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Report

MEL-28 Is Downstream of the Ran Cycle and Is Required for Nuclear-Envelope Function and Chromatin Maintenance

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Summary

Early embryonic development depends on the faithful execution of basic cell biological processes whose coordination remains largely unknown. With a global network analysis, we found MEL-28 to be associated with two types of complexes, one implicated in nuclear-envelope function and the other in chromatin organization [1]. Here, we show that MEL-28, a protein that shuttles between the nucleus and the kinetochore during the cell cycle, is required for the structural and functional integrity of the nuclear envelope. In addition, *mel-28(RNAi)* embryos exhibit defects in chromosome condensation, pronuclear migration, kinetochore assembly, and spindle assembly. This combination of *mel-28(RNAi)* phenotypes resemble those caused by depleting members of the Ran cycle in *C. elegans* [2], a conserved cellular signaling pathway that is required for mitotic spindle assembly, nuclear-envelope reformation after mitosis, and nucleocytoplasmic exchange (reviewed in [3–8]). Although MEL-28 localization to the nuclear periphery is not dependent on nuclear pore components, it is dependent on RAN-1 and other key components of the Ran cycle. Thus, MEL-28 is downstream of the Ran cycle and is required for both proper nuclear-envelope function and chromatin maintenance.

Results and Discussion

MEL-28 Has a Dynamic Localization Pattern during the Cell Cycle

A recent network-based analysis of *C. elegans* embryogenesis showed that the molecules driving early embryogenesis are organized in distinct groups or "modules" [1]. Although most proteins in this analysis could be classified within a single module, C38D4.3, which corresponds to genetic locus *mel-28* [9], could be grouped in at least two modules: one that shows functional connections to proteins involved in chromatin maintenance and a second module containing genes required for nucleocytoplasmic exchange. We thus decided to investigate whether MEL-28 contributes to both nuclear-envelope function and chromatin maintenance.

MEL-28 is a large (1784 amino acid) protein with two predicted adenine-thymine (AT) hooks (thought to bind AT-rich DNA) near the C terminus. RT-PCR using primers from the predicted start and stop of the protein confirmed the same open reading frame annotated in WormBase 150. We generated polyclonal antibodies against the MEL-28 protein and a transgenic line (strain PF402) that stably expresses a GFP::MEL-28 translational fusion (Supplemental Experimental Procedures in the Supplemental Data available online). Visualization of GFP::MEL-28 in living embryos and immunofluorescence labeling show the same localization patterns (Movie S1, Figure 1), which are abolished in *mel-28(RNAi)* animals (data not shown). MEL-28 shuttles dynamically between the nuclear periphery and the kinetochore during the cell cycles of early embryogenesis. During meiosis, MEL-28 is present on the nuclear periphery in the oocyte, on the condensing oocyte chromosomes (Figure S1), and on meiotic chromosomes (polar bodies, Figure 1) in the fertilized embryo. During interphase of the first mitotic cycle, MEL-28 is present at the nuclear periphery and as puncta within the nucleus. On the nuclear periphery, MEL-28 localization overlaps with the interior rim of nuclear-envelope marker mAB414 (which in *C. elegans* recognizes primarily nucleoporins NPP-9 [Nup358] and NPP-10 [Nup98/96] [10]). As the pronuclei meet, each nucleus shows a dynamic reorganization of the internal puncta (Movie S1). By metaphase, the scattered dots resolve into pairs of stripes on either side of the metaphase plate, a pattern indicative of holocentric kinetochore localization (Figure 1). The shuttling of MEL-28 between the nuclear envelope and the kinetochore is consistent with the idea that MEL-28 has roles in both structures.

