Beyond Tethering and the LEM domain: MSCellaneous functions of the inner nuclear membrane Lem2

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ABSTRACT

The nuclear envelope plays a pivotal role in the functional organization of chromatin. Various inner nuclear membrane (INM) proteins associate with transcriptionally repressed chromatin, which is often found at the nuclear periphery. A prominent example is the conserved family of LEM (LAP2-Emerin-MAN1) domain proteins that interact with DNA-binding proteins and have been proposed to mediate tethering of chromatin to the nuclear membrane. We recently reported that the fission yeast protein Lem2, a homolog of metazoan LEM proteins, contributes to perinuclear localization and silencing of heterochromatin.1 We demonstrate that binding and tethering of centromeric chromatin depends on the LEM domain of Lem2. Unexpectedly, this domain is dispensable for heterochromatin silencing, which is instead mediated by a different structural domain of Lem2, the MSC (MAN1-Src1 C-terminal) domain. Hence, silencing and tethering by Lem2 can be mechanistically separated. Notably, the MSC domain has multiple functions beyond heterochromatic silencing. Here we discuss the implications of these novel findings for the understanding of this conserved INM protein.

KEYWORDS

chromatin tethering; heterochromatin; MSC domain; LEM domain; perinuclear silencing

Introduction: Nuclear organization and heterochromatin

The chromatin of eukaryotic cells is organized into functional domains that are non-randomly distributed within the nucleus. Transcriptionally active chromatin (known as euchromatin) is mainly localized in the nuclear interior, whereas inactive chromatin regions that are transcriptionally silent (heterochromatin) are often found close to the nuclear membrane or at the nucleolus. This observation has raised the question whether these nuclear structures provide specialized subcompartments for gene repression. Yet sequestration alone is often not sufficient to induce gene repression and transcriptional silencing requires in addition the presence of repressive factors at the nuclear periphery. Thus, high local concentrations of repressive factors generally expressed at low levels may favor the establishment and maintenance of heterochromatin. Accordingly, 2 conditions need to be met to promote perinuclear silencing: (1) mechanisms that recognize and sequester heterochromatin at the nuclear periphery; (2) mechanisms that mediate the perinuclear recruitment and enrichment of silencing factors, which then act locally on the sequestered chromatin.

Potential candidates involved in sequestering include various nuclear membrane proteins identified in worm and vertebrates and known to interact with peripheral chromatin (reviewed in3). Many of these proteins are components of the nuclear lamina, a subnuclear structure that lies beneath the nuclear envelope and consists of a meshwork of intermediate filaments (lamins) and integral membrane protein (lamin-associated proteins or LAPs). Both, lamins and LAPs, are in direct contact with chromatin regions that are gene-poor and enriched for repressive histone modifications. Moreover, several LAPs contain specific structural domains known to interact with DNA or chromatin-associated proteins.4,5 A prominent example is the LEM domain, a 40-amino acid helix-
extension-helix (HEH) motif. This domain interacts specifically with so-called barrier-to-autointegration factors (BAFs), a family of sequence-independent DNA-binding factors involved in nuclear envelope assembly.6,7 However, the functional relevance of these interactions with respect to the peripheral sequestration and repression of heterochromatin remains mostly obscure. Major challenges in studying these functions are the large number of metazoan LAPs and their potential redundancy.6

**Fission yeast as a model for perinuclear heterochromatin**

The unicellular fission yeast *Schizosaccharomyces pombe* has proven to be a powerful model system for studying heterochromatin formation and nuclear organization.8-11 Unlike its distant kin *Saccharomyces cerevisiae* (budding or baker’s yeast), *S. pombe* comprises many of the conserved hallmarks of metazoan heterochromatin, as for example methylated lysine 9 of histone H3 (H3K9me) that marks repressed chromatin; members of the heterochromatin protein 1 (HP1) family that recognize and mediate the spreading of this heterochromatin mark; and the RNA interference (RNAi) machinery that is involved in the nucleation of heterochromatin. Conversely, *S. pombe* has a relative small genome of 12.3 Mb distributed over 3 chromosomes and contains distinct heterochromatic domains (pericentromeres, subtelomeres, silent mating type locus, and the rDNA loci) spanning 20-40 kb in length. These heterochromatin domains adopt a perinuclear distribution called the Rabl configuration: while the centromeres localize next to the spindle pole body (SPB, which is analogous to the metazoan centrosome and attached to the nuclear envelope), the telomeres form clusters at the opposite side of the nucleus. Importantly, 3 INM proteins—Lem2, Man1, and Ima1—with homology to metazoan LAPs have been identified in *S. pombe*,12 despite the fact that lamins and BAF proteins are not present in yeast. Lem2 and Man1 each contain an HEH domain considered ancestral to the metazoan LEM motif.6 Ima1 is homologous to human Samp1 and rat NET5.13,14 These INM proteins associate with chromatin and contribute to the integrity of the nuclear membrane, displaying partially overlapping functions.15-17 However, a role in establishing silent heterochromatin had not yet been examined.

