Inactivation of the *C. elegans* lipin homolog leads to ER disorganization and to defects in the breakdown and reassembly of the nuclear envelope

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Summary

The nuclear envelope (NE) is a dynamic structure, undergoing periods of growth, breakdown and reassembly during the cell cycle. In yeast, altering lipid synthesis by inactivating the yeast homolog of lipin, a phosphatidic acid phosphohydrolase, leads to disorganization of the peripheral ER and abnormal nuclear shape. These results suggest that lipid metabolism contributes to NE dynamics; however, since yeast undergo closed mitosis, the relevance of these observations to higher eukaryotes is unclear. In mammals, lipin has been implicated in adipose tissue differentiation, insulin resistance, lipid storage and obesity, but the underlying cellular defects caused by altering lipin levels are not known. Here, we identify the *Caenorhabditis elegans* lipin homolog (LPIN-1) and examine its affect on NE dynamics. We find that downregulating LPIN-1 by RNAi results in the appearance of membrane sheets and other abnormal structures in the peripheral ER. Moreover, *pin-1* RNAi causes defects in NE breakdown, abnormal chromosome segregation and irregular nuclear morphology. These results uncover cellular processes affected by lipin in metazoa, and suggest that lipid synthesis has a role in NE dynamics.

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Introduction

In metazoa, the nuclear envelope (NE) is comprised of a double membrane and an underlying nuclear lamina. The double membrane is peppered with nuclear pores that enable movement of cellular components between the nucleus and the cytoplasm, whereas the nuclear lamina forms contacts with chromosomes and contributes to overall nuclear architecture (reviewed by Crisp and Burke, 2008). During cell division, the NE undergoes dramatic changes (reviewed by Hetzer et al., 2005): the NE breaks down early in mitosis, allowing spindle microtubules to gain access to the chromosomes, and it reassembles around the segregated DNA masses during later stages of mitosis. The endoplasmic reticulum (ER) has an integral role in this cycle of NE breakdown (NEBD) and reassembly (Prunuske and Ullman, 2006): during NEBD, components of the NE, such as nuclear pore components and inner nuclear membrane proteins, are absorbed by the ER, and the ER itself becomes enriched in membrane tubules (Ellenberg et al., 1997; Puhka et al., 2007). During the initial stages of NE reassembly, ER tubules form contacts with chromosomes. These tubules then flatten to form an intact nuclear double membrane (Anderson and Hetzer, 2007). Given that the NE is an extensive membrane sheet, the formation of the NE requires a change in the ER topology, from tubules to sheets. Indeed, Anderson and Hetzer showed recently that the levels of reticulons, conserved proteins that stabilize ER tubules (Voeltz et al., 2006), affect the timing of NE formation: high levels of reticulons, which favor tubule formation, delay NE formation whereas low levels of reticulons, which increase the abundance of ER sheets, accelerate NE formation (Anderson and Hetzer, 2008).

Reticulons are not the only cellular component that affects ER shape. Studies in yeast have shown that deletion of the gene encoding the phosphatidic acid phosphohydrolase Pah1p (also known as Smp2p in budding yeast and Ned1p in fission yeast), which converts phosphatidic acid to diacylglycerol, leads to the appearance of ER sheets (Campbell et al., 2006; Han et al., 2006; Santos-Rosa et al., 2005; Siniossoglou et al., 1998; Tange et al., 2002). Budding yeast *pah1Δ* mutants exhibit elevated levels of phosphatidic acid, phosphatidylinositol and phosphatidylethanolamine and reduced levels of diacylglycerol and triacylglycerol. In addition, *pah1Δ* mutants and mutations in genes that regulate Pah1p (e.g. *sco7Δ, nem1Δ*) exhibit an abnormal nuclear morphology (Campbell et al., 2006; Han et al., 2006; Santos-Rosa et al., 2005; Siniossoglou et al., 1998; Tange et al., 2002). The effect of Pah1p inactivation on both ER and nuclear morphology is intriguing, because it further suggests a functional link between ER organization and NE dynamics. However, because both budding and fission yeasts undergo closed mitosis (i.e. the NE does not break down during mitosis), these model organisms are not suitable for examining whether Pah1p-dependent ER perturbations affect NEBD and reassembly.

**PAH1** is the yeast homolog of the mammalian lipin (Reue and Brindley, 2008); mammals have three lipin homologs: LPIN1, LPIN2 and LPIN3. In mice, inactivation of lipin-1 causes reduced adipose tissue mass, insulin resistance and lipid storage defects (Peterfy et al., 2001; Reue, 2007). The latter is consistent with a...
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defect in the synthesis of triacylglycerol, the major component of stored lipid. In addition, lipin-1 overexpression leads to obesity (Phan and Reue, 2005). In humans, mutations in LPIN2 cause Majeed syndrome (Ferguson et al., 2005), an autoinflammatory disorder, and mutations in either LPIN1 or LPIN2 are associated with metabolic syndrome and type-2 diabetes (Reue and Brindley, 2008). Little is known about the function of lipin-3. Although the physiological consequences of mammalian lipin inactivation and overexpression have been examined, the effect of mammalian lipin inactivation on ER structure and NE dynamics has not been explored. In this study, we took advantage of the nematode C. elegans, which has a single lipin gene, to examine what effect, if any, lipin has on ER organization and NE breakdown and reassembly.