MEL-28 Is Required for Nuclear-Envelope Structure and Function

In addition to its localization to the nuclear envelope, previous large-scale RNAi-based screens suggested that MEL-28 has a role in nuclear-envelope assembly ([1, 11, 12]; Movie S1). To directly test the role of MEL-28 in nuclear-envelope function, we used RNAi to deplete MEL-28 in strains expressing either GFP::tubulin [13] or PIE-1::GFP [14]. Normally, GFP::tubulin is excluded from nuclei, whereas PIE-1::GFP is enriched in the nucleus in the P1 cell (Movies S3 and S5, Figure 2). Strikingly, in *mel-28(RNAi)* embryos, GFP::tubulin is no longer excluded from pronuclei (n = 17/18, Movie S4, Figure 2) and PIE-1::GFP is not enriched in the P1 nucleus (n = 5/9, Movie S6, Figure 2). The simplest explanation to account for these observations is that *mel-28(RNAi)* nuclear envelopes are not capable of creating a barrier that effectively separates nuclear and cytoplasmic material.

Because the defects observed when GFP::tubulin and PIE-1::GFP are visualized in *mel-28(RNAi)* embryos are similar to those seen upon depletion of NPP-1 (the *C. elegans* homolog of mammalian nucleoporin Nup54 [10, 15]), we hypothesized that *mel-28(RNAi)* may directly affect the formation of the nuclear pore. To test this idea, we asked how depletion of MEL-28 affects localization of the mAB414 antigens. In wild-type embryos, the

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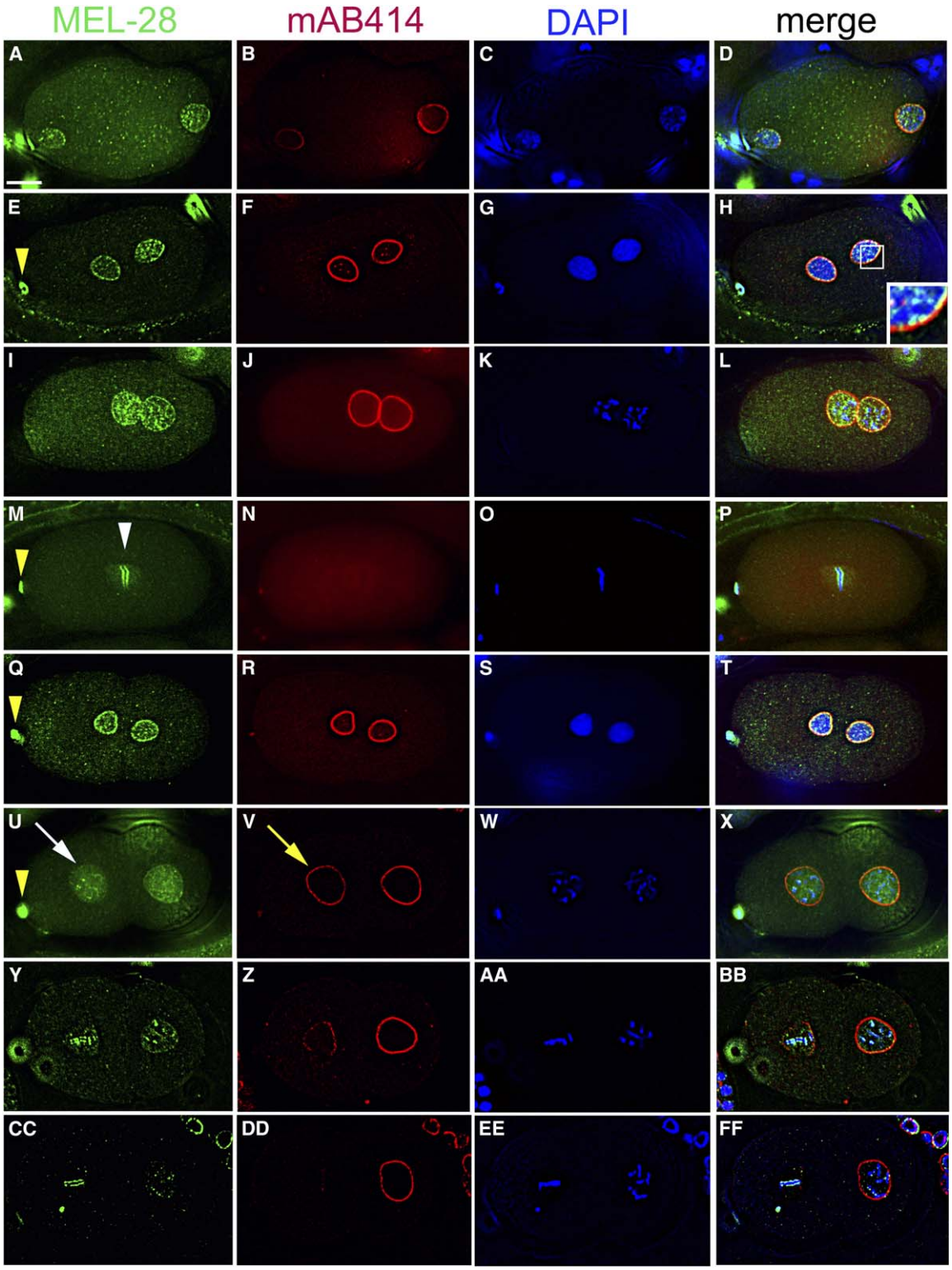

