LIN-41 inactivation leads to delayed centrosome elimination and abnormal chromosome behavior during female meiosis in Caenorhabditis elegans

Rieko Matsuura, Tomoko Ashikawa, Yuka Nozaki, and Daiju Kitagawa

ABSTRACT During oogenesis, two successive meiotic cell divisions occur without functional centrosomes because of the inactivation and subsequent elimination of maternal centrosomes during the diplotene stage of meiosis I. Despite being a conserved phenomenon in most metazoans, the means by which this centrosome behavior is controlled during female meiosis remain elusive. Here, we conducted a targeted RNAi screening in the Caenorhabditis elegans gonad to identify novel regulators of centrosome behavior during oogenesis. We screened 513 genes known to be essential for embryo production and directly visualized GFP–γ-tubulin to monitor centrosome behavior at all stages of oogenesis. In the screening, we found that RNAi-mediated inactivation of 33 genes delayed the elimination of GFP–γ-tubulin at centrosomes during oogenesis, whereas inactivation of nine genes accelerated the process. Depletion of the TRIM-NHL protein LIN-41 led to a significant delay in centrosome elimination and to the separation and reactivation of centrosomes during oogenesis. Upon LIN-41 depletion, meiotic chromosomes were abnormally condensed and pulled toward one of the two spindle poles around late pachytene even though the spindle microtubules emanated from both centrosomes. Overall, our work provides new insights into the regulation of centrosome behavior to ensure critical meiotic events and the generation of intact oocytes.

INTRODUCTION

The centrosome comprises a pair of centrioles surrounded by pericentriolar material (PCM) and serves as the major microtubule-organizing center (MTOC) in most animal cells (Nigg and Stearns, 2011; Bornens, 2012; Bornens et al., 2014; Gönczy, 2012). MTOC activity of centrosomes is crucial for proper chromosome segregation in mitosis and in meiosis during spermatogenesis. By contrast, during oogenesis in most metazoans, maternal centrosomes are inactivated because of the gradual loss of PCM components and are thereafter eliminated during meiotic prophase I; hence, two successive meiotic cell divisions occur without centrosomes (Sathananthan et al., 1996; Delattre and Gönczy, 2004; Manandhar et al., 2005; Schatten and Sun, 2009). The elimination of maternal centrosomes and the inheritance of paternal centrosomes to the progeny are crucial for maintaining the precise number of centrosomes in the fertilized zygote and thus for proper sexual reproduction.

The gonad of the Caenorhabditis elegans hermaphrodite is a well-suited model for analyzing the mechanisms governing centrosome behavior during oogenesis because all stages of oogenesis can be seen in a continuous manner within a single gonad (Hubbard and Greenstein, 2000). To reduce successfully the number of centrosomes in oocytes, as the first step, centrosomes lose the ability to nucleate microtubules around the transition zone (TZ) during meio- sis (Kemp et al., 2004; Mikelaźdre-Dvali et al., 2012). As the second step, elimination of centrioles occurs in the loop region of the gonad during the diplotene stage (Mikelaźdre-Dvali et al., 2012). The...
developing oocytes from which centrioles were previously eliminated arrest in meiotic prophase I until the resumption of meiotic maturation by the major sperm protein (MSP; Kim et al., 2013). During late prophase of meiosis I, the synaptonemal complex, a protein complex that mediates the pairing of homologous chromosomes during meiosis, disassembles before meiotic spindle formation begins (Zetka et al., 1999; Hansen et al., 2004). The cyclin-dependent kinase 1 (CDK-1)/cyclin B complex, a maturation-promoting factor (MPF), is required for both mitotic and meiotic spindle formation with or without centrosomes (McCarter et al., 1999; Burrows et al., 2006; Kim et al., 2010). Therefore, to prevent ectopic activation of centrosomes, CDK-1/cyclin B activity must be suppressed until centrosome elimination is completed in oocytes.

Although the molecular mechanisms responsible for the inactivation and elimination of centrosomes during oogenesis remain elusive, the RNA helicase CGH-1 has been reported to promote timely centriole elimination in C. elegans germ cells (Mikeladze-Dvali et al., 2012). Cosuppression of the CKD inhibitor Cki-2 has been shown to perturb centrosome elimination in the same model system (Dae and Roy, 2006). However, in germ cells of cki-2-null mutant hermaphrodites, centrosome elimination is rarely affected (Buck et al., 2009; Mikeladze-Dvali et al., 2012). Because little is known about how centrosomes are inactivated and eliminated in a timely manner during oogenesis, we sought to identify such regulators of centrosome behavior using a combination of a semicomprensive feeding RNA interference (RNAi) screening and direct visualization of centrosomes at all stages of C. elegans oogenesis.

LIN-41 is known to act in the heterochronic pathway that regulates the differentiation and development of somatic cells from larva to adult in C. elegans (Reinhart et al., 2000; Slack et al., 2000). A recent study claimed that LIN-41 prevents the precocious transition to embryonic differentiation and teratoma formation in developing oocytes (Tocchini et al., 2014). Another group demonstrated that LIN-41 regulates meiotic transition from prophase to M phase through the CDK-1 pathway in the female germ cells of C. elegans (Spike et al., 2014a). Although both studies showed the existence of centrosomes in the proximal gonad of lin-41 mutants, it is most likely that the lin-41 mutant oocytes entered M phase directly after the pachytene stage but did not pass through the diplotene stage of meiotic prophase I, during which centrosome elimination normally occurs. Therefore it is not fully understood whether LIN-41 functions in centrosome elimination during the diplotene stage. In our RNAi-based screening, we used mild inactivation of lin-41 to observe the effect of LIN-41 depletion on centrosome elimination in the diplotene and diakinesis stages and identified LIN-41 as a promoter of centrosome elimination during oogenesis. This regulation seems to be independent of the CDK-1 pathway. We also show that ectopic activation of centrosomes led to abnormal behavior of meiotic chromosomes during oogenesis upon LIN-41 depletion.