Results

C. elegans lipin is needed for lipid storage and development

The sequence of the C. elegans genome revealed that unlike mammals, which have three lipin genes, C. elegans has only one putative lipin homolog, H37A05.1 (Lykidis, 2007). Since mice lacking lipin-1 have no adipose tissue, we wished to determine whether the C. elegans lipin homolog, which we named lipin-1, affects the ability to accumulate and store fat. Unlike mammals, which store fat in adipose cells, worms accumulate fat in lipid droplets that form in intestinal and hypodermal cells. These droplets can be visualized by the vital dye Nile red (Ashrafi et al., 2003). To examine whether downregulation of LPIN-1 affected the accumulation of lipid droplets, embryos of wild-type (N2) worms were laid on bacteria expressing either control double-stranded RNA (dsRNA) or dsRNA against lipin-1 [henceforth lipin-1(RNAi)]. The bacterial cultures were mixed with Nile red, which was taken up by the worms during feeding. During the first 4 days after the embryos were laid, worms that hatched on lipin-1(RNAi) bacteria were indistinguishable from the control worms in both size and Nile red accumulation (Fig. 1A; and data not shown). However, differences between control and lipin-1(RNAi) worms became apparent as worms reached adulthood: at days 5 and 6, the lipin-1(RNAi) worms were smaller (Fig. 1B) than the control RNAi-treated worms (see also supplementary material Fig. S1). The reduced body size and decreased lipid droplet accumulation in the lipin-1(RNAi) worms was not a result of decreased food intake, as the pumping rate of lipin-1(RNAi) and control animals was similar (5.8±0.45 pumps/second and 4.96±0.01 pumps/second, respectively). The effect of lipin-1(RNAi) on lipid droplet accumulation was comparable to that of sbp-1(RNAi), which was found by Ashrafi and colleagues (Ashrafi et al., 2003) to induce a significant reduction in lipid droplet accumulation (supplementary material Fig. S1). These results suggest that, as in mammalian cells, downregulating the activity of the C. elegans LPIN-1 reduces the amount of neutral lipids in the worm and leads to a lipid-storage defect. Consistent with its role in lipid storage, a GFP::LPIN-1 fusion (see Materials and Methods) was present in the gut, where it localizes to the cytoplasm and the nucleus, as well as in other tissues such as head neurons, hypodermis and vulva muscles (Fig. 1C; and data not shown). Our gfp::lipin-1 construct was not expressed in the germline, but genetic evidence presented below suggests that LPIN-1 is present in the germline as well.

Despite their smaller size, lipin-1(RNAi) worms produced and laid embryos during the same time interval as control worms (4-6 days post-hatching), indicating that their smaller size was not due to a slower developmental program. However, lipin-1(RNAi) worms did have a smaller brood size (66.33±1.75 per worm) compared with controls (307.67±10.01 per worm). Moreover, although less than 1% of embryos laid by control worms failed to hatch (0.91±0.79% of total brood), nearly 50% of embryos from lipin-1(RNAi) worms were dead (46.67±15.90% of total brood), and embryonic lethality exceeded 80% for embryos laid on day 6 (82.78±13.37% of embryos laid on day 6 post-hatching). To determine whether the embryonic lethality stems from the downregulation of LPIN-1 expressed in the soma or the germline, hatching experiments were repeated with strains carrying ppw-1(pk1425) or rrf-1(pk1417), leading to defects in RNAi in the germline or soma, respectively (Tijsterman et al., 2002; Grishok et al., 2005). L4 larvae of these two strains, as well as an N2 wild-type control, were placed on bacteria expressing lipin-1 dsRNA or control dsRNA, and hatching of embryos laid 24-48 hours later was determined. As expected, hatching of control worms was nearly 100% for all three strains (n=300 for each of the three strains). By contrast, lipin-1(ok2761) induced embryonic lethality in N2 and the rrf-1(pk1417) strain [lethality, 91.18±6.04% (n=453) and 71.71±15.56% (n=365), respectively], whereas no lethality was observed for the lipin-1(RNAi) ppw-1(pk1425) strain (100% hatching in three independent experiments, n=358). Since rrf-1(pk1417) strains are defective in RNAi only in the soma, and ppw-1(pk1425) strains are defective in RNAi only in the germline, these results suggest that the embryonic lethality induced by lipin-1(RNAi) is probably due to downregulation of LPIN-1 present in the germline.

We also examined the phenotype of a small deletion in the lipin-1 gene, lipin-1(ok2761), which removed 518 bp near the 5’ end of the lipin-1 gene coding region, deleting most of the second exon and part of third exon. Worms homozygous for lipin-1(ok2761) hatched normally, but failed to progress past the L1 stage and died 2-3 days after hatching (see Materials and Methods). Given the RNAi results, it is likely that maternal LPIN-1 is required for embryogenesis, and that lipin-1(ok2761) homozygous worms, which are derived from heterozygous mothers, can develop until the maternal stores of LPIN-1 are depleted. Taken together, these results show that C. elegans lipin-1 is needed for the formation of lipid droplet and is important for development.

