Lem2 is retained at the nuclear envelope through its interaction with Bqt4 in fission yeast

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INTRODUCTION

In eukaryotic cells, the nuclear organization of chromosomes is mediated through their interactions with the nuclear envelope (NE). These interactions modulate the gene expression through the formation of heterochromatin beneath the NE (reviewed in Meekhail & Moazed, 2010; Towbin, Gonzalez-Sandoval, & Gasser, 2013; Van de Vosse, Wan, Wozniak, & Aitchison, 2011). The NE comprises the inner nuclear membrane (INM) and the outer nuclear membrane (ONM), wherein the INM proteins are considered to play key roles in the interaction of the NE with chromatin (reviewed in Czapiewski, Robson, & Schirmer, 2016; Harr, Gonzalez-Sandoval, & Gasser, 2016). Although a number of INM proteins have been identified in vertebrates (Korfali et al., 2012; Schirmer, Florens, Guan, Yates, & Gerace, 2003; Schirmer & Gerace, 2005), their roles with regard to chromosome function remain to be unveiled. Some of these INM proteins share redundant functions (Huber, Guan, & Gerace, 2009; Liu et al., 2003) and thus pose difficulties for functional studies. Conversely, the fission yeast Schizosaccharomyces pombe exhibits a relatively small number of INM proteins, and molecular genetic tools are available to separate their redundant functions. In S. pombe, it has been reported that among potentially redundant INM proteins, Lem2 displays a unique genetic interaction with another INM protein, Bqt4, which is involved in anchoring telomeres to the nuclear envelope. Double mutations in the lem2 and bqt4 genes confer synthetic lethality during vegetative growth. Here, we show that Lem2 is retained at the nuclear envelope through its interaction with Bqt4, as the loss of Bqt4 results in the exclusive accumulation of Lem2 to the spindle pole body (SPB). An N-terminal nucleoplasmic region of Lem2 bears affinity to both Bqt4 and the SPB in a competitive manner. In contrast, the synthetic lethality of the lem2 bqt4 double mutant is suppressed by the C-terminal region of Lem2. These results indicate that the N-terminal and C-terminal domains of Lem2 show independent functions with respect to Bqt4.

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growth, centromeres cluster at the spindle pole body (SPB; the equivalent of the centrosome in animals), and telomeres are attached to the NE through an interaction between telomere protein Rap1 and an INM protein Bqt4 (Chikashige et al., 2009). Upon entry into meiosis, the telomeres tether to the SPB, while the centromeres detach from the SPB. During this process of telomere tethering, the meiosis-specific Bqt1-Bqt2 protein complex binds to both Rap1 and Sad1, bridging the telomeres and the NE (Chikashige et al., 2006). Sad1 is a founding member of the SUN-domain (Sad1 and Lnc-84) family of conserved INM proteins (reviewed in Starr & Fridolfsson, 2010; Starr, 2011; Tzur, Wilson, & Gruenbaum, 2006). In S. pombe, Sad1 forms a complex with a KASH-domain protein Kms1 and the Sad1-Kms1 complex, together with the telomeres, is tethered to the SPB by the dynein microtubule motor protein (Chikashige et al., 2006; Miki et al., 2004; Shimanuki et al., 1997; Yoshida et al., 2013; reviewed in Chikashige et al., 2007; Hiraoka & Dernburg, 2009). Thus, INM proteins play an important role in chromosome organization within the nucleus.

In addition to Sad1 and Kms1, Ima1, Lem2 and Man1 have been identified as INM proteins in S. pombe (Gonzalez, Saito, & Sazer, 2012; Hiraoka et al., 2011; Steglich, Filion, van Steensel, & Ekwall, 2012) and have been confirmed by immunoelectron microscopy (Tange et al., 2016). The Ima1 protein of S. pombe is a homologue of the human Samp1 and the rat NET5 proteins (Buch et al., 2009; Schirmer & Gerace, 2005; Schirmer et al., 2003); however, no obvious homologues of these mammalian proteins have been identified in the budding yeast Saccharomyces cerevisiae. Both Lem2 and Man1 belong to the conserved LEM-domain INM protein family: the LEM domain was initially identified as ~40 amino acid residues that are shared between LAP2, gmerin and Man1 in the N-terminal nucleoplasmic region (Brachner, Reipert, Foisner, & Gotzmann, 2005; Laguri et al., 2001; Lin et al., 2000). In metazoans, the LEM-domain protein family has been extended to Lem2 (NET25), Lem3, Lem4 and Lem5 (reviewed in Barton, Soshnev, & Geyer, 2015; Lee & Wilson, 2004; Wagner & Krohne, 2007). Heh1 (also called Src1) and Heh2, identified in S. cerevisiae (Grund et al., 2008; King, Lusk, & Blobel, 2006), display the LEM-like helix-extension-helix (HEH) domain in their N-terminal region, and the MSC (MAN1/Src1C-terminal) domain in their C-terminal region, which are homologous to similar domains present in the metazoan Lem2 and Man1 proteins (Brachner & Foisner, 2011; Mans, Anantharaman, Aravind, & Koonin, 2004). The S. pombe Lem2 and Man1 proteins share a similar domain structure, containing HEH/LEM and MSC domains and two transmembrane domains (Barrales, Forn, Georgescu, Sarkadi, & Braun, 2016; Gonzalez et al., 2012; Hiraoka et al., 2011; Steglich et al., 2012; Tange et al., 2016). The chromatin is anchored to the nuclear periphery through the HEH/LEM domain (Gonzalez et al., 2012).