Figure 1. MEL-28 Shuttles between the Nuclear Periphery and the Kinetochore

Wild-type embryos were stained with anti-MEL-28 antibodies (first column, green), monoclonal anti-nucleoporin antibody mAB414 (second column, red), and DNA (DAPI, third column, blue). Merged images are shown in the fourth column. Before the onset of mitosis (A–L), MEL-28 is present at the nuclear periphery and within the nucleus. On the nuclear periphery, MEL-28 overlaps with the interior border of the mAB414 staining ([H], inset). At metaphase (M–P), MEL-28 decorates two stripes on either side of the metaphase chromosomes, coincident with the holocentric kinetochore ([M], white arrowhead). By early anaphase (Q–T), nuclei have already reconstituted their mAB414 and MEL-28 nuclear periphery localization. As the AB cell prepares to divide (U–FF), MEL-28 diminishes from the nuclear envelope and becomes enriched on the chromatin ([U], white arrow), although the mAB414 nuclear-envelope staining is still intact ([V], yellow arrow). Polar bodies (yellow arrowheads) mark anterior. The scale bar represents 10 μM.

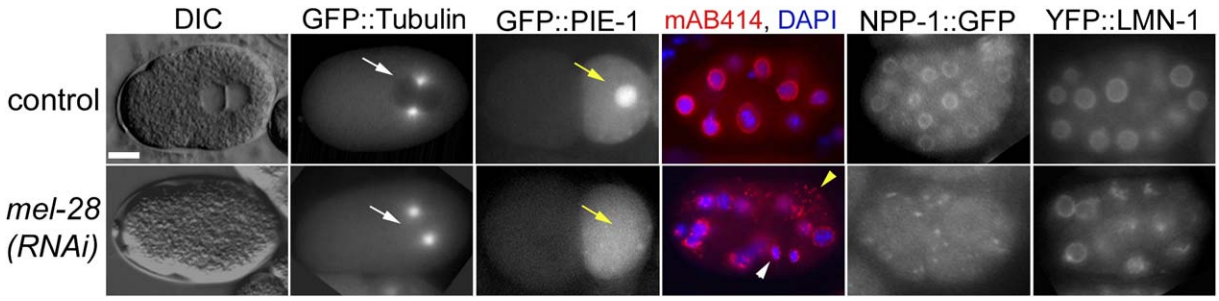

Figure 2. MEL-28 Is Required for Nuclear-Envelope Function and Proper Nuclear Pore and Nuclear Lamina Structure

Wild-type embryos (top row) were compared to *mel-28(RNAi)* embryos (lower row). In *mel-28(RNAi)* embryos, nuclei are indistinguishable from cytoplasm (DIC) and fail to exclude GFP::tubulin (white arrows) or become enriched for GFP::PIE-1 in the P1 nucleus (yellow arrows). *mel-28* (*RNAi*) embryos show aberrant nuclear pores (mAB414, DAPI, and NPP-1::GFP columns) and nuclear lamina (YFP::LMN-1). In *mel-28* (*RNAi*) embryos, nuclear-pore components either form aberrant puncta surrounding chromatin (white arrowhead) or form aggregates not associated with chromatin (yellow arrowhead). The scale bar represents 10 μM.

mAB414 nucleoporin antigens are distributed evenly around each nucleus, but in *mel-28* RNAi-treated animals, they form aberrant aggregates (Figure 2). We observed a similar aggregation phenotype when we depleted MEL-28 in an NPP-1::GFP line (Figure 2, Movies S7 and S8).