RESULTS
Identification of genes that participate in the regulation of centrosome behavior during oogenesis
To identify the genes that regulate the precise time of centrosome elimination during oogenesis, we performed RNAi screening in C. elegans gonads that expressed green fluorescent protein (GFP)-γ-tubulin as a centrosome marker. In this system, centrosome behavior during oogenesis can be readily monitored because all stages of female germline development proceed in a continuous manner within a single gonad. To judge the adequate time needed for centrosome elimination, we focused on the three proximal oocytes, −1 to −3 positions away from the spermatheca, which lack centrosomes in the wild type (Mikeladze-Dvali et al., 2012). Although genome-wide RNAi screening was performed using C. elegans early embryos (Gönczy et al., 2000; Sönntig et al., 2005), those screens did not identify any genes whose reduction leads to retention of maternal centrosomes in the embryos. It could be that pleiotropic defects in meiosis hindered such a phenotype in the early embryos. Alternatively, we hypothesized that if maternal centrosomes remain competent to nucleate microtubules during oogenesis, the processes for proper oogenesis and embryo production could be affected. On the basis of these considerations, we selected a set of 513 genes that are essential for proper embryo production in C. elegans (Green et al., 2011) and used RNAi feeding to test the effects of the inactivation of each gene on centrosome behavior during oogenesis.

In the first screening of each gene, we observed gonads of at least 10 adult worms that were treated with RNAi feeding for 24 h. We examined the time of disappearance of GFP-γ-tubulin foci as an indicator of centrosome elimination, which normally occurs around the diplotene stage in meiotic prophase I (Mikeladze-Dvali et al., 2012). We classified the phenotypes into two main categories depending on whether the disappearance of GFP-γ-tubulin foci in the female germline was delayed or accelerated. In wild-type gonads, the GFP-γ-tubulin signal representing centrosomes could be detected from the distal to the loop region of the gonad (Figure 1A). In the screening, the inactivation of 33 genes caused a significant delay in the disappearance of GFP-γ-tubulin foci (Figure 1B and Supplemental Table S1). These included genes related to a variety of biological processes, such as translation, mitotic–meiotic transition, and membrane trafficking. In particular, inactivation of 29 translation-related genes delayed centrosome elimination, which was accompanied by an obvious developmental delay.

To find specific regulators of centrosome elimination, we focused on the other candidate genes (four genes in the table of Figure 1B). GFP-γ-tubulin foci were consistently retained in proximal oocytes depleted of defect in germline development-1 (GLD-1), abnormal cell lineage-41 (LIN-41), ADP-riboseylation factor-1.2 (ARF-1.2), or N-myristoyl transferase-1 (NMT-1; Figures 1B and 2A). Of interest, in oocytes depleted of ARF-1.2 or NMT-1, numerous GFP-γ-tubulin foci were observed in the cytoplasm and, in particular, were found to accumulate around the nucleus in proximal oocytes (Figures 1B and 2A; 14 of 31 and 18 of 30 gonads, respectively). By contrast, earlier elimination of GFP-γ-tubulin foci at centrosomes was detected in the case of RNAi targeting against nine genes (Figure 1B). Eight genes encode γ-tubulin or one of the subunits of the chaperonin-containing TCP-1 (CCT) complex, which act together as a chaperone for tubulin family proteins (Lundin et al., 2008; Saegusa et al., 2014). In gonads depleted of C23G10.8, whose function is not known, most GFP-γ-tubulin foci were weakly stained even in the distal gonad and were not detectable in the TZ by fluorescence microscopy (Figure 2A and Supplemental Table S1; 10 of 10 animals).

In the second assay, we performed immunostaining of the gonads using antibodies against RME-2 (Grant and Hirsh, 1999), a marker of oocyte maturation, to exclude that the defects in centrosome elimination stem from the secondary effects of developmental delay. In the wild type, RME-2 was detected in the cytoplasm from the diplotene stage to the proximal diakinesis oocytes and accumulated particularly at the plasma membrane of the proximal oocytes (Supplemental Figure S1). Centrosomal GFP-γ-tubulin foci were never observed in control mature oocytes that were well stained with RME-2 antibodies (Supplemental Figure S1; 28 gonads). In glld-1(RNAi) animals, although centrosomal GFP-γ-tubulin foci remained in the proximal oocytes, these oocytes were small, round, and
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and centriolar proteins. In wild-type gonads, the PCM component SPD-5, which recruits \(\gamma\)-tubulin to centrosomes, localized to the vicinity of germ cell nuclei (GCN) from the distal gonad and disappeared at the diplotene stage, as observed for \(\gamma\)-tubulin distribution in the gonad (Figure 2B; wild-type, 31 of 31 gonads) (Mikeladze-Dvali et al., 2012). Of importance, both SPD-5 and \(\gamma\)-tubulin foci at centrosomes were detectable in the LIN-41–depleted diakinetic oocytes (Figure 2B; lin-41(RNAi), 73 of 73 gonads). By contrast, SPD-5 signal was not detectable in the ARF-1.2–depleted proximal oocytes even though these oocytes contained multiple clear \(\gamma\)-tubulin foci (Figure 2B; arf-1.2(RNAi), 23 of 23 gonads). A similar result was obtained for staining of SAS-4, a centriolar protein, in the gonads of lin-41(RNAi) and arf-1.2(RNAi) animals (Figure 2C; 20 of 20 gonads for lin-41(RNAi) and 30 of 30 gonads for arf-1.2(RNAi)).