Lem2 is considered to share some functions with Man1 and Ima1 in S. pombe: the depletion of any one of these three proteins causes no obvious growth defects, but the depletion of all three does, implying that their redundant functions are essential for growth (Hiraoka et al., 2011). However, recent studies have shown that Lem2 shows distinct functions: Lem2 has shown cell cycle-dependent enrichment at the SPB (Gonzalez et al., 2012; Hiraoka et al., 2011), and the loss of Lem2 increased the rate of minichromosome loss (Tange et al., 2016). Furthermore, the disruption of the bqt4 gene along with the lem2 gene, but not with the man1 gene, confers synthetic lethality (Tange et al., 2016). Thus, in spite of the structural similarity between Lem2 and Man1, Lem2 shows a unique genetic interaction with Bqt4. Here, we report the biochemical interaction between Lem2 and Bqt4 and the role of Bqt4 in Lem2 localization.

## RESULTS

### Lem2 and Man1 bind to Bqt4

To elucidate the biochemical basis of the genetic interaction between Lem2 and Bqt4, we examined the binding of Lem2 to Bqt4 in S. pombe cells using an immunoprecipitation assay. A full-length Lem2 protein tagged with FLAG and HA at its N- and C-terminus, respectively, and a GFP-tagged Bqt4 (GFP-Bqt4) protein were expressed under their native promoters, at physiological levels. The Lem2 protein was immunoprecipitated using an anti-FLAG antibody, followed by an anti-HA antibody to minimize the background, as the physiological level of Lem2 was low. Western blot analysis showed the binding of Lem2 to Bqt4 (Figure 1a).

Next, we examined the ability of Bqt4 to bind Lem2 and Man1. We expressed GFP-Bqt4 together with either a full-length Lem2 or Man1, tagged with FLAG and HA in S. pombe cells. In this experiment, we expressed Lem2 and Man1 under the nmt1 promoter, as the extraction efficiency of Man1 was much lower than that of Lem2 (compare the Lem2 and Man1 “Input” levels) and it was difficult to obtain enough amount of Man1. The proteins were immunoprecipitated using an anti-HA antibody, followed by an anti-HA antibody to minimize the background, as the physiological level of Lem2 was low. Western blot analysis showed the binding of Lem2 to Bqt4 (Figure 1b).

### Lem2 binds to Bqt4 through its N-terminal region adjacent to the transmembrane domain

To determine the Bqt4 binding site on Lem2, we examined the Bqt4-binding ability of Lem2 fragments, using in vivo
co-immunoprecipitation assays (Figure 2). We expressed the Lem2 fragments shown in Figure 2a in S. pombe cells expressing GFP-Bqt4; all of these Lem2 fragments contained transmembrane (TM) domains, and their localization at the NE was confirmed by fluorescence microscopy (Figure S1). We found that Bqt4 co-immunoprecipitated with fragments containing the full-length Lem2 (Lem2; #2 in Figure 2a,b) and the N-terminal nucleoplasmic region of Lem2 (Lem2N; #3 in Figure 2a,b), but surprisingly not with the C-terminal region of Lem2, which is the domain that had shown a genetic interaction with Bqt4 (Lem2LuC; #7 in Figure 2a,b). To narrow down the exact Bqt4-binding site on the Lem2N fragment, we examined three additional fragments: Lem2NΔLEM (Lem2N lacking the HEH/LEM domain; #4 in Figure 2a), Lem2NΔ100 (Lem2N, with amino acid residues 1-99 deleted; #5 in Figure 2a) and Lem2NΔ200 (Lem2N, with amino acid residues 1-199 deleted; #6 in Figure 2a). Bqt4 co-immunoprecipitated with all of these fragments (#4, #5 and #6 in Figure 2b). Thus, the region adjacent to the first TM in Lem2 (amino acid residues 200-307) is likely to be necessary for Bqt4 binding. We confirmed this hypothesis using Lem2 without the putative Bqt4-binding domain (Lem2Δ200-307). Co-immunoprecipitation assays showed that the binding of Bqt4 to Lem2 was greatly reduced in Lem2Δ200-307 (Figure 2c), confirming the role of amino acid residues 200-307 as the major binding site for Bqt4. Taking all data into consideration, we concluded that the region spanning amino acid residues 200-307 of Lem2 included the Bqt4-binding domain and that the HEH/LEM domain of Lem2 is not necessary for its binding to Bqt4.