**Identification of Lem2 as a novel factor in heterochromatin silencing**

In a recent study, we identified Lem2 through a genetic screen for mutants with defects in pericentromeric silencing in *S. pombe*.1 Although deletion of the *lem2* gene causes only a moderate but reproducible defect at pericentromeres, we considered this nuclear membrane protein an attractive candidate for studying the functional link between heterochromatin establishment and peripheral tethering. First, homologs of Lem2 in budding yeast and worm associate with heterochromatin and promote its localization to the nuclear periphery.16,22 Moreover, Lem2 also affects telomere positioning in *S. pombe*, and its HEH/LEM domain is crucial for anchoring chromatin to the nuclear envelope.16 Nonetheless, the role of Lem2 in silencing has remained enigmatic, particularly as silencing of the rDNA locus (in sharp contrast to its peripheral positioning) appears to be independent of Lem2 in *S. cerevisiae*.20 We systematically examined transcription of endogenously silent chromatin domains in *S. pombe* and found that Lem2 contributes to the repression of all major heterochromatic loci.1 Intriguingly, this role in silencing is unique to Lem2 and not shared with other LAP homologs (i.e. Man1, Ima1). On the other hand, the lack of Lem2 does not completely alleviate silencing and only modestly affects pericentromeric H3K9me, which was independently confirmed by 2 recent studies.23,24 In this respect, the silencing defect in *lem2Δ* cells differs substantially from the strong phenotypes seen for mutants deficient in heterochromatin establishment (e.g. H3K9me, RNAi). This may be one reason why *lem2* has been overlooked in previous genetic screens for heterochromatic defects. However, we hypothesized that the moderate silencing defect may actually arise from redundancy with other pathways that control heterochromatin.

**Redundant functions with other peripheral silencing pathways**

To uncover potential redundancy of Lem2 with other factors, we took advantage of genetic tools available in yeast that allow for the dissection of pathways by functional genomics. Systematic pairwise combinations of mutants can be obtained through large-scale genetic crosses using the Synthetic Genetic Array (SGA) method, originally developed in *S. cerevisiae*.25 Genetic interactions between mutant pairs can be determined using cellular growth rates indicative of ‘fitness’. These
can be conveniently assessed by measuring the size of yeast colonies. If two genes act redundantly in parallel pathways, then the loss of both genes will cause a more severe growth phenotype than predicted from the individual gene deletions (synthetic or negative interaction). Conversely, for 2 genes acting in the same pathway, the corresponding double mutant will display a non-aggravated phenotype compared to the single mutants (epistatic or positive interaction). Using growth as a functional output is tremendously powerful for determining genetic interactions, since this readout has no bias toward specific cellular functions and every single gene is considered to contribute to overall fitness. However, it has certain limitations when analyzing the role of genes with weak phenotypes and/or pleiotropic functions that contribute unequally to fitness. In this respect, it is noteworthy that many mutants with silencing defects in *S. pombe* produce only weak growth defects. To overcome this challenge, we decided to use the transcriptional activity of a reporter gene inserted into a heterochromosomal domain as a functional output instead of general fitness. Since the readout of this reporter assay is also based on growth (i.e., colony size), the workflow of the ‘conventional’ SGA method can be easily adapted. However, the functional readout is more sensitive and relies exclusively on defects in silencing.