We also found that centrosomal localization of SPD-5 and SAS-4 was detectable in nmt-1(RNAi) gonads only at the diplotene stage, RME-2–negative immature oocytes (RME-2 positive; 0 of 13 gonads). In the case of gld-1(RNAi), this is consistent with the fact that female germ cells of the gld-1 loss-of-function mutant reenter mitosis around the pachytene stage and escape from meiotic prophase (Francis et al., 1995). In nmt-1(RNAi) animals, the proximal oocytes seemed to be morphologically fully grown but lacked membrane RME-2 (RME-2 positive; one of 16 gonads). These results suggest that inactivation of gld-1 and nmt-1 indirectly caused centrosome elimination delay as a consequence of defective specification of female germ cells or delayed oocyte development. Of importance, coexistence of membrane RME-2 and GFP–\(\gamma\)-tubulin foci was frequently observed in the arf-1.2(RNAi) and lin-41(RNAi) proximal oocytes (Supplemental Figure S1; RME-2 positive; 18 of 31 gonads in arf-1.2(RNAi) and 23 of 39 gonads in lin-41(RNAi)).

In the third assay, we examined whether the persistent GFP–\(\gamma\)-tubulin foci in proximal oocytes contain other PCM components and centriolar proteins. In wild-type gonads, the PCM component SPD-5, which recruits \(\gamma\)-tubulin to centrosomes, localized to the vicinity of germ cell nuclei (GCN) from the distal gonad and disappeared at the diplotene stage, as observed for \(\gamma\)-tubulin distribution in the gonad (Figure 2B; wild-type, 31 of 31 gonads) (Mikeladze-Dvali et al., 2012). Of importance, both SPD-5 and \(\gamma\)-tubulin foci at centrosomes were detectable in the LIN-41–depleted diakinetic oocytes (Figure 2B; lin-41(RNAi), 73 of 73 gonads). By contrast, SPD-5 signal was not detectable in the ARF-1.2–depleted proximal oocytes even though these oocytes contained multiple clear \(\gamma\)-tubulin foci (Figure 2B; arf-1.2(RNAi), 23 of 23 gonads). A similar result was obtained for staining of SAS-4, a centriolar protein, in the gonads of lin-41(RNAi) and arf-1.2(RNAi) animals (Figure 2C; 20 of 20 gonads for lin-41(RNAi) and 30 of 30 gonads for arf-1.2(RNAi)). We also found that centrosomal localization of SPD-5 and SAS-4 was detectable in nmt-1(RNAi) gonads only at the diplotene stage.
FIGURE 2: Defects in centrosome behavior in the hermaphrodite gonad upon RNAi-mediated inactivation of candidate genes. (A) Representative fluorescence images of gonads expressing GFP–γ-tubulin and treated with RNAi against the indicated genes. GFP–γ-tubulin foci detectable at the most proximal site of the gonads are shown in magnified insets. Inactivation of C23G10.8 resulted in early elimination of GFP–γ-tubulin foci in the distal gonad. Insets are magnified threefold. Arrow shows the direction of germline development. (B, C) Immunostaining of hermaphrodite gonads expressing GFP–γ-tubulin in wild-type, arf-1.2(RNAi), and lin-41(RNAi) with antibodies against SPD-5 (red in B), SAS-4 (red in C), and GFP (green). DNA is shown in blue. GFP–γ-tubulin foci detected at the most proximal site of the gonads are shown in magnified insets. Insets are magnified threefold. Numbers show positions of oocytes from the spermatheca. Scale bars, 10 μm.
although tiny ectopic GFP–γ-tubulin foci were found in the proximal oocytes, which was similar to the phenotype seen in arf-1.2(RNAi) gonads (Supplemental Figure S2, A and B). These results suggest that, whereas centrosome elimination was affected markedly by LIN-41 depletion, only γ-tubulin distribution was affected upon ARF-1.2 depletion independently of the occurrence of centrosome elimination.

**Precocious γ-tubulin reduction affects gonad morphology but not PCM assembly and centriole integrity in female germ cells**

Next we set out to characterize the detailed phenotype of the GFP–γ-tubulin elimination advance found in the first screening (Figure 1B). To examine whether early disappearance of GFP–γ-tubulin foci in the gonad reflects precocious elimination of PCM and centrioles, we immunostained RNAi-treated gonads using antibodies against SPD-5 and SAS-4 (Figure 3A and Supplemental Figure S2B). In tbg-1(RNAi) animals, in which the characteristic localization of γ-tubulin at centrosomes and plasma membranes in the gonad was diminished, the width of the rachis and position of the GCN in the gonad were affected, as previously reported (Figure 3B; Zhou et al., 2009; Green et al., 2011). Whereas the GCN were well aligned at the periphery in wild-type gonads, they were randomly located within the central rachis in tbg-1(RNAi) gonads. This defect probably reflects the lack of nuclear anchoring of the GCN with the microtubule filaments formed from the membrane-associated γ-tubulin complexes (Zhou et al., 2009). By contrast, both the centrosomal SPD-5 and centriolar SAS-4 foci were normal in the vicinity of the GCN from the distal gonad to the diplotene stage (Figure 3A; 18 of 18 gonads; and unpublished data).