Downregulation of LPIN-1 results in disorganization of the peripheral ER

Thus far, the effect of lipin inactivation on ER structure has only been examined in yeast (Campbell et al., 2006; Han et al., 2006; Santos-Rosa et al., 2005; Siniossoglou et al., 1998; Tange et al., 2002). To determine whether lipin of a metazoan organism has an analogous function, L4-staged C. elegans larvae expressing the ER marker SP12::GFPI (Poteryaev et al., 2005) and histone H2B fused to Cherry Red (henceforth H2B::CR) were grown on bacteria expressing either control dsRNA or lipin-1 dsRNA. After 48 hours, ER structure was examined in young embryos at two focal planes: a central plane that traverses the nuclei and a peripheral plane that includes mostly peripheral ER. In control worms, the peripheral ER appears as a network of fine tubules with occasional small patches (Fig. 2A) (Poteryaev et al., 2005). lipin-1(RNAi) led to a disruption of ER structure, with the appearance of membrane sheets, patches and ring or vesicle-like structures (Fig. 2B; Fig. 3B). Although the morphology of the ER is known to change during the cell cycle (Poteryaev et al., 2005), the ER structures seen following lipin-1 dsRNA did not resemble an ER organization seen at any stage during a normal cell cycle. The abrogation of ER structure
in worms treated with lipin-1(RNAi) and in *pah1Δ* yeast suggests that the role of lipin in ER membrane organization is evolutionarily conserved.

Downregulation of LPIN-1 leads to abnormal nuclear morphology

Since lipin inactivation was shown to affect nuclear shape in cells that undergo closed mitosis, we wanted to determine what effect lipin-1(RNAi) has on the NE of cells undergoing open mitosis. To address this question, L4-staged larvae expressing the nuclear pore protein NPP-1 (Schetter et al., 2006) tagged with GFP (NPP-1::GFP) and H2B::CR were grown on bacteria expressing either control dsRNA or lipin-1 dsRNA, and nuclear morphology was examined in embryos after 24-48 hours of RNAi treatment. Unlike embryos from control worms, which displayed round nuclei (Fig. 3A, left panel), embryos from lipin-1(RNAi) worms displayed abnormal nuclear phenotypes that could be classified into one of two general categories: a ‘mild’ phenotype, in which one or more cells had paired, round nuclei (Fig. 3A, middle panel), and a ‘severe’ phenotype, in which nuclei had an uneven size and shape, the spacing between nuclei was abnormal, and foci of NPP-1::GFP appeared in the cytoplasm (Fig. 3A, left panel; supplementary material Fig. S2). The mild phenotype was typically seen after 24-36 hours of lipin-1(RNAi) treatment at 20°C, whereas the severe phenotype was seen after longer exposure to lipin-1(RNAi) (typically 48 hours at 20°C or more than 24 hours at 24°C). To examine whether the nuclear morphology following lipin-1(RNAi) treatment corresponds to a particular ER morphology, we compared the ER

**Fig. 1.** RNAi of the *C. elegans* homolog of lipin results in reduced body size and defects in lipid storage. (A) Adult wild-type (N2) worms laid embryos for 1 hour on plates seeded with bacteria expressing either control RNAi or lipin-1(RNAi). The adult worms were then removed and the embryos were followed for the next 6 days. Each day, the length and width of at least 20 worms from the control plates (black line) or lipin-1(RNAi) plates (gray line) was determined as described in the Materials and Methods. A typical worm from each treatment (taken at day 6 after the embryos were laid) is shown. (B) Adult worms were placed on control or lipin-1(RNAi) plates as described above, except that the bacteria were mixed with Nile red (Ashrafi et al., 2003). Worms were imaged daily. Examples of a typical worm from each condition, taken on day 6, are shown. The bright white dots are lipid droplets that accumulated Nile red. The boxed areas are enlarged below. To quantify the Nile red fluorescence, average pixel intensity as a function of distance from the tip of the worm’s pharynx (on the left of the graph) was determined for eight worms of each condition from day 6. Note that the average pixel intensity for control RNAi (black traces) are greater than the average pixel intensity for worms that hatched on lipin-1(RNAi), indicating the downregulation of LPIN-1 disrupted the accumulation of lipid droplets. (C) An adult worm expressing GFP::LPIN-1. A small region showing the pattern of GFP::LPIN-1 in the gut is enlarged.
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morphology in embryos with mild versus severe nuclear phenotypes (Fig. 3B). Embryos with a mild nuclear phenotype typically exhibited a ‘stringy’ ER morphology, with an abundance of ER sheets in place of the tubules network. Embryos with a severe nuclear phenotype exhibited sheet-like ER structures, as well as patches and vesicle-like structure that did not contain DNA. Thus, the severity of nuclear phenotypes correlated with the extent of disruption of the ER morphology. Interestingly, although the oocytes of worms that laid embryos exhibiting a mild or severe phenotype had a disorganized ER similar in pattern to the embryos, the nuclei of these oocytes appeared normal in both size and shape (Fig. 3C). Since these oocytes are arrested in prophase of meiosis I, these results suggest that the abnormal nuclear phenotype is due to a post-fertilization event, such as NE breakdown or NE reassembly (see below). Thus, lipin activity is needed for normal nuclear structure in dividing cells.