2.3 HEH/LEM domain assists the association of Lem2 with the centromeric region by binding to DNA

It is known that Lem2 binds to chromatin through its conserved LEM domain (Gonzalez et al., 2012) and that the S. pombe Lem2 also affects chromatin functions (Banday, Farooq, Rashid, Abdullah, & Altaf, 2016; Barrales et al., 2016; Gonzalez et al., 2012; Tange et al., 2016). Thus, we investigated the DNA-binding ability of Lem2 using an on-bead DNA-binding assay (Figure 3). GST-Lem2 fragments were prepared in Escherichia coli cells (Figure 3a,b) and conjugated to glutathione-Sepharose 4B beads (see Experimental Procedures). The beads conjugated to Lem2 fragments were incubated with salmon sperm DNA, and following incubation, DNA was stained with a DNA-specific fluorescent dye (4′,6-diamidino-2-phenylindole; DAPI). The beads were then observed under a confocal microscope (Figure 3c, top panels), and the DAPI signal on the beads was quantified (Figure 3c, bottom graph). All control beads without DNA were negative for DAPI staining (“−DNA” in Figure 3c). The peripheral staining of the bead-bound Lem2 and Lem2ΔTM fragments containing the N-terminal region (#2 and #3, respectively, in Figure 3c) indicated that those fragments bound to DNA, although Lem2ΔTM, in which the HEH/LEM domain was deleted, failed to bind DNA (Lem2ΔTMΔLEM; #4 in Figure 3c). Although the fragment containing amino acid residues 11-44 (LEM11-44, assigned as the HEH/LEM domain in the database) failed to bind DNA (#8 in Figure 3c), a slightly longer fragment corresponding to amino acids

FIGURE 1 Lem2 and Man1 bind to Bqt4. (a) FLAG-HA (control) and FLAG-Lem2-HA (Lem2) constructs were co-expressed with GFP-Bqt4 in Schizosaccharomyces pombe lem2Δ cells, under their native promoters (strains H1N255 and H1N259) (Input). FLAG-Lem2-HA was immunoprecipitated using an anti-FLAG antibody, followed by an anti-HA antibody. The coprecipitated GFP-Bqt4 was detected using an anti-GFP antibody (IP, top). The precipitated Lem2 was detected using an anti-FLAG antibody (IP, middle). The total protein levels were detected using amido black staining (bottom). Molecular weight makers are shown on the left. (b) FLAG-HA (control), FLAG-Lem2-HA (Lem2) and FLAG-Man1-HA (Man1) constructs were expressed in S. pombe lem2Δ cells together with GFP-Bqt4 (strains H1N389, H1N390 and H1N391). Expressed proteins were immunoprecipitated using an anti-HA antibody. The coprecipitated GFP-Bqt4 (top) was detected using an anti-GFP antibody, whereas Lem2 and Man1 (middle) were detected with an anti-FLAG antibody. The total protein levels were detected using amido black staining (bottom). Molecular weight makers are shown on the left.
1-60 (LEM60) bound to DNA (#9 in Figure 3c). As some of the amino acids forming the second α-helix (Brachner & Foisner, 2011) are deleted in LEM11-44, the helix-extension-helix conformation appears to play a key role in its DNA-binding ability. On the contrary, the C-terminal fragments Lem2LuC, Lem2LuΔTM and Lem2CΔTM (#5, #6 and #7, respectively, in Figure 3c) failed to bind DNA, even though Lem2LuC displayed the complete MSC domain—the DNA-binding domain in other species. Thus, the HEH/LEM domain is likely a unique DNA-binding site in Lem2 in S. pombe. As shown in Figure 2b, the Lem2 binding to Bqt4 is still retained by Lem2NΔLEM. Thus, we concluded that the HEH/LEM domain of Lem2 plays a role in DNA binding, but does not mediate the binding of Lem2 to Bqt4.

To investigate the role underlying its DNA-binding activity, we evaluated the association of Lem2ΔLEM with the centromeric region, using chromatin immunoprecipitation (ChIP) (Figure 3d). Although the full-length Lem2 associated with the core centromeric region where the CENP-A chromatin is formed, as previously reported (Barrales et al., 2016; Tange et al., 2016), this association was partially, but significantly reduced by the deletion of the HEH/LEM domain (Lem2ΔLEM in Figure 3d). This suggested that the DNA-binding activity of the HEH/LEM domain assists the association of Lem2 with the centromeric region.