We then asked whether MEL-28 had a broader role in nuclear-envelope organization by examining the nuclear lamina in *mel-28(RNAi)* embryos. We found that YFP::LAMIN-1 [10] also localizes aberrantly in *mel-28(RNAi)* embryos, forming aggregates (Figure 2, Movies S9 and S10). In contrast, depleting nucleoporins NPP-1, NPP-3, NPP-12, or NPP-13 does not lead to detectable LMN-1 defects [10, 15, 16]. These observations suggest that MEL-28 is not only required for correct nuclear pore assembly but plays a more extensive role in organizing the nuclear envelope.

MEL-28 Is Required for Proper Chromatin Morphology and Positioning

To study the role of MEL-28 in chromatin maintenance, we RNAi-depleted MEL-28 in embryos carrying a GFP::Histone2B fusion and in embryos carrying both GFP::Histone2B and GFP::tubulin markers. In untreated embryos, pronuclei are 9.9 μM (± 0.7 μM) in diameter, and the GFP signal is diffuse throughout most of each pronucleus, with bright puncta appearing during late prophase (Figure 3A). In *mel-28(RNAi)* embryos, however, pronuclei are much smaller (4 ± 1 μM) (Figure 3A), and the maternal pronucleus typically appears uniformly hypercondensed whereas the paternal pronucleus is uniformly diffuse.

Paternal and maternal chromatin masses are not positioned correctly in *mel-28(RNAi)* embryos. In the wild-type, the maternal pronucleus migrates toward the paternal pronucleus, which is closely associated with the two centrosomes (Movie S11, Figure 3A). As the paternal and maternal pronuclei meet in the posterior, the centrosomes are positioned at the junction between the two pronuclei. The centrosomes rotate 90 degrees and the chromatin condenses, resulting in the localization of all pronuclear DNA between the two asters (Figures 3A and 3B) at metaphase. In contrast, in time-lapse analyses of *mel-28(RNAi)* embryos, we observed that the maternal and paternal pronuclei usually (n = 19/22) did not meet (Movie S12). Consequently, maternal and paternal chromatin remained separate and each often associated with just one of the asters (Figure 3B).

To ask whether nuclear-envelope problems alone could cause the chromatin defects we observed, we RNAi-depleted NPP-1, a protein required for integrity of the nuclear envelope in *C. elegans* [15], in embryos carrying GFP::Histone2B and GFP::tubulin (Movie S13, Figure 3A). Although these embryos often have chromatin bridges during the first cell division, they do not show the early chromatin morphology and positioning defects exhibited in *mel-28(RNAi)* embryos.

These observations indicate that MEL-28 is required for both proper chromatin morphology and correct positioning of the pronuclei during the first cell division. The chromatin defects we observe in *mel-28(RNAi)* strongly resemble chromatin morphology and positioning phenotypes observed upon depletion of Ran-cycle components in *C. elegans* [2].

MEL-28 Interacts with the Kinetochore Assembly Pathway

Localization studies showed that MEL-28 is present at the kinetochore early during its formation, leading us to explore the role of MEL-28 in kinetochore assembly. Formation of the kinetochore requires the histone H3 variant CENP-A, which is encoded by *hcp-3* in *C. elegans* [17, 18]. We examined whether MEL-28 localization to the kinetochore is dependent on HCP-3 by using immunostaining and recordings of GFP::MEL-28 dynamics in *hcp-3(RNAi)* embryos. Immunolocalization of MEL-28 showed that the association of MEL-28 with the nuclear periphery was not affected by HCP-3 depletion (data not shown), but analysis of MEL-28 at the kinetochore was complicated by the aberrant metaphase chromatin morphology in these embryos. Analysis of GFP::MEL-28 dynamics showed that it localized to the nucleus during interphase as in wild-type embryos but did not relocalize to chromatin during mitotis, and instead disappeared (Figure S2; compare Movie S14 to the untreated embryo in Movie S1). To confirm that MEL-28 localization to metaphase chromatin was dependent on HCP-3, we used a temperature-sensitive metaphase-arrest mutant *emb-30(tn377)* [19]. At the restrictive temperature, embryos from *emb-30(tn377)* homozygous mothers arrested with condensed chromosomes that are decorated by MEL-28 antibody (Figure 3C).