We found that depletion of the CCT complex was similar to that observed in tbg-1(RNAi) gonads in terms of the localization of SPD-5 and SAS-4 and gonad morphology; including the arrangement of the GCN and membranes (Figure 3B and unpublished data). It is therefore possible that the CCT complex regulates the integrity and distribution of γ-tubulin in the gonad, which is crucial for maintaining proper gonad morphology (Melki et al., 1993; Lundin et al., 2008; Saegusa et al., 2014). In C23G10.8(RNAi) animals, the signal intensity of SPD-5 foci seemed not to be affected, and these foci colocalized with a weak GFP–γ-tubulin signal in the distal gonad (Figure 3A; 14 of 15 gonads). Although GFP–γ-tubulin was precociously depleted from the vicinity of crescent nuclei in the TZ (Figure 3A; 15 of 15 gonads), the staining of membrane GFP–γ-tubulin, centrosomal SPD-5, and centriolar SAS-4 and the overall gonad morphology were not significantly affected in C23G10.8(RNAi) animals (Figure 3, A and B, and Supplemental Figure S2B). These results suggest that C23G10.8 protein specifically regulates the maintenance of centrosomal γ-tubulin in oogenesis. From these findings, we conclude that precocious reduction of γ-tubulin does not affect the PCM or centriole stability during oogenesis and that membrane γ-tubulin is crucial for the maintenance of proper gonad morphology.

**Depletion of LIN-41 leads to a delay in centrosome elimination and to centrosome separation**

Although previous studies reported that centrosomes remain in the proximal region of the gonads of the lin-41-null mutant (Spive et al., 2014a; Tocchini et al., 2014), the GCN that retained centrosomes in the proximal gonad did not appear to be in diplotene or diakinesis. This was presumably because the GCN precociously entered M phase from the pachytene stage without passing through diplo- tene and diakinesis. In addition, in the most proximal gonad of the lin-41-null mutant, the germ cells undergo somatic-like differentiation and form teratomas. Therefore, to identify a potential role of LIN-41 in centrosome elimination during diplotene in female meiosis, we used several RNAi conditions to analyze the detailed phenotype elicited by LIN-41 depletion. The penetrance seemed to depend on the duration of feeding lin-41(RNAi). Whereas the oocyte morphology and meiotic chromosomal behavior were generally not affected in the proximal gonads treated with lin-41(RNAi) for 12 h, the oocytes tended to be smaller and formed teratomas upon longer treatment with lin-41(RNAi) (Supplemental Figure S3A; Spive et al., 2014a; Tocchini et al., 2014). We also observed RME-2–positive mature oocytes in the proximal gonads treated with lin-41(RNAi) for 12 h. By contrast, in the severe RNAi condition (e.g., for 24 h), RME-2 localization was no longer detected in the proximal gonads, as reported in previous studies (Spive et al., 2014a; Tocchini et al., 2014). Accordingly, to investigate the precise time of centrosome elimination in lin-41(RNAi) oocytes, we used an antibody to intermediate filament antigen (IFA), a centriole marker in C. elegans, and the mild RNAi condition (12 h) to examine the centrioles in the oocytes. Remarkably, centrioles were frequently detectable even in diakinetic oocytes in the LIN-41-depleted proximal gonads (Figure 4, A and B; 50, 40, and 10% at –4, –3, and –2 oocytes, respectively, in lin-41(RNAi), compared with 2, 0, and 0% in wild type). However, we found no centrioles in the most proximal (–1) oocytes.

It was previously reported that disengaged centrioles exist adja- cent to each other after meiotic entry and are simultaneously elimi- nated without separation at the diplotene stage (Mikeladze-Dvali et al., 2012). However, intriguingly, centrosome separation occurred in LIN-41–depleted oocytes around late pachytene (Figure 4C; 88% in lin-41(RNAi) gonads [n = 33], compared with 0% in wild-type gonads [n = 28]). We then examined whether the anomalous centrosome behavior in lin-41(RNAi) gonads depends on the PCM. We treated lin-41(RNAi) on top of the spd-5(or213) mutant background, whose PCM assembly is defective. In this situation, the phenotype of centrosome elimination delay was not significantly altered in the proximal oocytes (Figure 4, A and B; centrioles detected by 52, 22, and 7%, respectively, at –4, –3, and –2 oocytes in spd-5(or213);lin-41(RNAi) animals). We still detected diakinetic oocytes that harbored remaining centrioles (Figure 4A). By contrast, the ectopic centrosome separation induced upon LIN-41 depletion was partially suppressed by concomitant inactivation of spd-5 (Figure 4C; 88% in lin-41(RNAi) gonads [n = 33], compared with 38% in spd-5(or213);lin-41(RNAi) gonads [n = 26]). Overall, these results suggest that LIN-41 regulates the time of centrosome elimination in developing oocytes in a PCM-independent manner. On the other hand, the ectopic centrosome separation induced by LIN-41 depletion might be PCM dependent, at least in part.

**Depletion of LIN-41 induces ectopic centrosome activation during oogenesis**

Recent studies showed that, in the proximal gonad of lin-41–null mutants, ectopic centrosome activation occurs at the GCN that precociously proceeded in M phase or in the somatically differentiated state (Spive et al., 2014a; Tocchini et al., 2014). To test whether the milder depletion of LIN-41 also induces ectopic centrosome activation when meiotic progression of GCN appears not to be significantly affected, we monitored the MTOD activity of centrosomes in the gonads treated with mild lin-41(RNAi). In the wild type, in which the centrosomes have already lost the MTOD activity around the TZ, signals from TAC-1, a PCM marker that should reflect the MTOD activity of centrosomes, were never observed in the loop region of the gonads (Figure 5, A and B; 42 of 42 gonads). By contrast, a
FIGURE 3: Precocious γ-tubulin reduction affects gonad morphology but not PCM assembly and centriole integrity in female germ cells. (A) Immunostaining of hermaphrodite gonads expressing GFP–γ-tubulin in wild-type, tbg-1(RNAi), and C23G10.8(RNAi) with antibodies against GFP (green) and SPD-5 (red). DNA is shown in blue. Representative centrosomes and nuclei are indicated in magnified insets. Insets are magnified twofold. TZ, transition zone. 