### 2.4 Intracellular localization of Lem2 is affected by Bqt4

We evaluated the effect of Bqt4 on the intracellular localization of Lem2 (Figure 4a). As previously reported (Barrales et al., 2016; Hiraoka et al., 2011), the full-length Lem2 tagged with GFP (Lem2-GFP) localized to the NE, in addition to its colocalization with Sid4-mRFP (used as a marker for the SPB), in the presence of Bqt4 (“bqt4 +” in Figure 4a). To quantify fluorescent signals accumulated at the SPB, we measured a fluorescence intensity ratio at the SPB vs NE (SPB/NE ratio) as described in Experimental Procedures (Figure 5e). The Lem2-GFP signal at the SPB was 1.6-fold stronger than that at the NE (SPB/NE ratio of 1.6) (“bqt4 +” in Figures 4a and 5e), although GFP-Bqt4 localized only to the NE with no additional accumulation at the SPB (SPB/NE ratio of 0.97) (Figures 4b and 5e) as previously reported (Chikashige et al., 2009). In contrast, Lem2-GFP accumulated almost exclusively at the SPB with reduction in the NE signal in the absence of Bqt4 (“bqt4Δ” in Figure 4a); a signal at the SPB was 4.9-fold stronger than that at the NE (SPB/NE ratio of 4.9) (Figure 5e), suggesting that the NE localization...
FIGURE 3  The HEH/LEM domain binds to DNA. (a) Schematic representation of the GST-fused Lem2 protein fragments. (b) GST-fused Lem2 fragments prepared in Escherichia coli cells were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Molecular weight markers are shown on the left. The GST-fused Lem2 fragments were attached to the beads and used for DNA-binding assay in (c). (c) On-bead binding assay. Beads bound to GST and GST-fused Lem2 fragments were incubated with (+DNA) or without (−DNA) salmon sperm DNA. DNA was stained using DAPI, and the beads were observed using confocal microscopy. The DNA signals (DAPI, top panels) and bead positions (DIC, middle panels) are shown. The fluorescence intensity of the DAPI-stained DNA on the n independent beads was quantified, and the mean intensities are plotted with standard deviations; a.u., arbitrary unit (bottom). (d) ChIP analysis of Lem2. (Left) Schematic diagram of the centromere 1 locus. The black lines below the diagram indicate the position of the PCR products in the ChIP assay (cnt, imr#3, dg and dh). (Right) FLAG-Lem2-HA (“Lem2,” black bar), FLAG-Lem2ΔLEM-HA (“Lem2ΔLEM,” gray bar) and FLAG-HA (“lem2Δ,” white bar) were ectopically expressed in lem2Δ cells (strains H1N253, H1N257 and H1N315) and thus immunoprecipitated using an anti-FLAG antibody. After ChIP-qPCR, the values were normalized and represented as values relative to that detected in the wild type at the cnt region. The mean values from n independent experiments, with three technical replicates for each experiment, are shown with standard deviations. The p-values were obtained by Student’s t test.
of Lem2 depends on its binding to Bqt4. Localization of GFP-Man1 at the NE was unaffected by the presence or absence of Bqt4 (SPB/NE ratio of 0.93 or 0.94, respectively) (Figures 4c and 5e), suggesting that the NE localization of Man1 is independent of Bqt4. As it was reported that Lem2, but not Man1, shows SPB localization (Hiraoka et al., 2011), it is likely that its affinity for the SPB is an intrinsic property of Lem2.

2.5 | N-terminal region of Lem2 is necessary for the Bqt4-dependent NE localization

We further compared the localization of the GFP-tagged Lem2 fragments in the presence or absence of Bqt4 (Figures 5 and S2). In these experiments, we introduced Lem2 fragments in the presence of the endogenous Lem2 because some of the Lem2 fragments do not rescue the synthetic lethality conferred by the absence of both Lem2 and Bqt4; these Lem2 fragments were expressed under the native lem2 promoter, at an ectopic genomic locus. We also examined the localization of the GFP-tagged Lem2 fragments in the absence of endogenous Lem2 when possible and obtained basically the same results of localization (Figure S3). The protein expression levels varied among the Lem2 fragments, as measured by Western blot, but were approximately the same in the presence or absence of Bqt4 for each fragment (Figure S4). As some of the Lem2 fragments showed lower protein expression levels compared with the full-length Lem2 (“lem2p” in Figure S4), we also constructed strains expressing Lem2 fragments from the bqt4 promoter for higher expression levels to consider the effect of protein expression levels to