We applied this advanced SGA approach to screen a mutant library of non-essential genes using a reporter inserted into the silent pericentromeric region. This led to the identification of many mutants causing aggravated silencing defects in combination with *lem2A*, implying the existence of multiple redundant pathways. Quite remarkably, several of these genes encode proteins that localize, like Lem2, close to the SPB or at the nuclear envelope. For instance, we found the centromere-clustering factor Csi1 that associates with the SPB as well as several RNAi factors that interact with the INM protein Dsh1. These similarities in localization prompted us to test whether Lem2 also acts redundantly with other silencing pathways at the nuclear periphery. Taz1 is a telomere-binding protein that protects the telomeric ends and also contributes to subtelomeric silencing and anchoring of telomeres. We found that cells lacking both Lem2 and Taz1 indeed display a synthetic silencing defect for subtelomeric genes, which becomes even more pronounced when this double mutant is combined with a deletion of the RNAi recruitment factor Dsh1, leading to an almost complete de-repression. Taken together, these findings indicate that Lem2 is part of a complex network of redundant pathways that cooperate at the nuclear envelope. We suspect that limiting multiple repressor pathways to the nuclear periphery ensures proper silencing of specific domains, while it lowers the risk of ectopic silencing at non-appropriate genomic sites.

**Correlation between silencing and localization**

Given that these redundant silencing pathways all have in common that they originate from the nuclear envelope, we wondered whether they also cooperate in the peripheral recruitment of heterochromatin. While telomeres are more frequently detached from the nuclear envelope in cells lacking Lem2, no such defect has been observed in mutants deficient for RNAi. However, we observed that combining both deficiencies resulted in a significant increase in telomere delocalization, recapitulating the synthetic defect in telomere silencing. A similar observation was made for centromeres, which cluster together next to the SPB at the nuclear envelope. Deletion of Lem2 only mildly affects centromere clustering, whereas Csi1 plays a more critical role. Nonetheless, even in *csi1Δ* cells, centromeres are not completely delocalized and at least one of the 3 centromeres remains associated with the SPB. Yet, in the double mutant, we found that centromeres are entirely detached from the nuclear envelope in about 20% of cells. Importantly, the contribution of Lem2 toward centromere localization becomes only manifest when Csi1 is absent, explaining why a role for Lem2 in centromere localization has not been reported before. Declustered centromeres often remain associated with the nuclear membrane in the *csi1Δ* mutant. However, in the double mutant they localize more frequently to the nuclear interior. This finding implies that Lem2 contributes directly to the peripheral attachment (but not clustering) of centromeres, underscoring the importance of redundant mechanisms in centromere localization. Given the striking correlation of functional redundancy between heterochromatin silencing and localization at centromeres as well as telomeres, we wondered whether there is a causal relationship between these 2 functions of Lem2.

**Dissecting the functions of Lem2**

Addressing the fundamental question whether silencing is a direct consequence of tethering is challenging,
as it requires identification of the modules mediating these functions and testing whether they can be functionally separated. Lem2 contains 2 conserved structural domains that are potentially involved in interactions with DNA or chromatin: the N-terminal LEM-like domain and the C-terminal MSC domain containing a winged-helix fold known to interact with nucleic acids. Interestingly, the MSC domain appears to have evolved earlier than the LEM domain, suggesting that its function is more conserved. Both domains face the nucleoplasm and are separated by 2 transmembrane domains. To investigate their involvement in heterochromatin silencing and localization, we generated truncated versions of Lem2 that lack either domain and performed functional complementation assays by expressing these constructs in a lem2Δ strain. Chromatin immunoprecipitation (ChIP) experiments showed that Lem2 associates with chromatin and specifically binds to centromeres. We found that the N-terminal part comprising the LEM domain is necessary and sufficient for the interaction with centromeric chromatin. Intriguingly, the domain is also required for the proper localization of centromeres. In contrast, the MSC domain is dispensable for these functions. From these results we concluded that the LEM domain mediates tethering of centromeres, confirming the previous notion that this domain is crucial for Lem2 interaction with chromatin. We then explored the relevance of the LEM domain for silencing: if tethering is a prerequisite for silencing, we expect a similar requirement for this domain. Surprisingly, we found the exact opposite: silencing of all heterochromatin domains—including the centromeres—depends exclusively on the presence of the MSC domain. Thus, the mechanisms for chromatin tethering and silencing can be functionally separated, at least for centromeres (see below and Fig. 1A).