The paired nuclei seen in the lpin-1(RNAi)-induced mild phenotype persists through cell division

To gain a better understanding of what caused the lpin-1(RNAi)-induced mild phenotype, we carried out live-cell imaging of worms expressing NPP-1::GFP and H2B::CR. L4-staged larvae were treated with control RNAi or lpin-1(RNAi), as described above, and embryos were imaged at the indicated time intervals (Fig. 4). As described previously (Gorjánácz et al., 2007; Lee et al., 2000), in embryos from control worms (Fig. 4A, arrow), early in mitosis the chromosomes condensed and aligned on a metaphase plate, and the nuclear pores dissociated from the NE. The chromosomes separated during anaphase, and at telophase the nuclear pores reappeared as the NE reassembled. The initial steps of mitosis in nuclei exhibiting the mild phenotype were similar to a normal mitosis (Fig. 4B, arrow): the chromosomes condensed, the DNA aligned on a metaphase plate and the nuclear pores surrounding the adjacent DNA masses disappeared. However, following chromosome segregation, instead of forming one nucleus in each daughter cell, the NE assembled around two separate DNA masses (Fig. 4B, 240 seconds time point). To determine how the two paired nuclei formed, we followed spindle elongation in a two cell stage embryo from a lpin-1(RNAi) worm expressing tubulin::GFP and

Fig. 2. lpin-1(RNAi) disrupts ER morphology. Embryos from L4-staged worms (strain OCF5) expressing SP12::GFP (green in the merged panel) and histone H2B::CR (red in the merged panel) that were grown on bacteria expressing either control dsRNA (panel A) or lpin-1 dsRNA (panel B) were imaged after 48 hours using confocal microscopy. Individual sections from either a central plane (left and middle columns) or a peripheral plane (right column) are shown.

Fig. 3. lpin-1(RNAi) disrupts nuclear morphology. (A) L4-staged worms (strain OCF3) expressing NPP-1::GFP (green) and histone H2B::CR (red) were grown on bacteria expressing either control dsRNA (left) or lpin-1 dsRNA (middle and right) as described for Fig. 2, and embryos were imaged after 36-48 hours. Shown are merged images from multiple focal planes spanning each embryo. Note that in the case of embryos exhibiting a mild phenotype, some cells can have paired nuclei (gray arrow) whereas others can have a normal nuclear morphology. (B) Comparison of ER and nuclear morphologies following lpin-1(RNAi) treatment: worms expressing SP12::GFP and H2B::CR treated as described in A. For ER morphology (as detected by SP12::GFP) and histone H2B::CR grown on bacteria expressing either control dsRNA or lpin-1 dsRNA for 36-48 hours. Oocytes were imaged by confocal microscopy at either a central plane or a peripheral plane, as indicated. The designation of mild vs severe phenotypes was made based on the phenotype of the embryos from the same worms (not shown).
H2B:CR (Fig. 4C). In the embryo shown, one cell contained a single nucleus (upper right) whereas the other contained a pair of nuclei (lower left), which is typical of cells exhibiting the *lpin-1*(RNAi)-induced mild phenotype. Invariably, in this type of cells, the spindle formed along the interface of the paired nuclei (Fig. 4C, 40 second time point, arrow). As the spindle in the cell with the paired nuclei elongated and the chromosomes began to separate, the spindle appeared to be wider than the spindle in the cell with a single nucleus [Fig. 4C, compare time points 280 seconds (single nucleus) and 480 seconds (paired nuclei), arrowheads]. Moreover, the spindle associated with the paired nuclei appeared to be made of two side-by-side spindles (Fig. 4C, time points 440-520 seconds), although each spindle pole contained only a single centrosome, as determined by observing the centrosomal protein SPD-2 fused to GFP (data not shown). Therefore, although the two DNA masses of paired nuclei align on a common metaphase plate, the chromosomes of each DNA mass do not mix, leading to the formation of two separate nuclei once mitosis is completed (Fig. 4C, time point 760 seconds).

Since the chromosomes of paired nuclei caused by *lpin-1*(RNAi) treatment appeared to segregate on two side-by-side spindles, we reasoned that there is a physical barrier between the two parallel spindles that prevented chromosome mixing. Given that *lpin-1*(RNAi) treatment disrupted the ER membrane, one possibility was that the barrier is a membranous structure. However, the abundance of ER membrane around spindles of normally dividing cells made it difficult to determine unequivocally whether there was an enrichment of ER membrane between the two parallel spindles. Therefore, we used the *C. elegans* lamin protein, LMN-1, fused to YFP [YFP::lmn-1 (Galy et al., 2008)]. Worms have a single lamin gene that is homologous to lamin B, and it is associated with the NE (Liu et al., 2000). Therefore, if in cells exhibiting the *lpin-1*(RNAi)-induced mild phenotype the barrier between the two parallel spindles was residual nuclear membrane, it might also be