(B) Immunostaining of the loop region of hermaphrodite gonads expressing GFP–γ-tubulin in wild-type, tbg-1(RNAi), C23G10.8(RNAi), and cct-2(RNAi) with antibodies against GFP (green). DNA is shown in blue. The dashed arrow shows the width of rachis at the pachytene stage. Note that the arrangement of GCN in the gonad was not affected in C23G10.8(RNAi) (n = 13) but was severely affected in tbg-1(RNAi) (9) and cct-2(RNAi) (26). Arrowheads indicate the membrane-bound GFP-γ-tubulin. Arrow shows the direction of germline development. Scale bars, 10 μm.
frameshift that is predicted to result in a premature stop codon, a stronger phenotype was detected. This contrasts with ma104, which does not behave as a null allele but instead as a reduction-of-function allele (Slack et al., 2000). We also examined whether progression through the pachytene stage is needed to trigger these events in lin-41(RNAi) gonads. To arrest meiotic progression in the pachytene stage, we used an mpk-1(ga111) mutant in conjunction with lin-41(RNAi) treatment. In the mpk-1(ga111) background, overall lin-41(RNAi) phenotype was not detected (Supplemental Figure S3C; 12 of 12 gonads), indicating that reduction of LIN-41 leads to ectopic activation of centrosomes and microtubule nucleation after the pachytene stage in oogenesis.

We also tested whether the heterochronic genes let-7 and lin-29 are implicated in the function of LIN-41 in oogenesis, because lin-41 is known to act in the heterochrony pathway in somatic cells (Reinhart et al., 2000; Slack et al., 2000). As previously reported, the ectopic centrosome activation and other accompanying phenotypes were not rescued in the let-7(mg279);lin-41(RNAi) (24 of 24) animals, which exhibited small and disorganized oocytes with condensed nuclei and activated centrosomes in the proximal gonad, in addition to the GCN with activated centrosomes just after pachytene (lin-41(24hr) in Figure 5A). A similar phenotype was also found in the lin-41 mutants ma104 and n2914, as previously reported (Supplemental Figure S3B; Spike et al., 2014a; Tocchini et al., 2014). The MTOC activity of centrosomes at the loop or proximal region of the gonad was observed in lin-41(n2917) (100%, n = 9) mutants (Supplemental Figure S3B). Because lin-41(n2914) is a null allele that leads to a substantial number of activated centrosomes with ectopic TAC-1 signals were present around the loop region of the gonad under mild lin-41(RNAi) (Figure 5, A–C). Although the activated centrosomes were detectable just after late pachytene stage (Figure 5A), centrosomes in diakinetic oocytes seemed to be inactive in the proximal gonad.

The number of ectopic TAC-1 foci increased with increasing duration of RNAi treatment (Figure 5B). Under the severe RNAi condition, lin-41(RNAi) animals frequently exhibited small and disorganized oocytes with condensed nuclei and activated centrosomes in the proximal gonad, in addition to the GCN with activated centrosomes just after pachytene (lin-41(24hr) in Figure 5A). A similar phenotype was also found in the lin-41 mutants ma104 and n2914, as previously reported (Supplemental Figure S3B; Spike et al., 2014a; Tocchini et al., 2014). The MTOC activity of centrosomes at the loop or proximal region of the gonad was observed in lin-41(n2917) (100%, n = 9) mutants (Supplemental Figure S3B). Because lin-41(n2914) is a null allele that leads to a substantial number of activated centrosomes with ectopic TAC-1 signals were present around the loop region of the gonad under mild lin-41(RNAi) (Figure 5, A–C). Although the activated centrosomes were detectable just after late pachytene stage (Figure 5A), centrosomes in diakinetic oocytes seemed to be inactive in the proximal gonad.

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before NEBD (Figure 6A). After NEBD, formation of a bipolar spindle occurred, and this appeared to attempt to capture and segregate homologous chromosomes (Figure 6A).

To investigate further the abnormal chromosomal behavior during pachytene, we carefully monitored the synapsed homologous chromosome pairs, nuclear membrane, microtubule nucleation, and centrosomes in \textit{lin-41(RNAi)} gonads. In wild-type gonads, HIM-3, which is a synaptonemal complex component, accumulated between sister chromatids, specifically during meiosis from the TZ to the proximal oocytes (Supplemental Figure S4C). In \textit{lin-41(RNAi)} gonads, before NEBD, as TAC-1 foci began to accumulate on the surface of the nuclear membrane, HIM-3–positive chromosomes gathered into a cluster in the nucleus (Figure 6B). Of interest, during or after NEBD, all six chromosomes started to be pulled toward one of the two spindle poles (Figure 6B). At this stage, HIM-3 signals were retained on the chromosomes and gradually declined as bipolar spindle formation proceeded (Figure 6, C and D; HIM-3 absent in \(\sim 12\%\) of GCN before NEBD, \(\sim 35\%\) of GCN in metaphase-like, and \(\sim 54\%\) of GCN after metaphase-like). After the metaphase-like event, although we could not detect properly segregated chromosomes, all chromosomes were in a blob and eventually captured by microtubules emanating from one of the two spindle poles (Figure 6, B, C, and E). Although the reason that all the synapsed

or \textit{lin-29(n482);lin-41(RNAi)} (23 of 23) gonads compared with \textit{lin-41(RNAi)} gonads (30 of 31; Supplemental Figure S3D; Spike et al., 2014a). Although we cannot exclude the possibility that the residual activity of LET-7 or LIN-29 in these mutants might suffice to exert the heterochronic pathway, these data strongly suggest that the heterochronic pathway is not implicated in centrosome behavior in oogenesis.