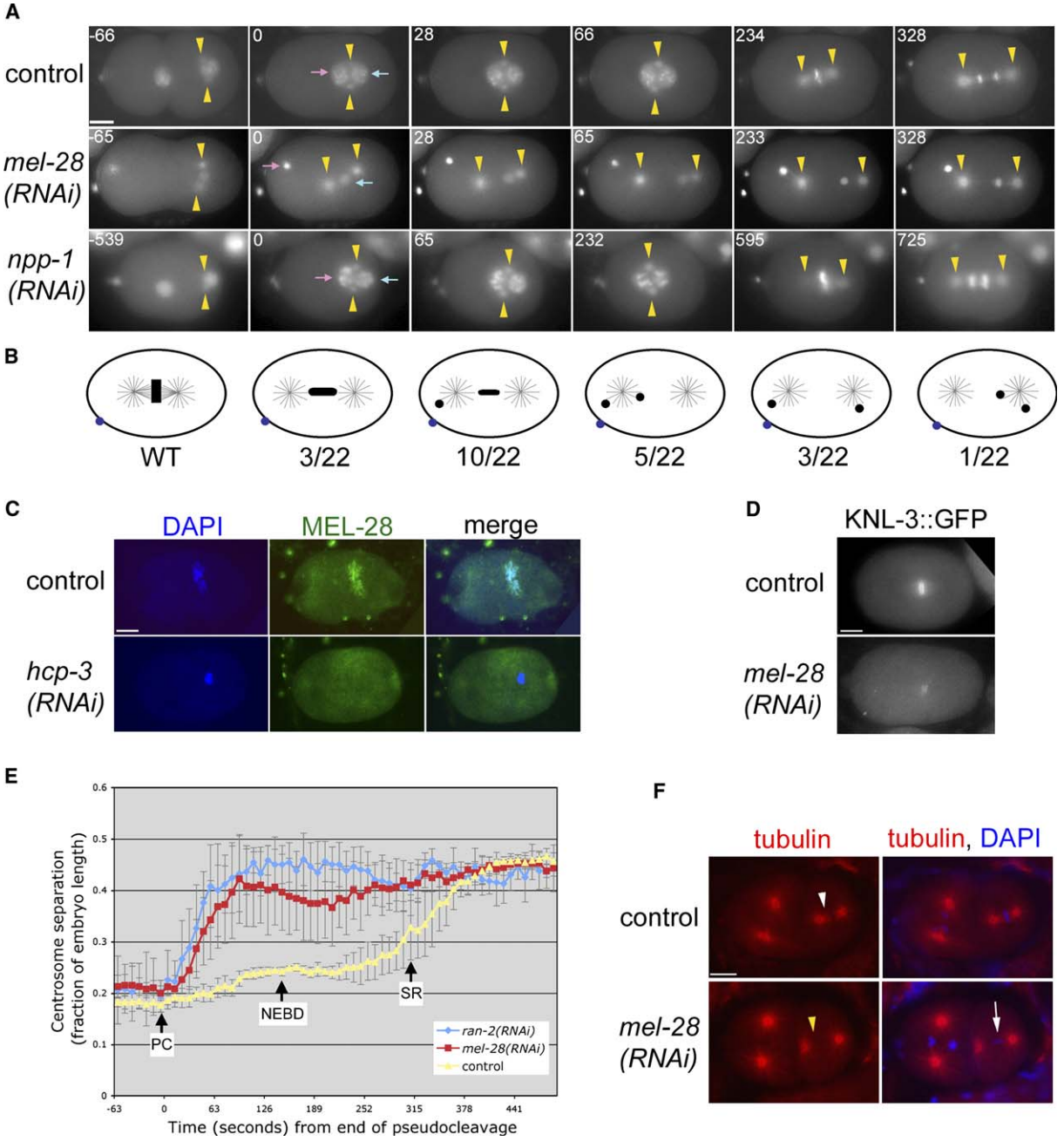

Figure 3. *mel-28(RNAi)* Embryos Show Defects in Chromatin Morphology, Pronuclear Meeting, Centrosome Separation, Kinetochore Assembly, and Spindle Assembly