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**Fig. 4.** Nuclei exhibiting the *lpin-1*(RNAi)-induced mild phenotype give rise to paired nuclei following mitosis because of defects in NEBD. (A) L4-staged worms (strain OCF3) expressing NPP-1::GFP (green) and histone H2B::CR (red) grown on bacteria expressing control dsRNA for 48 hours. Live imaging of a four-cell-stage embryo from such a worm using confocal microscopy. Merged images of multiple focal planes from the indicated time point (in seconds) are shown. The nucleus labeled with an arrow is in prometaphase at time 0 seconds, in metaphase at 80 seconds, and in anaphase by 160 seconds. (B) L4-staged worms (strain OCF3) expressing NPP-1::GFP (green) and histone H2B::CR (red) grown on bacteria expressing *lpin-1* dsRNA for 36 hours and a four-cell embryo with cells exhibiting a mild phenotype (for example, see arrow) was imaged at the indicated time points, as described above. The timing of prometaphase, metaphase and anaphase is similar to that shown in A. (C) L4-staged worms (strain OCF2) expressing tubulin::GFP (green) and histone H2B::CR (red) grown on bacteria expressing *lpin-1* dsRNA for 36 hours and embryos were taken for live imaging as described above. In the example shown, a two-cell-stage embryo contains one cell with a normal nucleus (top right) and one cell with paired nuclei (bottom left). Chromosome segregation in the normal nucleus precedes that of the paired nuclei. In the case of the paired nuclei, the spindle poles align on both sides of the interface between the two DNA clusters before spindle elongation (arrow). Note the greater width of the spindle in the cell that has paired nuclei (arrowhead, time 480 seconds) compared with the spindle in the cell that has a single nucleus (arrowhead, time 280 seconds). (D,E) L4-staged worms (strain OCF4) expressing YFP::LMN-1 (green) and histone H2B::CR (red) were grown on bacteria expressing either control dsRNA (D) or *lpin-1* dsRNA (E) for 24 hours at 24°C, and embryos from these worms were imaged by live microscopy as described above. In the case of the control RNAi, the nuclear lamina is at the nuclear periphery; it dissociates at the 160 second time point, during the early stages of anaphase. By contrast, in the case of *lpin-1*(RNAi) the lamina is present at the periphery of each of the paired nuclei, and is also hanging off the edges of the interface between the two nuclei (time point 520 seconds, arrowhead). As mitosis progresses, the nuclear lamina persists, and it is stretched as the distance between the segregating chromosomes increases. Note that the nuclear lamina appears to separate the DNA that had originated from each of the paired nuclei, presumably preventing their mixing.
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To examine this possibility, L4-staged larvae expressing YFP::LMN-1 and H2B::CR were treated with control RNAi or lipin-1(RNAi). In embryos from control worms, YFP::LMN-1 could be detected until early anaphase; it disassembled in late anaphase and reassembled around the segregated chromosomes in telophase (Fig. 4D) (Liu et al., 2000). By contrast, in embryos from lipin-1(RNAi) worms, YFP::LMN-1 was present throughout mitosis (Fig. 4E, follow cell labeled with arrow), and in particular between the DNA masses of the paired nuclei. In fact, the lamina that failed to disassemble during mitosis persisted into the next cell cycle, creating thread-like structures that were attached to the daughter nuclei (Fig. 4E, see arrowhead at the 520 second time point). Thus, the downregulation of LPIN-1 resulted in a defect in nuclear lamina disassembly.

Defects in nuclear lamina disassembly that resulted in a paired nuclei phenotype were previously described (Audhya et al., 2007), as a result of RNAi against reticulons and the RAB-5 GTPase, and by Galy and colleagues (Galy et al., 2008), who used RNAi against a nuclear pore component, gp210. In both cases, the paired nuclei were a result of a failure in nuclear lamina disassembly during pronuclear fusion of the first mitotic division. Consequently, the maternal and paternal chromosomes failed to mix and remained encapsulated in separate nuclei. In the cases of lipin-1(RNAi)-induced paired nuclei, at least some paired nuclei were also generated because of a failure in pronuclear fusion [see supplementary material Movie 1 (control RNAi) and Movie 2 (lipin-1(RNAi))]. However, in some cases, we observed cells with three associated nuclei, where the three DNA masses remained separated through mitosis (supplementary material Movie 3). The presence of more than two nuclei cannot be readily explained by pronuclear fusion failure, and it could be a consequence of an aberrant mitosis later in development.

Whether a reduction in LPIN-1 activity can lead to the formation of paired nuclei de novo in later stages of embryonic division remains to be determined.

Downregulating LPIN-1 activity can lead to defects in chromosome segregation, mislocalization of nuclear pore proteins and aberrant NE reassembly

Unlike the mitosis of nuclei exhibiting the lipin-1(RNAi)-induced mild phenotype, dividing cells exhibiting a severe phenotype usually undergo an abnormal mitosis. In some cases, the consequences of downregulating LPIN-1 are so severe that cell cycle progression ceases altogether. An example of nuclear division in cells exhibiting a lipin-1(RNAi)-induced severe phenotype is shown in Fig. 5A and supplementary material Movie 4. Although nuclear pores disassembled as cells progressed through the cell cycle, chromosome segregation itself was abnormal: nuclei appeared to converge before nuclear pore disassembly, chromosomes failed to align on a proper metaphase plate and chromosome movement was irregular. Moreover, in some cases, at the end of mitosis the NE reassembled around individual chromosomes or clusters of chromosomes (Fig. 5A, time point 25 minutes 20 seconds, boxed region), possibly explaining the source of the irregularly shaped nuclei (Fig. 3A; supplementary material Fig. S2). The aberrant chromosome...
segregation is consistent with abnormal spindle structures seen in

\textit{lpin-1(RNAi)} cells exhibiting a severe phenotype (Fig. 5B).