Ectopic spindles tried but failed to segregate the synapsed meiotic chromosomes after pachytene in \textit{LIN-41–depleted gonads}

Next we set out to investigate the effect of ectopic centrosome activation on meiotic events in \textit{lin-41(RNAi)} gonads. In wild-type gonads, nuclear envelope breakdown (NEBD) was never observed in the loop region because only the most proximal oocyte that receives the MSP signal for meiotic maturation induces NEBD (Harris et al., 2006; Kim et al., 2013). Although ectopic NEBD and condensed chromosomes were observed in the loop region of \textit{LIN-41–depleted} gonads, presumably because of precocious M-phase entry (Supplemental Figure S4, A and B; Spike et al., 2014a; Tocchini et al., 2014), it is not clear how ectopic formation of spindles affects NEBD and chromosome condensation. We found that ectopic microtubule nucleation was detectable in late pachytene nuclei even before NEBD (Figure 6A). After NEBD, formation of a bipolar spindle occurred, and this appeared to attempt to capture and segregate homologous chromosomes (Figure 6A).

To investigate further the abnormal chromosomal behavior during pachytene, we carefully monitored the synapsed homologous chromosome pairs, nuclear membrane, microtubule nucleation, and centrosomes in \textit{lin-41(RNAi)} gonads. In wild-type gonads, HIM-3, which is a synaptonemal complex component, accumulated between sister chromatids, specifically during meiosis from the TZ to the proximal oocytes (Supplemental Figure S4C). In \textit{lin-41(RNAi)} gonads, before NEBD, as TAC-1 foci began to accumulate on the surface of the nuclear membrane, HIM-3–positive chromosomes gathered into a cluster in the nucleus (Figure 6B). Of interest, during or after NEBD, all six chromosomes started to be pulled toward one of the two spindle poles (Figure 6B). At this stage, HIM-3 signals were retained on the chromosomes and gradually declined as bipolar spindle formation proceeded (Figure 6, C and D; HIM-3 absent in \(\sim 12\%\) of GCN before NEBD, \(\sim 35\%\) of GCN in metaphase-like, and \(\sim 54\%\) of GCN after metaphase-like). After the metaphase-like event, although we could not detect properly segregated chromosomes, all chromosomes were in a blob and eventually captured by microtubules emanating from one of the two spindle poles (Figure 6, B, C, and E). Although the reason that all the synapsed
were probably in a somatically differentiated state, because they were found mainly in teratoma-like small oocytes.

CDK-1/cyclin B regulates ectopic centrosome activation but not timely centrosome elimination and centrosome separation upon LIN-41 depletion

The CDK-1/cyclin B complex, which is the MPF, is required for both mitotic and meiotic spindle formation with or without centrosomes (McCarter et al., 1999; Burrows et al., 2006; Kim et al., 2010). Because a recent study showed that the phenotype in the female germline of the lin-41-null mutant was rescued by inactivation of chromosome pairs were pulled together toward one of the two centrosomes is unclear, these results indicate that, in lin-41(RNAi) gonads, ectopically nucleating microtubules at centrosomes affect the behavior of meiotic chromosomes after the late pachytene stage. It is possible that the physical association of homologous chromosomes via the remaining synaptonemal complexes perturbs proper chromosome segregation.

In the severe lin-41(RNAi) condition or on the lin-41-mutant background, condensed chromosomes without HIM-3 signal were segregated equally by ectopic spindles in the proximal gonads, as previously reported (Spike et al., 2014a). We assume that such GCN were probably in a somatically differentiated state, because they were found mainly in teratoma-like small oocytes.

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the gonads of cyb-1(gk35);lin-41(RNAi) (Figure 7, A and D, and Supplemental Figure S5, A–D). As a consequence, the HIM-3 diminution seen in lin-41(RNAi) gonads was not observed in the gonads of cyb-1(gk35);lin-41(RNAi) (Figure 6B and Supplemental Figure S5B).

cdk-1 (Spike et al., 2014a), we next focused on centrosome behavior upon LIN-41 depletion in the cyb-1/cdk-1 mutant background. As previously reported, M phase–like events, including NEBD, chromosome condensation, and centrosome activation, were suppressed in the gonads of cyb-1(gk35);lin-41(RNAi) (Figure 7, A and D, and Supplemental Figure S5, A–D). As a consequence, the HIM-3 diminution seen in lin-41(RNAi) gonads was not observed in the gonads of cyb-1(gk35);lin-41(RNAi) (Figure 6B and Supplemental Figure S5B).
To examine whether LIN-41 affects CDK-1 activity during oogenesis, we stained LIN-41–depleted gonads with phospho–CDK-1 (pCDK-1) antibody, which specifically detects the inactive form of CDK-1 (Figure 7E and Supplemental Figure 5S; D’Angiolella et al., 2007; Kim et al., 2010). In wild-type gonads, pCDK-1 accumulated in the GCN from late pachytene to proximal oocytes (25 of 25). Although pCDK-1 initially accumulated in the GCN at late pachytene in LIN-41–depleted gonads, the signal disappeared around the loop region where condensed chromosomes were detected (Supplemental Figure 5S; 23 of 38 gonads). Of interest, centrosome separation was already recognizable in nuclei that contained pCDK-1 (Figure 7E; 14 of 21 gonads), suggesting that CDK-1 activation is dispensable for the centrosome separation found in lin-41(RNAi) gonads. We also found that pCDK-1 was absent from the nuclei that showed highly condensed chromosomes and microtubule asters (Figure 7E and Supplemental Figure 5S; 33 of 33 GCN).

Of importance, we found that the delay in centrosome elimination and centrosome separation were not rescued in cyb-1(gk35);lin-41(RNAi) animals (Figure 7, A–C; centrioles detected, 65, 54, and 39%, respectively, at –4, –3, and –2 lin-41(RNAi) oocytes vs. 50, 46, and 23% at –4, –3, and –2 cyb-1(gk35);lin-41(RNAi) oocytes; centriole separation, 88% in lin-41(RNAi) gonads vs. 81% in cyb-1(gk35);lin-41(RNAi) gonads). The remaining centrioles were found in diakinetic oocytes in the proximal region of cyb-1(gk35);lin-41(RNAi) gonads (Figure 7A). The same was also true when we tested lin-41(RNAi) in the cdk-1(ne2257) loss-of-function mutant background (Supplemental Figure 5S; 29 gonads). Taken together, these results suggest that whereas precocious M-phase events depend on CDK-1 activity, the delay in centrosome elimination and centrosome separation occurs independently of CDK-1 activity in lin-41(RNAi) gonads (Figure 7F).