What is the function of the MSC domain in silencing? Whereas the MSC domain of human MAN1 interacts with DNA in vitro through a stretch of positively charged residues, this motif is absent in fission yeast Lem2. Yet, it is noteworthy that winged-helix domains have also been reported to mediate protein-protein interactions. Furthermore, in marked contrast to the LEM domain, we did not detect any interactions of the MSC domain with heterochromatin under identical experimental conditions, raising doubts whether this domain associates with chromatin. Thus, rather than being directly involved in tethering chromatin, we propose that the MSC domain contributes to the enrichment of factors critical for silencing at the nuclear periphery. This is reminiscent of silencing mechanisms described in budding yeast, worm and mammalian cells. In support of this idea, we found that Lem2 is critical for maintaining a proper balance on heterochromatin between the antagonistic factors SHREC and Epe1. SHREC is a repressor complex homologous to the mammalian NuRD complex, whereas Epe1 is a member of the Jumonji family with similarities to H3K9me demethylases that prevents heterochromatin spreading. In particular, the absence of Lem2 causes a decrease in the abundance of SHREC on chromatin, and they both act in the same pathway in telomeric silencing. Hence, the simplest hypothesis is that Lem2 binds directly to one of the SHREC subunits via its MSC domain that may provide a binding interface for interaction partners. However, using co-immunoprecipitation experiments we have been unable to detect a physical interaction between Lem2 and Clr3, the HDAC subunit of SHREC, so far. Interestingly, other pathways involving Lem2 seem to act independently of SHREC (see below). It is conceivable that Lem2 interacts with a variety of binding partners through its MSC domain, possibly in a transient manner that is difficult to detect. Thus, to understand the function of the MSC domain, further work will be needed to identify the physical binding partners of Lem2.

Functions of Lem2 beyond heterochromatin silencing

Repression by Lem2 is not constrained to H3K9me-marked heterochromatin but also seen at the centromeric core domain and several subtelomeric long-terminal repeat (LTR) elements, all of which are mostly devoid of H3K9me. This type of silencing is also mediated by the MSC domain of Lem2. Interestingly, a recent study by the Hiraoka lab reported that suppressor mutations arise spontaneously in lem2Δ cells (see below). These suppressor mutants often display duplications of genomic sequences flanked by LTRs, implying that Lem2 also contributes to genome stability by repressing LTR transcription and
recombination. Silencing of LTRs by Lem2 may take place analogously by recruiting silencing factors. However, while Lem2 acts together with SHREC at heterochromatin, this repressor complex plays only a minor role in LTR silencing (our unpublished results).

Other functions of the MSC domain beyond gene repression have also been described (Fig. 1B). Notably, we found that telomere anchoring is independent of the LEM motif. Instead, it requires the MSC domain, indicating that centromeres and telomeres employ different mechanisms for recruitment to the nuclear periphery. However, we cannot exclude the possibility that telomere anchoring is a consequence of silencing, for example by being coupled to the recruitment of SHREC or other HDACs. Hiraoka and co-workers reported that expression of the MSC domain also suppresses the slow growth phenotype and minichromosome loss observed in lem2Δ cells. Moreover, they found that combined mutations in Lem2 and the INM protein Bqt4—which is also involved in telomere anchoring—are synthetically lethal but can be rescued by expression of the MSC domain. Interestingly, the redundant functions of Lem2 and Bqt4 in cell survival seem to be independent of telomere anchoring. Support for this notion comes from the observation that the deletion of another telomere-associated protein, Rap1, is not synthetically lethal in combination with lem2Δ, even though Rap1 acts together with Bqt4 in telomere anchoring. As for heterochromatin silencing, the underlying molecular mechanisms of these repression-independent functions remain unclear and the MSC domain may have multiple interaction partners.