**FIGURE 4** Lem2 is retained at the NE through its interaction with Bqt4. GFP-tagged Lem2 (a), Bqt4 (b), Man1 (c) (represented as green on each panel) and the Sid4-mRFP SPB marker protein were co-expressed in lem2" bqt4" cells (strains YK92, H1N361 and H1N371 for Lem2, Bqt4 and Man1, respectively) and lem2" bqt4Δ cells (strains YK95 and H1N372 for Lem2 and Man1, respectively) and observed under a fluorescence microscope. Insets represent enlarged images of the nucleus. Dashed lines indicate the outline of the cell. Bars represent 5 μm
its localization. The Lem2 fragments increased protein expression levels when expressed from the \textit{bqt4} promoter (“\textit{bqt4p}” in Figure S4). Quantification of the localization for each construct (Figures 5e and S2 for the \textit{lem2} promoter; Figures 5f and S5 for the \textit{bqt4} promoter), as described below in detail, showed that the localization patterns of Lem2

**FIGURE 5**  Lem2N is necessary for retaining Lem2 at the NE. (a–d) Localization of the GFP-tagged Lem2 fragments in \textit{lem2}^+ \textit{bqt4}^+ cells (strains YK209, YK93, YK514 and YK167 for Lem2NLu, Lem2LuC, Lem2A200-307 and Lem2ΔLEM, respectively, top) and \textit{lem2}^+ \textit{bqt4}Δ cells (strains YK211, YK96, YK515 and YK179 for Lem2NLu, Lem2LuC, Lem2A200-307 and Lem2ΔLEM, respectively, bottom), observed as described in Figure 4. Dashed lines indicate the outline of the cell. Bars represent 5 μm. (e) Quantification results. SPB/NE ratios measured in \textit{n} independent cells. The number of cells examined (\textit{n}) is indicated below each bar graph. The means of those ratios are shown with standard deviations. Asterisks * and ** represent \( p < 0.01 \) and \( p < 0.001 \), respectively; ns represents no significance (\( p > 0.05 \)). Black asterisks indicate the statistical significance of SPB/NE ratios for each fragment in \textit{bqt4}^+ vs \textit{bqt4}Δ cells (Mann–Whitney U test). Blue and orange asterisks indicate the statistical significance of SPB/NE ratios for Lem2 fragments vs Lem2 in \textit{bqt4}^+ (blue) and \textit{bqt4}Δ (orange) cells (Steel–Dwass test). (f) Quantification results for Lem2 fragments expressed from the \textit{bqt4} promoter. Symbols and statistics are same as in (e)
fragments were not affected by their protein expression levels in our experimental conditions.

The localization pattern for the Lem2 fragment lacking the C-terminal region (Lem2NLu, amino acid residues 1-574) resembled the Bqt4-dependent localization and was similar to the one observed for the full-length Lem2 (Figure 5a,e; compare with Figure 4a): NE localization with a slight SPB accumulation in bqt4Δ cells (SPB/NE ratio of 1.3) and an exclusive SPB accumulation in bqt4Δ cells (SPB/NE ratio of 4.7). Thus, the Lem2C region has basically no or little effect on Lem2 localization. Conversely, the Lem2 fragment lacking the N-terminal region (Lem2LuC; amino acid residues 308-688) localized at the NE and SPB in the presence or absence of Bqt4, losing the exclusive SPB accumulation in the absence of Bqt4 (Figure 5b,e); Lem2LuC also dispersed in membrane structures outside the nucleus, such as ER, vacuoles and other cytoplasmic membranes, independently of Bqt4 (Figures 5b and S2). The Lem2LuC fragment showed a comparable SPB/NE ratio observed for the full-length Lem2 in the presence of Bqt4 (Figure 5e). These results suggest that both the Lem2NLu and Lem2LuC regions have an affinity for the SPB. However, the SPB affinity of these regions is regulated in a different manner: Bqt4 dependent and Bqt4 independent for the Lem2NLu and Lem2LuC regions, respectively.

The Lem2Δ200-307 fragment lacking the Bqt4-binding region showed localization biased to the SPB even in the presence of Bqt4 (Figure 5c,e), leading to the SPB/NE ratio of 2.6, higher than the SPB/NE ratio of 1.6 observed for the full-length Lem2 in the presence of Bqt4. This result suggests that this region of Lem2 contributes to the Bqt4-dependent NE localization. In contrast, in the absence of Bqt4, Lem2Δ200-307 showed a reduced accumulation at the SPB (SPB/NE ratio of 3.3), compared with the exclusive SPB accumulation observed for the full-length Lem2 (SPB/NE ratio of 4.9) (Figure 5c), suggesting that this region of Lem2 also contributes to the Bqt4-independent SPB localization, in addition to the Bqt4-dependent NE localization. Taken together, our results suggest that the Lem2NLu region has a Bqt4-independent affinity for the SPB and a Bqt4-dependent affinity for the NE and that the Bqt4-binding region of Lem2 is necessary for its localization at the NE. In contrast, the Bqt4-dependent localization of Lem2 was unaffected by the deletion of the HEH/LEM domain (Lem2ΔLEM; Figure 5d,e), suggesting a minor role for the HEH/LEM domain in the localization of Lem2 to the NE or the SPB.