**DISCUSSION**

We sought to identify critical genes that control in the behavior of centrosomes in oogenesis by using RNAi-mediated reduction of the maternal expression of essential genes in *C. elegans*. Although the RNAi screening failed to show retention of maternally contributed centrosomes to the zygotes as a phenotype, we found that inactivation of some essential genes resulted in a significant delay in centrosome elimination during *C. elegans* oogenesis. Given that, in almost all cases, centrosomes eventually disappeared in the most proximal (–1) oocyte, it might be that meiotic maturation in the –1 oocyte somehow involves a surveillance system for centrosome elimination. Alternatively, it is also possible that multiple factors redundantly promote centrosome elimination, which would make it difficult to completely block centrosome elimination solely by inactivation of a single gene. It has been reported that the XX karyotype of germ cells is critical to the timely removal of centrosomes during oogenesis (Mikeladze-Dvali et al., 2012). This raises the possibility that the transcription of genes on the X chromosomes in late pachytene and early diplotene may be implicated in centrosome elimination. Alternatively, it is also possible that multiple factors redundantly promote centrosome elimination, which would make it difficult to completely block centrosome elimination solely by inactivation of a single gene. It has been reported that the XX karyotype of germ cells is critical to the timely removal of centrosomes during oogenesis (Mikeladze-Dvali et al., 2012). This raises the possibility that the transcription of genes on the X chromosomes in late pachytene and early diplotene may be implicated in centrosome elimination. It will be interesting to examine the influence of inactivation of a single or combination of X chromosome genes on centrosome elimination.

In most metazoans, γ-tubulin is critical for microtubule nucleation at centrosomes and regulation of microtubule organization. In *C. elegans* germ cells, γ-tubulin proteins localize not only to centrosomes but also at the plasma membrane in the syncytial gonad (Bobiniec et al., 2000; Zhou et al., 2009). Our screening identified several regulators of the unique subcellular localization of γ-tubulin in the germ cells of *C. elegans*. RNAi-mediated depletion of ARF-1.2 or NMT-1 led to the ectopic accumulation of γ-tubulin foci in the oocyte cytoplasm. By contrast, depletion of CCTs or C23G10.8 accelerated the elimination of γ-tubulin foci at centrosomes but not that of other PCM and centriole components. Although the exact function of C23G10.8 in the regulation of γ-tubulin in germ cells remains to be elucidated, it is possible that cytoplasmic chaperonin is required for the folding of γ-tubulin proteins, as reported in in vitro systems (Melki et al., 1993). Thus the behavior of γ-tubulin proteins at centrosomes seems to differ from that of other PCM and centriole components.

LIN-41 functions as a conserved heterochronic gene during the differentiation and development of somatic cells (Reinhart et al., 2000; Slack et al., 2000; Zou et al., 2013). Recent studies showed the novel function of LIN-41 in the female germline of *C. elegans* (Spike et al., 2014a; Tocchini et al., 2014). Using loss-of-function mutants of lin-41, Tocchini et al. (2014) showed that LIN-41 regulates the germ-cell soma transition and normally suppresses precocious somatic gene expression in developing oocytes. In line with this, LIN-41 is expressed substantially in the cytoplasm of female germ cells from late pachytene to proximal oocytes. It is also likely that the function of LIN-41 as an E3 ubiquitin ligase is dispensable for the regulation of gene expression in the gonad. Tocchini et al. (2014) reported that RNAi-mediated depletion of LIN-29, which is believed to be epistatic to lin-41 in the heterochronic pathway, did not suppress the defects in the female germline of the lin-41 mutant. Thus it is likely that LIN-41 functions through distinct targets and/or mechanisms in germ and somatic cells.

Spike et al. (2014a,b) showed that LIN-41 has an opposing function against OMA-1 to regulate the prophase-to-M-phase meiotic transition in female germ cells of *C. elegans*. Precocious meiotic M-phase entry through the CDK-1 pathway was observed in LIN-41–compromised developing oocytes. They claimed that the ectopic somatic gene expression detected in the lin-41 loss-of-function mutant might result from a defect in meiotic progression. Using cyb-1 loss-of-function mutant, we confirmed that the similar phenotypes of precocious M-phase entry in LIN-41–compromised oocytes depend on ectopic CDK-1/CYB-1 activation. In addition, we found direct evidence of CDK-1 activation in oocytes, using a pCDK-1 antibody that specifically detects inactive CDK-1.

Of importance, we found that the significant delay in centrosome elimination in lin-41(RNAi) gonads appears to be largely independent of the CDK-1/cyclin B pathway and PCM assembly. This suggests that LIN-41 somehow plays a role in promoting centrosome elimination in developing oocytes through unknown function(s). To identify the time of centrosome elimination in oocytes, we carefully optimized the mild lin-41(RNAi) condition to not affect meiosis progression and oocyte maturation. Spike et al. (2014a,b) suggested that centrosomes need to undergo the diplotene stage for their elimination, but in our mild lin-41(RNAi) condition, a significant delay in centrosome elimination was observed in mature diakinetic oocytes after passage through the diplotene stage. However, we cannot exclude the possibility that shortening the diplotene stage or defects in oocyte growth might have indirectly delayed centrosome elimination in lin-41(RNAi) germ cells. Based on these results, it is tempting to speculate that LIN-41 regulates, at least in part, the gene expression required for timely centrosome elimination around the diplotene stage in female germ cells. This may represent a conserved system for the process in most metazoans.