(A) Dynamics of centrosome and chromosome movement during the first mitosis in wild-type one-celled embryos, *mel-28(RNAi)* embryos, and *npp-1(RNAi)* embryos visualized by GFP::tubulin (yellow arrowheads) and GFP::Histone (pink arrow and blue arrow, maternal and paternal pronuclei, respectively). Numbers at the upper-left corner of each panel indicate time (s) from the end of pseudocleavage. In *mel-28(RNAi)* embryos, chromatin is hypercondensed and not positioned properly, and the centrosomes separate prematurely. *npp-1*(RNAi) embryos are smaller and slower than the wild-type, but show normal pronuclear meeting, centrosome segregation, and chromatin condensation. [The anterior centrosome is out-of-focus in the metaphase frame of the *npp-1(RNAi)* embryo.] For *mel-28(RNAi)* and wild-type embryos, frames representing equivalent time points are shown. Because *npp-1(RNAi)* embryos are slower than the wild-type, equivalent stages to those of the wild-type were chosen on the basis of chromatin morphology.

(B) Summary of metaphase figures from 22 time-lapse recordings. The maternal and paternal pronuclei often (19/22 cases) failed to meet in *mel-28(RNAi)* embryos. In the three cases where they did meet, they met in abnormal location more centrally in the embryo, and their combined chromatin was pulled apart unevenly, resulting in chromatin bridges. When pronuclei failed to meet, usually this was because the maternal pronucleus did not migrate toward the posterior (18/19 cases). The paternal chromatin then either became sheared as a result of chromatin bridging between the two asters (ten cases) or was associated exclusively with the anterior (five cases) or posterior (three cases) aster.

(C) MEL-28 localization to the kinetochore requires HCP-3. *emb-30(tn377)* animals were placed at 25°C to arrest them at metaphase and RNAi-depleted of *hcp-3* (lower row) or left untreated (upper row). MEL-28 decorates metaphase chromatin in arrested *emb-30(tn377)* embryos, but not if HCP-3 has been RNAi-depleted.