The role of the N-terminal region for the localization of Lem2 at the NE was also supported by mobility measurements of Lem2 and Lem2LuC using fluorescence recovery after photobleaching (FRAP). Lem2LuC, which shows a similar localization pattern to the full-length Lem2 but does not interact with Bqt4, displayed a higher mobility than Lem2 in the NE (Figure 6), suggesting a minor role for the HEH/LEM domain in the localization of Lem2 to the NE through its interaction with Bqt4.

**DISCUSSION**

Lem2 is associated with chromatin functions, such as the maintenance of the centromeres and the telomeres (Banday et al., 2016; Barrales et al., 2016), genome stability and
long-terminal repeat recombination (Tange et al., 2016), as well as checkpoint signaling (Hayles et al., 2013; Xu, 2016). These Lem2 functions, at least in part, are distinct from those of Man1, as man1Δ mutants show phenotypes that are distinct from those of lem2Δ mutants (Barrales et al., 2016; Gonzalez et al., 2012; Tange et al., 2016). In this report, we show that the localization of Lem2 is modulated by its interaction with Bqt4 in S. pombe. To dissect Lem2 functions, we characterized the Lem2 domains involved in its intracellular localization and its binding to Bqt4. In conclusion, the NLu region of Lem2 is necessary for the Bqt4-independent localization of Lem2 at the SPB, and the Bqt4-binding domain competes with Bqt4 (Figure 7).

3.1 Molecular mechanisms that determine Lem2 localization

We have previously shown the genetic interaction between Bqt4 and Lem2 (Tange et al., 2016). The present study confirmed their biochemical interaction and suggested that the localization of Lem2 is determined by at least two types of interactions: a Bqt4-dependent NE localization and a Bqt4-independent SPB localization (Figure 7). Whereas the localization of Lem2 to the NE and SPB was observed in the presence of Bqt4, Lem2 displayed an exclusive accumulation at the SPB in the absence of Bqt4 (Figure 4). Thus, Lem2 shows strong affinity for the SPB, but Bqt4 exerts a stronger regulatory effect to retain Lem2 at the NE. This affinity of Lem2 for Bqt4 depends on the Bqt4-binding domain (amino acid residues 200-307), located in the N-terminal region of Lem2 (Figure 7a).

In the absence of Bqt4, a strong accumulation at the SPB was observed in Lem2, Lem2NLu and Lem2ΔLEM (Figure 5). Thus, there exists an unidentified Lem2-binding partner, located at the SPB, that interacts with the Lem2NLu region excluding the HEH/LEM domain (Figure 7a). This putative Lem2-binding partner is considered to compete with Bqt4 to promote an equilibrium between the localization of Lem2 to the NE and SPB. In contrast, the HEH/LEM domain plays a minor role in the localization of Lem2 (Figure 5d), but assists in the association of Lem2 with the centromeric locus through its DNA-binding activity (Figure 3d). The DNA-binding ability of the HEH/LEM domain is likely sequence independent, as the N-terminal Lem2 fragment lacking the transmembrane domain disperses throughout the nucleus, with no specific localization on the chromatin (Barrales et al., 2016). These results suggest that yet-unidentified Lem2-binding factors at the SPB primarily localize Lem2 to the SPB and that the HEH/LEM domain consolidates the association of Lem2 with the centromeric regions adjacent to the SPB.
In these experiments, the Lem2 fragments were expressed in a \textit{lem}2+ genetic background, thus it is possible that the localization of Lem2 fragments may be affected by the endogenous Lem2. As some of the FLAG-tagged Lem2 fragments can rescue \textit{lem}2Δ \textit{bqt4Δ} cells, we tested the localization of those fragments in \textit{lem}2Δ and \textit{lem}2Δ \textit{bqt4Δ} cells. The Bqt4-dependent and Bqt4-independent localization patterns of these Lem2 fragments were not affected by the presence or absence of the endogenous Lem2 (Figure S3), suggesting that the endogenous Lem2 is unlikely to interfere with the Lem2-GFP fragments. We also confirmed that the localization patterns of GFP-tagged Lem2 fragments are not affected by their protein levels expressed from the different promoters (Figures 2e and S2 for the \textit{lem}2+ promoter; Figures 2f and S5 for the \textit{bqt4} promoter).