One of the characteristics of the \textit{lpin-1(RNAi)}-induced severe
phenotype is the appearance of cytoplasmic NPP-1::GFP clusters.
Interestingly, these clusters disappeared at the same time as the
nuclear pores dissociated from the NE (Fig. 5A, compare time points
12 minutes and 20 minutes; supplementary material Movies 4 and
5), suggesting that the NPP-1::GFP cytoplasmic clusters are either
intact or subassemblies of nuclear pores that are still subjected to
cell cycle regulation. What causes the assembly of these clusters
away from the nuclear membrane is not known, but given the
disordered nature of the ER, it is possible that the abnormal presence
of sheet-like ER membrane following \textit{lpin-1(RNAi)} treatment
misdirects the targeting of nuclear pore proteins and competes with
the nuclear membrane for nuclear pore assembly. The distribution
of the nuclear lamina protein LMN-1 and the inner nuclear
membrane protein LEM-2 was also altered in embryos exhibiting a
severe nuclear phenotype, but unlike NPP-1, we did not detect
any LMN-1 or LEM-2 cytoplasmic foci. Instead, these proteins
appear to localize, at least in part, to structures that resemble the
disrupted ER that is associated with the severe phenotype (Fig. 5C).
Taken together, these results demonstrate that in its extreme form,
reduction in LPIN-1 activity leads to aberrant chromosome
segregation, defects in NE assembly and mislocalization of nuclear
pores (or subassemblies thereof) and other NE-associated proteins.

\textbf{Discussion}

In this study, we have shown that reducing lipin activity in \textit{C. elegans}
leads to defects in lipid storage, which is similar to the phenotype of
LPIN1-null mice. This observation is significant because it suggests
that the function of lipin is conserved, and therefore that the cellular
\textit{lpin-1(RNAi)} phenotypes we observe in worms, namely
disorganization of the ER, perturbation of NE disassembly, disruption of chromosome segregation and defects in NE assembly,
might be relevant to the consequences of lipin inactivation in
mammals. The GFP::LPIN-1 fusion protein localizes to numerous
tissues throughout the worm, and it is present in both the nucleus
and cytoplasm, as was previously described for human lipin-1 and
lipin-2 (Grimsley et al., 2008). Our GFP::LPIN-1 fusion is not
detectable in the germline, but our genetic evidence suggests that
LPIN-1 is expressed in the germline, and this is further supported
by immunolocalization data (Gorjánácz and Mattaj, 2009). Although
lipin was previously shown to affect nuclear morphology in cells
that undergo a closed mitosis (Campbell et al., 2006; Santos-Rosa
et al., 2005; Siniossoglou et al., 1998; Tange et al., 2002), this is
the first demonstration that lipin is important for NE dynamics and
ER structure in higher eukaryotes. Specifically, we show that in its
‘mild’ phenotype, downregulation of LPIN-1 results in NE
breakdown failure, as evidenced by the persistence of lamin around
the DNA throughout mitosis. In the accompanying manuscript,
Gorjánácz and Mattaj show that following \textit{lpin-1(RNAi)} treatment,
other NE-associated proteins, such as LEM-2, gp210 and emerin,
also remain associated with the nuclear periphery and fail to
disassemble (Gorjánácz and Mattaj, 2009). Whether lipin affects
ER structure and NE dynamics independently, or whether the NE
defects are a consequence of ER disruption, remains to be
determined. We favor the latter possibility because of the observation
that disrupting reticulin function leads to aberrations in NE
dynamics (Anderson and Hetzer, 2008; Audhya et al., 2007),
pointing to an intimate link between ER structure and NE
organization.

Much like LPIN-1 downregulation, inactivation of reticulons
also leads to the formation of ER membrane sheets at the expense
of membrane tubules (Voeltz et al., 2006). Although the
morphology of the ER following \textit{lpin-1(RNAi)} is not identical to
ER morphology caused by downregulation of reticulin activity
(Audhya et al., 2007), in both cases the relative amount of ER
tubes is reduced. Audhya and co-workers proposed that the
disruption in ER structure due to reticulin inactivation prevents
inner nuclear membrane proteins from being absorbed by the ER
during NEBD, resulting in the persistence of the nuclear lamina
throughout mitosis and causing a failure in pronuclear fusion
(Audhya et al., 2007). The similarity between the reticulin RNAi
phenotype and the \textit{lpin-1(RNAi)}-induced mild phenotype is
consistent with the possibility that both treatments lead to a related
ER defect that disrupts NEBD. Therefore, we postulate that when
lipin is downregulated, either an excess of ER membrane in the
form of sheets counteracts the activity of reticulins, or ER
membrane composition is altered such that the reticulons cannot
induce the formation of ER tubules. In either case, the balance
between ER tubules and ER membrane sheets shifts in favor of
the latter, and we speculate that this leads to the persistence of the
nuclear lamina throughout mitosis, a failure in pronuclear fusion
and the formation of paired nuclei.