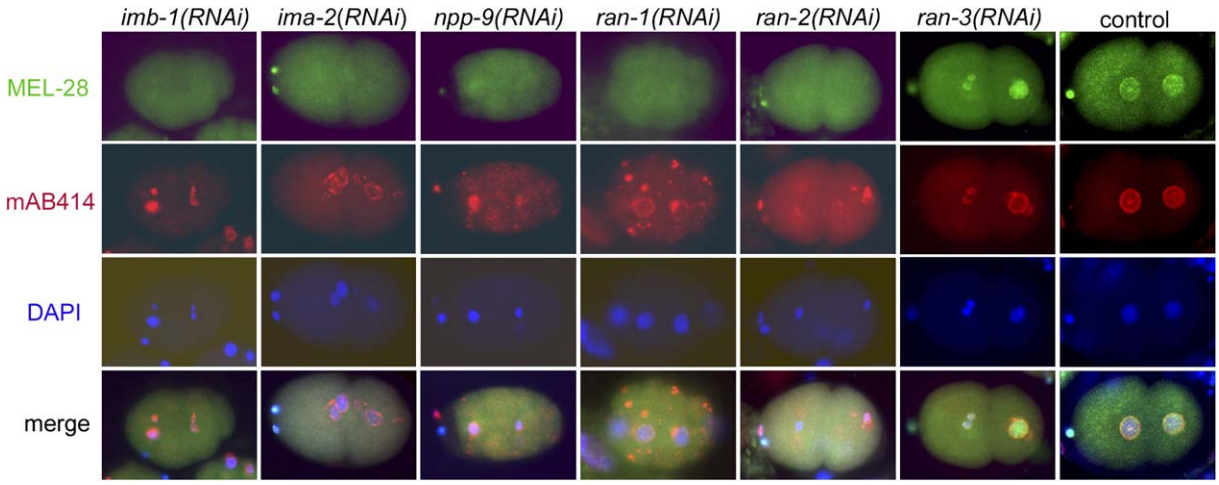

Figure 4. Components of the Ran Cycle Affect Localization of MEL-28

Control embryos and embryos RNAi-depleted of *imb-1* (importin β), *ima-2* (importin α), *npp-9* (RanBP2), *ran-1* (Ran), *ran-2* (RanGAP), and *ran-3* (RCC-1) were stained for MEL-28 (green), the nuclear envelope (mAB414, red), and DNA (DAPI, blue). All treatments except *ran-3(RNAi)* disrupted MEL-28 localization to the chromatin and the nuclear envelope. mAB414 staining shows aberrant nucleoporin aggregates in these embryos. Merged images are shown in the fourth row. The scale bar represents 10 μM.

However, in *emb-30(tn377); hcp-3(RNAi)* embryos, MEL-28 no longer localized to the chromosomes. Thus, HCP-3 is required for the localization of MEL-28 to the kinetochore but not to the nuclear envelope.

We then asked whether localization of kinetochore components was dependent upon MEL-28. In fixed embryos, immunolocalization studies using antibodies against CENP-A and CENP-C were inconclusive, suggesting that we needed to follow kinetochore assembly in live embryos. KNL-3 localizes to the kinetochore early and is required for its assembly ([20]; Movie S15). KNL-3::GFP localization at the kinetochore was reduced, although incompletely, in *mel-28(RNAi)* embryos. In time-lapse recordings of *mel-28(RNAi)* embryos, KNL-3::GFP was not visible until after the end of pseudocleavage, a delay of over a minute compared with wild-type embryos, and was weaker and more diffuse than in the wild-type (Figure 3D, Movie S16).

MEL-28 Is Required for Proper Centrosome Migration and Spindle Formation

The improper positioning of chromatin we observed in *mel-28(RNAi)* embryos could be explained in part by spindle or centrosome malfunction. To examine how MEL-28 affects spindle and centrosome behavior, we depleted *mel-28* from lines expressing a GFP::tubulin fusion in the early embryo [2, 13]. We observed that centrosomes separate prematurely in *mel-28* RNAi-treated embryos (Figure 3A, compare yellow arrowheads in the middle row to the upper-most row).

Precocious centrosome separation defects have also been described in *C. elegans* for essential kinetochore components *hcp-3*, *hcp-4*, *knl-3*, or *knl-1* and for the Ran cycle members *ran-1* or *ran-2*, although with different kinetics [2, 18, 20, 21]. To explore the kinetics of centrosome separation, we measured the distance between centrosomes at 9 s intervals during early embryogenesis in untreated and *mel-28(RNAi)* embryos. We found that centrosome separation in *mel-28(RNAi)* embryos initiates earlier and proceeds more rapidly than in control embryos, beginning at the end of pseudocleavage and reaching peak separation about 1.5 min later (Figure 3E). The kinetics of this separation differ from those observed upon kinetochore disruption [18, 20, 21] but are indistinguishable from those observed upon depletion of Ran or RanGAP (*ran-2* in Figure 3F, [2]). To determine whether a spindle malfunction could be contributing to the precocious centrosome separation, we localized tubulin in *mel-28(RNAi)* embryos (Figure 3F, Figure S3, and Movies S3 and S4) and found that although MEL-28-depleted embryos contain asters that appear wild-type, they fail to concentrate spindle microtubules. We also observed chromatin bridges (Figure 3F), which can indicate a failure of proper interactions between the chromatin and microtubules, as when the kinetochore [18, 20, 21] or spindle checkpoint [22] are impaired. Importantly, neither the precocious

(D) KNL-3 requires MEL-28 to accumulate at the kinetochore. KNL-3::GFP fusion embryos were RNAi-depleted of MEL-28. KNL-3::GFP does not accumulate to wild-type levels in *mel-28(RNAi)* embryos. Early anaphase for the control (upper) and *mel-28(RNAi)* (lower) is shown.