**The nuclear membrane—a general scaffold for factors controlling nuclear organization and gene repression?**

Among the factors that display redundancy with Lem2 in silencing, we found several proteins that localize to the nuclear periphery but have not yet been ascribed a function in heterochromatin formation. These
include Csi1, several cytoskeleton-associated proteins (e.g., Mto1, Alp14), or the ER protein Lnp1 (Lunapark). Interestingly, Lnp1 was identified by the Hiraoaka lab as a multi-copy suppressor of various phenotypes associated with lem2Δ (i.e. growth defect, minichromosome loss, H3K9me decrease). Multiple suppressor mutations resulted from spontaneous duplications of LTR-flanked genomic sequences that all contained the bnp1+ gene. Remarkably, duplication of the single bnp1+ locus is sufficient to restore these functions to the wild-type situation, even though Lnp1 is not thought to directly replace Lem2. Lunapark stabilizes 3-way junctions of tubules within the polygonal ER network and specifically localizes to these structures in budding yeast and mammalian cells, but it is also found at the nuclear membrane in S. pombe. Interestingly, altering the cellular level of Lnp1 affects the balance between tubules and peripheral sheets of the ER network. Thus, these structural changes may also affect the flux of integral membrane proteins within the ER network and other compartments like the nuclear membrane. Given that a 2-fold upregulation is sufficient to compensate for the silencing defect in lem2Δ cells, Lnp1 may affect the composition of the (inner) nuclear membrane and in particular the abundance of other integral membrane proteins (for instance Dsh1) that act redundantly with Lem2 in silencing. Notably, the synthetic lethality of lem2Δ bqt4Δ cells cannot be rescued by overexpressing Lnp1, providing further evidence that Lem2 contributes to different functional pathways.

We observed that other peripheral factors also cause heterochromatin defects without being necessarily in direct contact with the chromatin domain that is de-repressed. For example, the lem2Δ csi1Δ double mutant triggers synthetic silencing defects at pericentricromeric and subtelomeric chromatin. However, in mitotically growing cells, telomeres are unlikely to come in contact with Csi1, which is constrained to the SPB position at the nuclear membrane opposite of the telomere clusters. We suspect that the absence of Csi1, and maybe other peripheral factors, may cause indirect silencing defects through an abnormal morphology (e.g. fluidity, curvature) or composition of the nuclear membrane similar to Lnp1, which in turn could affect the abundance or activity of INM proteins. Given that many ‘direct’ silencing factors, like Lem2 (which controls SHREC and Epe1) or Dsh1 (which recruits the RNAi machinery) are integral components of the nuclear envelope, such a scenario seems plausible. Indeed, we observe that nuclear size and structure are altered in the lem2Δ csi1Δ mutant. In this regard, it is also tempting to speculate about whether silencing defects caused by lamin mutations in metazoans could derive from morphological changes of the nuclear lamina or envelope. It is clear that the interaction between the nuclear membrane and chromatin is multifaceted and complex, which may have various functional consequences for gene repression. This idea is reinforced by the observation that, vice versa, defects in heterochromatin establishment, like the loss of H3K9 methyltransferases, can induce morphological changes in the nuclear membrane.

**Outlook**

In conclusion, these novel findings reveal a complex role of Lem2 in controlling both chromatin localization and silencing, yet by utilizing different functional domains. According to our current model (Fig. 1A), Lem2 mediates centromere tethering through its LEM domain, whereas anchoring of telomeres and repression of heterochromatin in general require its MSC domain. In addition, Lem2 contributes to other functions that are not directly linked to its role in tethering and repression (Fig. 1B). For these different tasks, Lem2 cooperates with multiple redundant pathways that also localize to the nuclear periphery and whose functions in silencing and localization are well characterized. However, the molecular role that Lem2 plays in these functions is still poorly understood. Although our genetic data clearly indicate that heterochromatin silencing by Lem2 acts in part through SHREC and Epe1, the exact mechanisms and interplay between these factors remain elusive. Thus, future work needs to focus on identifying the physical binding partners and downstream factors of Lem2. Given that the LEM and MSC domains are highly conserved and that both domains are present in various metazoan LAPs, such studies in fission yeast will advance our understanding of how these domains may be involved in development and human disease. Until recently, the focus has been set primarily on the LEM domain, assuming that this domain would be key for deciphering the role of LEM proteins in repression mostly because of its interaction with chromatin. Hence, the finding that
many functions of Lem2 including silencing depend in fact on its MSC domain adds an unanticipated developing perspective to this conserved family of proteins.

Disclosure of potential conflicts of interests
No potential conflicts of interests were disclosed.

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