3.2 | Functional dissection of the Lem2-Bqt4 complex

We have previously reported that Bqt4 plays an important role in tethering telomeres to the NE (Chikashige et al., 2009). In addition, it has been reported that the C-terminal region of Lem2 mediates telomere tethering and silencing (Barrales et al., 2016), suggesting that the Lem2-Bqt4 region of Lem2 mediates telomere tethering and silencing (Barrales et al., 2016), suggesting that the Lem2-Bqt4 region of Lem2 mediates telomere tethering and silencing. In addition, it has been reported that the C-terminal region of Lem2 bears functional relationship, and that the dysfunction of the Lem2-Bqt4 complex results in synthetic lethality. However, it is unlikely that the synthetic lethality of the \textit{lem}2Δ \textit{bqt4Δ} double mutants resulted from the telomere-related functions of Bqt4 as discussed in our previous report, as synthetic lethality was not observed in the \textit{lem}2Δ \textit{rap1Δ} double mutants (Tange et al., 2016). Thus, Bqt4 may show unknown non-telomere-related functions, mediated through the C-terminal region of Lem2.

In this study, we have shown that Bqt4 binds to amino acid residues 200–307 of Lem2, within the N-terminal nucleoplasmic region. This is puzzling because the synthetic lethality observed in the \textit{lem}2Δ \textit{bqt4Δ} double mutant is complemented by the C-terminal region of Lem2, containing the MSC domain (Lem2LuC). Thus, we propose that the essential functions of Lem2 that may overlap with Bqt4 are independent of its interaction with Bqt4. This may be explained if we hypothesize that the roles of the N-terminal and C-terminal regions of Lem2 are independent of each other (Figure 7b). Bqt4 and the C-terminal region of Lem2 bear functional relationship, and their synergistic effect is independent of their physical interaction, that is, Bqt4 can function as a trans-acting factor for the C-terminal region of Lem2. Thus, we conclude that the N-terminal region of Lem2 binds to Bqt4 and that the Lem2-Bqt4 interaction promotes the localization of Lem2 to the NE, whereas Bqt4 and the C-terminal region of Lem2 play critical roles in cell viability through independent pathways (Figure 7b).

4 | EXPERIMENTAL PROCEDURES

4.1 | Yeast strains and media

All \textit{S. pombe} strains used in this study are listed in Table S1. All strains, except for the \textit{lem}2Δ strains, were maintained in YES medium (Moreno, Klar, & Nurse, 1991). The \textit{lem}2Δ strains were maintained in EMMG medium, to avoid the undesired genome reorganization of \textit{lem}2Δ cells, which can occur in YES medium (Tange et al., 2016). In addition, EMMG was used for microscopic observation and protein induction. The chemical reagents used in this study were purchased from Nacalai Tesque (Kyoto, Japan) and Wako (Osaka, Japan).

4.2 | Constructs

All plasmids were constructed using the In-Fusion (Takara Bio Inc., Kusatsu, Japan) and NEBuilder (New England BioLabs, MA, USA) systems, according to the manufacturer’s instructions. To express FLAG- and HA-tagged proteins in \textit{S. pombe}, we generated plasmids, as described below. We first inserted the FLAG and HA coding sequences, spanned by the \textit{Bam}HI and \textit{Bgl}II sites, into the \textit{pCST3} vector (Chikashige, Kurokawa, Haraguchi, & Hiraoka, 2004) between the \textit{NdeI} and \textit{Bgl}II sites (pCST3-FLAGHA). The original \textit{Bgl}II site on the \textit{pCST3} vector was deleted. The DNA sequences of the Lem2 and Man1 fragments were amplified via PCR, and cloned into the \textit{pCST3}-FLAGHA vector, between the \textit{Bam}HI and \textit{Bgl}II sites. For the expression of the FLAG-Lem2-HA construct at physiological levels, the thiamine-repressible \textit{nmt1} promoter of pCST3-FLAGHA was replaced with the native \textit{lem}2+ promoter (the −1 to −1,000 bp region, from its start codon) between the \textit{PstI} and \textit{NdeI} sites. The plasmid used to express Lem2-GFP, using the \textit{lem}2+ promoter and terminator, has been previously reported (Tange et al., 2016). The GFP-tagged Lem2 fragments were obtained by the replacement of the Lem2 DNA sequence on the plasmid with the PCR-amplified Lem2 fragments. To express the GFP-tagged Lem2 fragments under \textit{bqt4} promoter, DNA sequence for each fragment was digested out from FLAG-Lem2-HA plasmids with \textit{Bam}HI and \textit{Bgl}II sites, and the inserts were cloned into pCSS41, which is a backbone vector for pCSS42 (Chikashige et al., 2009), at the \textit{Bam}HI site. For the GST fusions, the PCR-amplified Lem2 fragments were inserted into the pGEX 5X-1 vector (GE Healthcare Inc., IL, USA), between the \textit{EcoRI} and \textit{XhoI} sites. All insertions were confirmed by sequencing. The plasmids used for the expression of GFP-Bqt4 (pCSS80), \textit{Sid4}-mRFP and GFP-Man1 have been previously reported (Chikashige et al., 2009, 2014; Tange et al., 2016).

