D. gri, *D. grimshawi*. (B, C) The

N-terminus is essential for HOAP telomere localization in S2 cells. HA-HipHop was co-transfected with FLAG-HOAP WT or deletion constructs. Cells were immunostained without detergent treatment (B) or treated with detergent before fixation (C). All cells were visualized by α-FLAG (red), α-HA (green) and DAPI (cyan). Scale bars: 10 μm. Arrowheads indicate HOAP-HipHop co-localization signals. Arrows indicate HipHop telomere signals. (D) N-terminus of HOAP interacted with Nbs. Immunoprecipitations were performed with α-FLAG antibody using Sf21 cell extracts infected with the indicated constructs, and then tagged proteins were immunoblotted with α-FLAG and α-HA antibodies. (E) Summary of IFs and IPs of the HOAP deletion constructs. Diagram of HOAP deletion constructs is shown on the left. Red regions indicate highly conserved regions and aa number was shown above. The right table summarizes the telomere localization in IFs (TL) and Nbs interaction in IPs (Nbs). '+' indicates a positive localization or interaction, and '−' indicates a lack of the localization or interaction.

Fig. 1

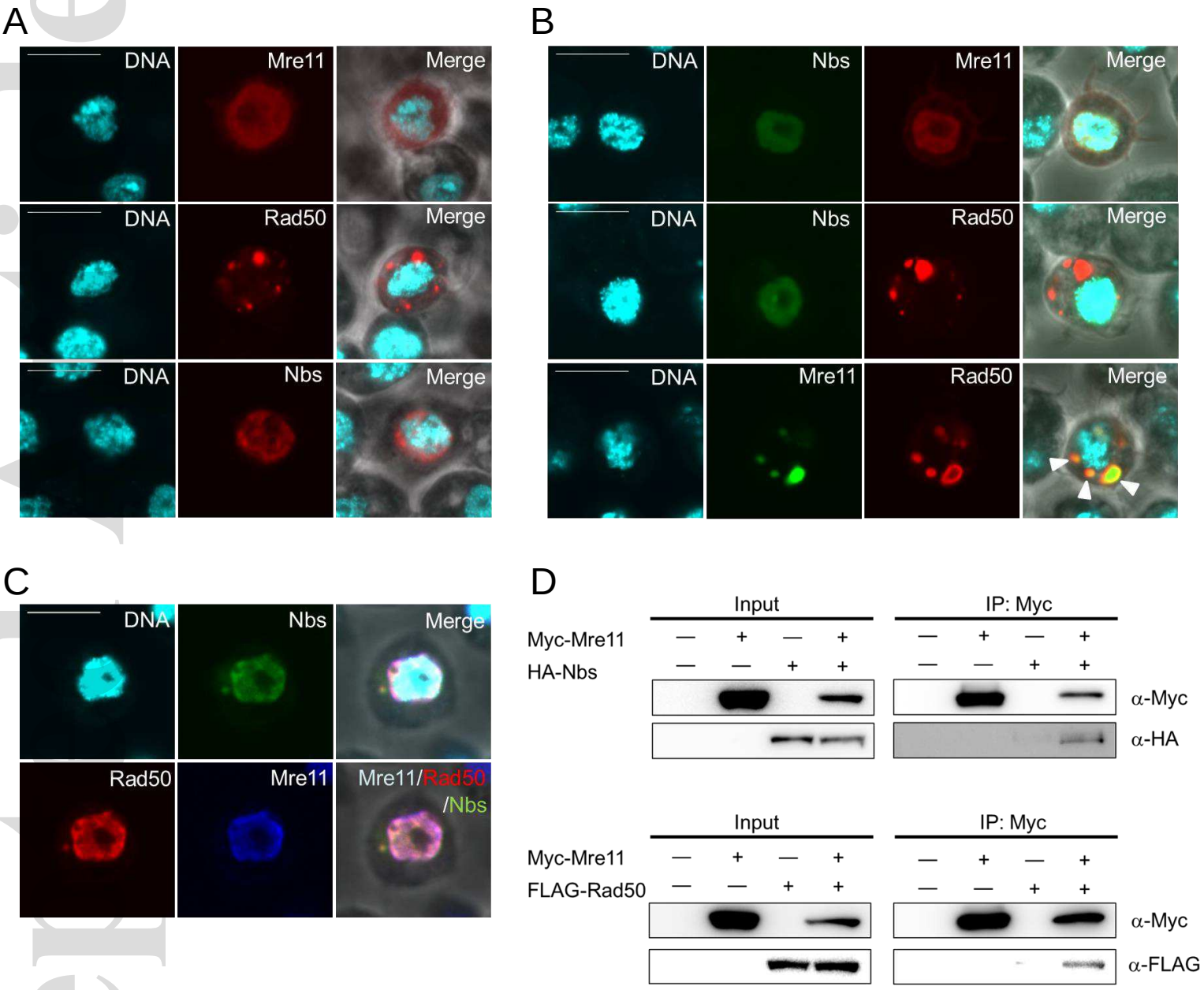

Fig. 2

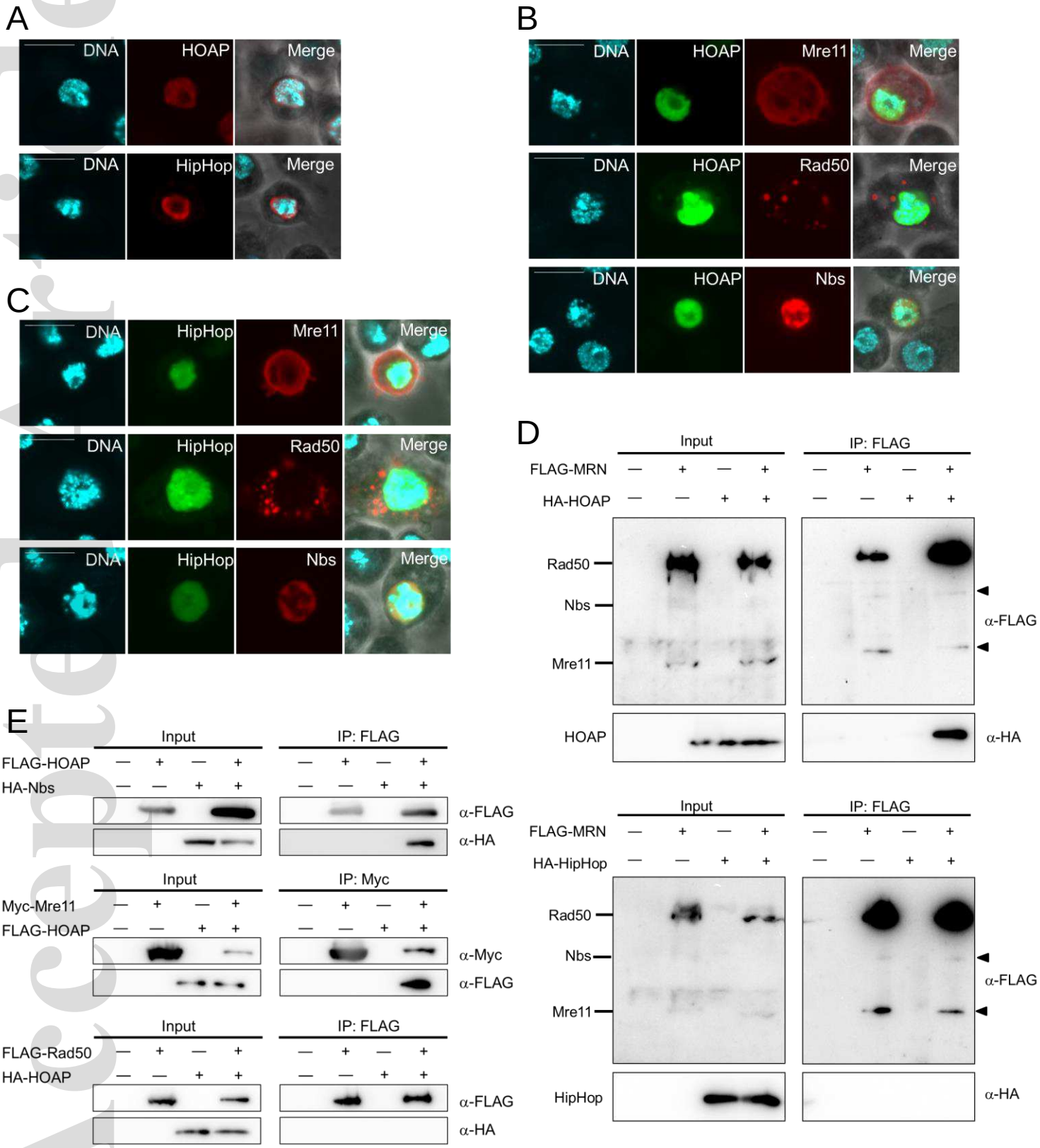

Fig. 3

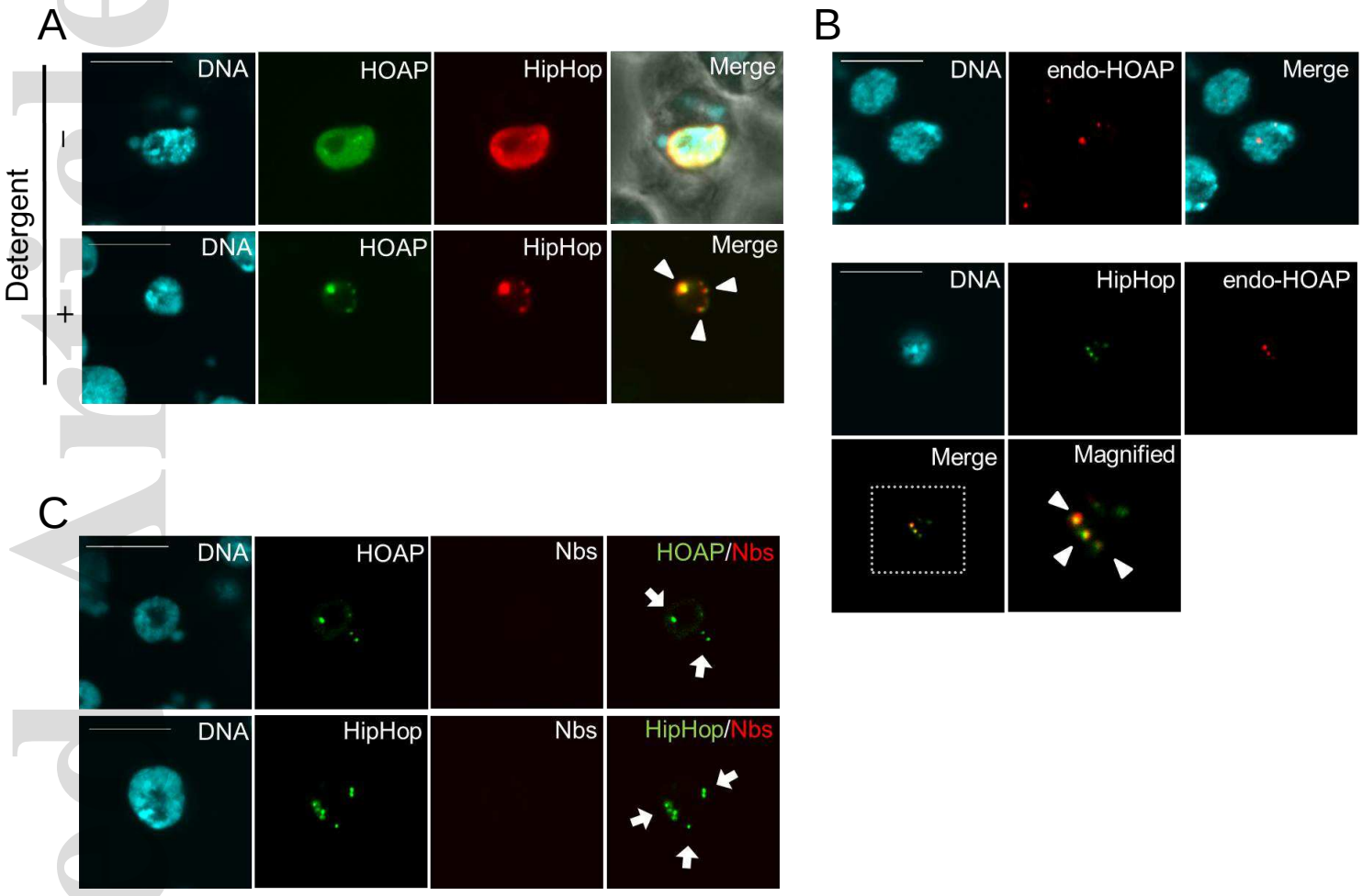

Fig. 4