The situation in cells exhibiting a \textit{lpin-1(RNAi)}-induced severe
phenotype is likely to be more complex. Although some embryos
exhibiting this phenotype are likely to be dead, the fact that we are
able to observe nuclear divisions, albeit abnormal, in a subset of
these embryos suggests that the severe phenotype is a consequence
of LPIN-1 downregulation rather than a non-specific death
phenotype. Moreover, qualitatively, the severity of the nuclear
phenotype appears to correlate with the degree of ER disruption,
and the severity of both the ER and NE phenotypes increases as a
function of the length of RNAi treatment, suggesting that the
increase in severity of the \textit{lpin-1(RNAi)}-induced phenotypes is a
consequence of the reduction in LPIN-1 activity over time. What
might be the cause of the \textit{lpin-1(RNAi)}-induced severe phenotype?
During normal cell cycle progression, the ER in \textit{C. elegans}
forms a sheath-like structure around the spindle and centrosomes
(Poteryaev et al., 2005). The abnormal chromosome segregation
and the accompanying defects in spindle structure observed in the
severe phenotype could be a result of ER membrane that fails to
‘get out of the way’ and consequently obstructs proper spindle
assembly and/or chromosome movement. According to this
hypothesis, the cell cycle changes that are observed in ER structure
serve not only to absorb NE components, but also to reduce the
risk of ER membrane interfering with chromosome segregation.
When LPIN-1 is downregulated, the abnormal structures that form
in the ER might not be readily amenable to tubulation by reticulons,
and as a result, they are not cleared from the region where the spindle
assembles and the chromosomes subsequently segregate. Thus,
conceivably, the abnormal ER structures caused by LPIN-1
downregulation could interfere directly with spindle assembly and
chromosome movement.

In cells exhibiting the \textit{lpin-1(RNAi)}-induced severe phenotype,
we observed that the NE sometimes forms around clusters of
chromosomes rather than all the chromosomes. This raises the
question of how, under normal conditions, the NE reassembles at
the end of mitosis around all the chromosomes, rather than forming
separate NEs around subsets of chromosomes. Clearly the proximity
of chromosomes has a role, because stray chromosomes will form
micronuclei, and the inactivation of the chromokinesin Kid in mice
results in the formation of multinucleated cells, at least during the first embryonic divisions (Ohsumi et al., 2008). The severe phenotype observed following lpin-1(RNAi) treatment raises another possibility, that the NE itself has a role in the formation of a single nucleus. According to this view, normally the amount of ‘flat’ ER membrane (not sequestered into tubules by reticulons, for example) could be limiting. This would create an unfavorable condition for the formation of multiple nuclei around closely positioned chromosomes, because the surface-area-to-volume ratio of multiple nuclei will be greater than that of a single nucleus. Thus, a limiting amount of flat membrane would favor the formation of a single nucleus. However, if flat ER membrane was abundant, which could be the case when lipin is inactivated, the amount of membrane available for the formation of nuclear membrane would not be limiting, thereby facilitating the formation of multiple nuclei. Thus, we propose that by regulating the activity of lipin, cells ensure that the amount of membrane available for the reformation of the nucleus is not excessive, thereby avoiding the formation of multiple or malformed nuclei.

Previous studies on lipin proteins in yeast examined the effect of lipin inactivation on ER and nuclear organization, whereas studies in mice focused on organismal physiology. Our investigation of the single lipin gene in C. elegans, which we showed to have functions shared with both yeast and mammalian lipin, provides the missing link between lipin studies in unicellular eukaryotes and higher eukaryotes. Specifically, our results show that NE dynamics depends not only on proteins that remodel the ER, but also on regulated lipid biosynthesis via lipin. The results described in this study should be taken into account when trying to elucidate the underlying causes of lipin-related diseases. For example, although the insulin sensitivity of LPIN1-null mice could be explained by the lack of adipose tissue, it is possible that in certain tissues or in cells carrying mutations in any of the three lipin genes, the aberrant ER contributes to disease by disrupting vesicular transport (e.g. James, 2005) or by causing ER stress that subsequently leads to inflammation (Eizirik et al., 2008). Further understanding of the relationship between the regulation of lipid synthesis and ER or nuclear membrane dynamics in C. elegans is likely to shed light on analogous processes in humans.