The gene disruptions for \textit{bqt4}+ and \textit{lem}2+ were performed using the direct chromosome integration method, as
Previously described (Bähler et al., 1998; Wach, 1996). The correct disruptions and integrations were confirmed using genomic PCR from both the 5′ and 3′ ends.

4.3 Immunoprecipitation

For the co-expression of GFP-Bqt4 and Lem2 fragments, cells were pre-cultured in EMMG medium supplemented with 2 μM thiamine, followed by incubation in EMMG medium without thiamine for 17 hr, at 30°C to induce protein expression. After expression, cells (5.0 × 10^7) were resuspended in 100 μl of CSK-HEPES buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM MgCl2, 300 mM sucrose, 1 mM EDTA and 0.5% Triton X-100) supplemented with 2 mM PMSF and 5% protease inhibitor cocktail (P8215, Sigma-Aldrich) for 2 hr at 4°C. After five rounds of washing, the bound proteins were eluted with CSK- Tris buffer and 5% protease inhibitor cocktail for 1 hr at 4°C. After incubation, beads were washed thrice with DNA-binding buffer and stained with 100 ng/ml DAPI for 10 min at 4°C. After washing thrice with DNA-binding buffer, beads were observed under a confocal microscope (LSM510META, Carl Zeiss, Jena, Germany), equipped with a 40× C-Apochromat objective lens (numerical aperture [NA] = 1.2). The total fluorescence intensity and diameter of each bead on an optical section containing the bead equator were measured using the Fiji software (Schindelin et al., 2012). Fluorescence intensities normalized to the bead diameters were plotted with standard deviations.

4.5 Microscopic observations

_Schizosaccharomyces pombe_ cells were observed using a DeltaVision OMX system (GE Healthcare Inc.) equipped with an EM-CCD camera (Cascade II, Photometrics, AZ, USA) and a 100× UPlanSApo silicone-immersion objective lens (NA = 1.35; Olympus, Tokyo, Japan). Cells were cultured overnight in EMMG medium at 30°C, to attain the logarithmic growth phase. The intracellular localization of GFP and mRFP fusion proteins was observed in living cells. The chromatog aberration and the geometry of the two EM-CCD cameras were corrected using the in-house “Chromagnon” software (Kraus et al., 2017), using transmission images of _S. pombe_ cells simultaneously taken in both the green and red channels as a reference. The images are presented after deconvolution, using the built-in SoftWoRx software. The brightness of the images was changed using the Adobe Photoshop CS6 software, for better visualization, without changing the gamma settings.

For the quantification of fluorescent signals at the SPB, NE and outside of the nucleus (We termed it “ON,” see also Figure S2a), regions of the SPB (marked by Sid4-mRFP, so that they are distinguishable from the NE signal) and of the NE and ON in the original unprocessed image were manually marked as regions of interest (ROIs) using the Fiji software. After the flat fielding to normalize the illumination intensity of the field and the subtraction of the background level measured outside the cells, the fluorescent intensities were summed within the ROI and divided by the number of pixels for each ROI. The SPB/NE, SPB/ON and NE/ON ratios were calculated for each cell, and means of these ratios were calculated over the number of cells indicated for each experiment.

For FRAP analysis, _S. pombe_ cells were attached on a 35-mm glass-bottom dish (MatTek Corp., MA, USA) coated with soybean lectin (L1395; Sigma-Aldrich). The mobility of Lem2 was analyzed using a confocal microscope (LSM780;
Carl Zeiss) equipped with a 63× Plan-Apochromat objective lens (NA = 1.4). Five images were collected before bleaching (approximately 2% transmission of a 488-nm argon laser, 380 ms per frame, 1.27 μs per pixel, 512 × 200 pixels, 48 μm pinhole, 10X zoom); a region of the NE opposite to the SPB (marked by Sid4-mRFP) was bleached using 100% of a 488-nm laser with five iterations, followed by the capture of a further 95 images, using the original setting. The fluorescence intensity in the NE was quantified using Fiji software. Photobleaching during imaging was monitored and normalized before drawing the recovery curve. The curve was plotted as a relative value set the average fluorescence intensity before bleaching as 1 so that the difference of the protein expression level is normalized. Presented images were rolling averaged ones generated from five raw images using a plugin “Running Z Projector” in the Fiji software.

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