(E) Centrosome-centrosome distance was measured in recordings from untreated (yellow triangles), *mel-28(RNAi)* (red squares), and *ran-2(RNAi)* (blue diamonds) GFP::tubulin embryos. Recordings were time-aligned by the completion of pseudocleavage, which is soon after the pronuclei meet in the wild-type. Each measurement was divided by the length of the embryo, and the mean relative centrosome separation was plotted. Completion of pseudocleavage (PC), nuclear-envelope breakdown (NEBD), and spindle rocking (SR) time points are noted on the wild-type curve. Each data point represents average and standard deviation (bars) from five independent experiments.

(F) Wild-type and *mel-28(RNAi)* embryos were stained with tubulin antibodies (red) to visualize the spindle and DAPI to visualize DNA (blue). A 2-cell-stage embryo is shown where the P1 cell is at metaphase. The white arrowhead points to spindles in the wild-type, and the yellow arrowhead shows a lack of spindle formation in *mel-28(RNAi)*. The white arrow shows a chromatin bridge. The scale bar represents 10 μM.

centrosome segregation defect nor the lack of concentration of spindle microtubules is seen when nucleoporins *npp-1*, *npp-3*, *or npp-13* are depleted (Figure 3A, our unpublished observations, [10]), indicating that these defects are not necessarily a consequence of a problem with the nuclear pore.

MEL-28 Localization Is Dependent on the Ran Cycle

We reasoned that genes that give rise to an RNAi-depletion phenotype similar to that of *mel-28* might function in the same pathway, so we tested 28 candidates for their affect of MEL-28 localization. We found that *npp-8* (Nup155) had a variable effect on MEL-28 localization, whereas *ran-1* (Ran), *ran-2* (RanGAP), *npp-9* (Nup358 or RanGAP cofactor RanBP2), *imb-1* (importin β), and *ima-2* (importin α) severely disrupted MEL-28 localization in the early embryo (Table S1). Thus, all major components that we tested of the Ran cycle except *ran-3*, the *C. elegans* ortholog of the RanGEF *RCC-1*, prevented MEL-28 localization to both chromatin and nuclear membranes (Figure 4). None of the other genes we tested hindered MEL-28 localization to the nuclear periphery, and only *hcp-3* (CENP-A) and *hcp-4* (CENP-C) RNAi depletions affected MEL-28 localization at the kinetochore (Table S1).

The Ran cycle is required for nuclear-envelope function, chromosome positioning, and spindle assembly, and there is mounting evidence that at least some of these functions are executed independently of the others [3–8]. MEL-28 is also required for these processes in *C. elegans*. Although nuclear-envelope defects can give rise to chromosome segregation problems such as chromatin bridges (Figure 3A, [23]), the severe and early defects we describe here in chromatin positioning, chromatin organization, and spindle assembly do not arise upon depletion of NPP-1, NPP-3, or NPP-13 (Figure 3A, [10, 15]). Similarly, depleting the essential kinetochore component KNL-3 gives rise to gross chromosome segregation defects but does not affect nuclear-envelope integrity [20]. Thus, although chromatin maintenance and nuclear-envelope function can be genetically separated, MEL-28 and the Ran cycle are required for both. Together, these observations are consistent with the idea that MEL-28 plays roles in nuclear-envelope integrity and chromatin maintenance and does so in a Ran-dependent manner.

Conclusions

By examining integrated functional genomic data, we hypothesized that MEL-28 contributed to both chromatin maintenance and nucleocytoplasmic exchange [1]. We found that this protein is essential for proper nuclear-envelope function and chromatin maintenance during the cell cycle. The *mel-28(RNAi)* phenotype strongly resembles loss-of-function phenotypes of genes in the Ran cycle in *C. elegans* [2, 24], including defects in nuclear-envelope function, spindle formation, pronuclear movement, and centrosome separation kinetics. Consistent with these phenotypes, proper MEL-28 localization is dependent on Ran-cycle components *ran-1*, *ran-2*, *npp-9*, *imb-1*, and *ima-2*, but is generally unaffected by removal of other proteins required for nuclear-envelope function. Taken together, our data indicate that MEL-28 has fundamental roles in both interphase and mitosis and is a likely downstream effector of the Ran pathway in *C elegans*.

Supplemental Data

Supplemental Data include sixteen movies, Experimental Procedures, three figures, and one table and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/17/1757/DC1/.

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