**MATERIALS AND METHODS**

Nematode strains

Wild-type (N2), spc-5(or213) (Tsai and Ahringer, 2007), lin-41(ma104) (Slack et al., 2000), lin-41(n2914) (Slack et al., 2000), let-7(mg279)
RNA interference and screening procedure
RNAI-mediated inactivation was done essentially as described (Delattre et al., 2006), using feeding strains from the Ahringer library (Kamath et al., 2003). We targeted available 513 genes out of 554 sterile genes known to be essential for proper embryo production and maintenance of gonad morphology (Green et al., 2011). In the first screen, at least 10 L4 GFP–γ-tubulin animals were placed on each feeding RNAI plate at 25°C for 24 h. Then, using fluorescence microscopy, the time of disappearance of GFP–γ-tubulin foci as an indicator of centrosomes in the gonad was analyzed. Each gonad morphology in general was roughly described using reference from Green’s phenotypic categories. As a lethality assessment, the percentage of embryos that fail to hatch was calculated from at least 50 embryos for each RNAI. The candidate genes that showed defects in GFP–γ-tubulin behavior in the gonad were subsequently assessed in the second screening (RME-2 staining for testing oocyte maturation) and third screening (SPD-5 and SAS-4 staining for testing PCM and centriole integrity). For the 4 candidate genes (Figure 1B), the same experiments were performed three times. We also confirmed the vectors encoding the candidate genes for feeding RNAI by DNA sequencing. For lin-41(RNAI), inactivation was achieved by placing L4 larvae at 25°C for 12–24 h. For details of the screening, see Supplemental Table S1.

Antibodies
The following primary antibodies were used in this study: rabbit polyclonal antibodies against SAS-4 (1:1000; Santa Cruz Biotechnology, Dallas, TX), SPD-5 (1:2000; a gift from Bruce Bowerman, University of Oregon, Eugene, OR), RME-2 (1:200; a gift from Barth Grant, Rutgers University, Piscataway, NJ), CDK-1 phosphorylated-Tyr15 (1:100; Merck, Darmstadt, Germany), HIM-3 (1:500; Novus, Littleton, CO), TAC-1 (1:1000; Bellanger and Gonczy, 2003), α-tubulin (1:500; ab18251; Abcam, Cambridge, UK); and mouse monoclonal antibodies against mAB414 (1:1000; Abcam), IFA (1:50; American Type Culture Collection, Manassas, VA), GFP (1:50; a gift from Vierstra Simians, EPFL, Lausanne, Switzerland), and α-tubulin (1:3000, DM1α; Sigma-Aldrich, St. Louis, MO). We used the secondary antibodies Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG; H+L) and Alexa Fluor 568 goat anti-rabbit IgG (H+L), both at 1:500 (Thermo Fisher Scientific, Waltham, MA).

Indirect immunofluorescence
Hermaphrodite gonads were fixed and stained essentially as described (Leidel et al., 2005). Briefly, gonads were dissected in 20 μM levamisole in phosphate-buffered saline (PBS) on polylysine-coated slides and then an equal amount of 4% paraformaldehyde was added for 2 min. Slides were transferred onto the frozen metal plate and then fixed in ice-cold methanol for 10 s. Slides were washed with PBS for 10 min and PBS/0.05% Tween 20 (PBST) for 10 min. Then the slides were incubated for blocking in 2% bovine serum albumin for 30 min before incubation with primary antibodies overnight at 4°C. The slides were washed with PBST for 10 min three times and incubated with secondary antibodies for 1 h at room temperature. The slides were washed with PBST for 10 min three times and counterstained with 1 μg/ml Hoechst 33258 (Sigma-Aldrich) to reveal DNA for 5 min. Immunofluorescence experiments were repeated at least three times unless otherwise specified.

Microscopy
For the microscopic analysis, worms were placed in a drop of M9 buffer containing 1 mM levamisole on 2% agar pad and covered with a coverslip (Kimura and Kimura, 2012). Fluorescence images were obtained on a Zeiss Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany) with a 100×/1.4 numerical aperture plan-Apochromat objective. Indirect immunofluorescence was imaged on a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Optical sections were acquired every 0.3 μm. Images were processed using ImageJ (National Institutes of Health, Bethesda, MD) and Photoshop (Adobe Systems, San Jose, CA), preserving relative image intensities within a series.

ACKNOWLEDGMENTS
We are grateful to Bruce Bowerman for SPD-5 antibodies, Barth Grant for RME-2 antibodies, the Caenorhabditis Genetic Center (University of Minnesota, Saint Paul, MN) for strains, Hitoshi Sawa, Ritsuko Arai, and Akatsuki Kimura for technical advices on the experiments, and Tamara Mikeladze-Dvali and Akshari Gupta for discussion and critical reading of the manuscript. We thank Pierre Gönczy for antibodies, fruitful discussion, and critical reading of the manuscript. This work was supported by a Grant-in-Aid for Young Scientists (A) and for Scientific Research on Innovative Areas from the Ministry of Education, Science, Sports and Culture of Japan, by an Improvement of Research Environment for Young Researchers Grant from the Japan Science and Technology Agency, by the Takeda Science Foundation, and by the NAITO foundation.

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(Reinhart et al., 2000), lin-29(n482) (Newman et al., 2000), cyb-1(gk35) (CGC VC50), cdk-1(ne2257) (Shirayama et al., 2006), mpk-1(ga111) (Lee et al., 2007), and transgenic animals expressing GFP–γ-tubulin (TBS-G1-GFP; Hannak et al., 2001) were maintained according to standard procedures (Brenner, 1974).
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