Materials and Methods

Worm strains

Worm strains expressing histone H2B fused to cherry Red and different GFP-tagged proteins were generated by crossing strain OCF1 (pie-1::mCherry::his-58) with the following strains: LW1092: unc-119(ed4); jls1092[pNUTI npp-1::gfp + unc-119(+);] jls25 [pAZ132; pie-1::mCherry]; hts37 [pAK64; pie-1::mCherry::his-58 + unc-119 (+);] hts25 [pAZ132; pie-1::mCherry::his-58 + unc-119 (+);] and B1 GFP R1 (GGGGACAACTTTGTATAGAAAAGTTGaagctgtaatatcaacctgcg) and B4 LPN F1 (GGGGACAACTTTGTATAGAAAAGTTGaagctgtaatatcaacctgcg) and B4 LPN F1 (GGGGACAACTTTGTATAGAAAAGTTGaagctgtaatatcaacctgcg). These PCR products were annealed and amplified with the outside primers to generate a PCR product that was then used to produce a Gateway BP reaction. The final product was designated pAG-103. A Gateway LR reaction with pCR319, pAG109, and pAG-108 was performed to produce pAG-126. The plasmid pCR319 contains the unc-119(+)/+ gene for transformation rescue of unc-119(ed3) mutants.

For RNAi, worms were fed bacterial strains expressing a control dsRNA against smd-1(RNAi) or lpin-1(RNAi). Both E. coli strains are commercially available from MRC GeneService (Fraser et al., 2000). The control smd-1(RNAi) was indistinguishable from an empty vector control. Feeding was done on MYOB plates containing IPTG and Carbenicillin at either 20°C (for all strains except OCF4), or at 24°C (for strain OCF4). For lipid droplet visualization, Nile red was added to the bacteria as described (Ashrafi et al., 2003). To determine brood size, embryos were hatched on individual plates containing bacteria expressing either control dsRNA (n=6) or lpin-1 dsRNA (n=20). The worms were passed daily to fresh RNAi plates and the eggs that were laid were counted and scored for hatching. For experiments with strains carrying ppp-1[ppk1425] and rpf-1[ppk1417], three larvae of the two strains, along with N2, were placed on individual plates seeded with bacteria expressing lpin-1 dsRNA. The worms were passed 24 hour to new lpin-1(RNAi) plates, and removed on the following day. Hatching was scored 24 hours later. As a control, two L4 larvae from each strain were placed on smd-1(RNAi) plates and treated as described above.

Determining the phenotype of lpin-1(ok2761)

A strain carrying the lpin-1(ok2761) allele (VC2114) was obtained from the Caenorhabditis Genetic Center and backcrossed ten times to a strain carrying sert-3(scs) unc-61(e228), which flanks the lpin-1 locus. The presence of the lpin-1(ok2761) mutation was confirmed by PCR. After ten backcrosses, second day (day 0) lpin-1(ok2761)/sqt-3(sc8) unc-61(e228) adult worms were allowed to lay eggs for 5 hours at 20°C (‘day 1’), after which the adults were removed and the F1 progenesis were followed. Second day + sert-3(scs) unc-61(e228) adult worms were used as a control. The percentage of hatching of the F1 progeny from the two strains (determined on day 2) was nearly identical (93.3±2.1% for the control (n=76) and 95.0±2.7% for the lpin-1(ok2761)/sqt-3(scs) unc-61(e228) strain (n=168)). On day 3, a very small percentage of the F1 of the control strain was still at the L1 stage (2.63±0.1%), whereas nearly a quarter of the F1 progeny of the lpin-1(ok2761)/sqt-3(scs) unc-61(e228) strain remained as an L1 larvae (22.52±7.37%). This suggests that lpin-1(ok2761) homozygous worms fail to develop past the L1 stage. The percentage of F1 progeny that were homozygous for sert-3(scs) unc-61(e228) was determined on day 4 and was found to be around a quarter of the progeny for both strains (23.36±2.8% and 26.06±1.77% for F1 of the control and lpin-1(ok2761)/sqt-3(scs) unc-61(e228) strain, respectively, as expected.

Microscopy

Fluorescent images (except for the Nile red experiment) were captured by spinning-disk confocal microscopy, using a Nikon Eclipse TE2000U microscope equipped with a 60× 1.4 NA Plan Apo objective. This system is outfitted with a Spectral Applied Research LMM5 laser merge module to control the output of four diode lasers (excitation at 405, 491, 561 and 655 nm), a Yokogawa CSU10 spinning-disc unit, and a Hamamatsu C9100-13 EM-CCD camera. Confocal images were acquired using OpenLab 4.0 and processed with Adobe Photoshop CS. Whole worm and Nile red images were taken using a Nikon E800 microscope equipped with a 10× Plan Apo objective, PerkinElmer Ultraspin LCI CSU10 scanning unit, an argon/krypton ion laser (Meller Grist, Carlsbad, CA), and an ORCA ER-cooled-charge-coupled device camera (Hamamatsu Photonics, Hamamatsu, Japan), operated by OpenLab 3.0 software. In the case of the Nile red images, care was taken to ensure that the exposure times were identical throughout the experiment, and no image adjustments (e.g. brightness, contrast) were performed. Most worms after stage L3 had to be captured by eye or three overlapping images. For length and width measurements, full-length worm images were assembled using Adobe Photoshop CS, and measurements were done using Image J 1.38X software. Width measurements were done at the vulva or around the midsection if the vulva was not detectable. Length measurements were done by drawing a line along the midline of the worm. Over 20 measurements were taken for each time point and the experiment was repeated several times; the results of a typical experiment are shown. Nile red intensity was measured using the PloT profile function of the ImageJ software.

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