主論文の要旨

EML4 promotes the loading of NUDC to the spindle for mitotic progression

（EML4 は NUDC を細胞分裂期の紡錘体に誘導する）

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<Background>

The proliferation of living cells requires an accurate distribution of duplicated genetic materials to daughter cells. Multiple proteins localized to the mitotic spindle regulate the cell division. Although extensive studies have been performed to elucidate the function of these microtubule-associated proteins, their exact functions remain unclear.

Echinoderm microtubule-associated protein (EMAP) is the most abundant microtubule-associated protein in sea urchin. The EML (EMAP-like) protein family is composed of mammalian homologs of EMAP. Six EML proteins (EML1-6) have been identified, and all the EML proteins have a HELP (hydrophobic echinoderm microtubule-associated protein-like protein) domain, and multiple WD40 domains. Although EML proteins are highly similar in sequence and localize to the microtubules, these proteins have little sequence homology with other microtubule-associated proteins, and their exact functions are largely unknown. In this report, we studied the role of EML4 in mitotic progression. We demonstrated that EML4 is required for the organization of the mitotic spindle and for the proper attachment of spindle microtubules to kinetochores. We also showed that EML4 is essential for the recruitment of NUDC, which is a critical factor for mitotic progression, to the mitotic spindle.

<Materials and methods>

HeLa cells were transfected with siRNAs using Lipofectamine RNAiMAX for 24 h, and then monitored for 48 h using a time-lapse microscope system, or for 72 h to be fixed and immunostained with antibody to discover the phenotype of spindle in metaphase cell. Flag-EML4 and its binding proteins were purified from Hela cell constituted expressing Flag EML4 for mass spectrometry analysis using LC-MS/MS system. The proteins were identified using the Mascot software package.

<Results>

EML4 knockdown delays the progression of cytokinesis

EML4 accumulated at the MTOC during prophase and localized to the mitotic spindle during metaphase. During telophase, EML4 concentrated to the either side of the midbody (Fig. 1A). We tried to characterize the defects induced by EML4 knockdown in mitosis. We used time-lapse microscopy. Control siRNA-transfected cells showed prompt chromosome alignment at the metaphase plate. In contrast, chromosomes were unstable and fractions of chromosomes were dispersed in metaphase of EML4-depleted cells (Fig.1B,C). Meanwhile most of control cells entered anaphase in 50 mins after Nuclear Breakdown. However, EML4 depleted cells took about 100 mins for the initiation of anaphase (Fig.1D). Time duration from NEBD to anaphase of EML4-knockdown cells was

significantly reduced by co-depletion of MAD2 (Fig.1D).

EML4 depletion inhibits spindle organization and kinetochore-microtubule attachment

We immune-stained the Hela cells transfected with control siRNA or EML4 siRNA with anti-tubulin and Hoechst. Most of control cells showed organized bipolar spindle and properly alignment chromosomes in metaphase. In contrast, about 60% EML4 depleted cells showed un-congressed chromosomes and disformed spindles (Fig.2A). EML4 depletion significantly reduced the number of metaphase cells with stable mitotic spindle in the cold treatment (Fig.2B). With monastrol treatment, most kinetochores in control cells attached to the end of microtubules; In contrast, many kinetochores in EML4-depleted cells attached to the side of microtubules (Fig.2C).

HELP domain and adjacent WD40 domains are required for mitotic progression

EML4 has a coiled-coil region and a HELP domain, and multiple WD40 domains. As depicted in Fig. 3A, EML4 deletion mutants were generated, and the cells that constitutively expressed GFP-tagged mutants were established by retroviral infection (Fig. 3B). Neither the N-terminal region that contained the coiled-coil region and HELP domain (aa 1-298) nor the WD40 repeats (aa 299-981) localized to the mitotic spindle (Fig. 3C); Neither of them could rescue the mitotic defect induced by endogenous EML4 knockdown (Fig.3D). However, an EML4 deletion mutant that contained the N-terminal region and adjacent partial WD40 repeats (aa 1-478) clearly localized to the mitotic spindle (Fig. 3C) and clearly reduced the number of cells with disorganized spindles or with uncongressed chromosomes (Fig. 3D). These results indicated that the N-terminal HELP domain and adjacent partial WD40 repeats are required for the localization and functionality of EML4.

EML4 associates with NUDC

By immunostaining, both EML4 and NUDC were localized to the mitotic spindle and concentrated to the both sides of the midbody in telophase (Fig.3E). We confirmed that Flag-NUDC was co-precipitated with GFP-EML4 by using immune-precipitation (Fig.4A). Meanwhile, we also confirmed the binding between endogenous NUDC and endogenous EML4 by immune-precipitation with anti-EML4 antibody. EML4 co-precipitated with NUDC but not IgG (Fig.4B,C).

EML4 is required for the loading of NUDC to the mitotic spindle

We checked which regions of EML4 and NUDC were required for the association. We made delete mutations of EML4 and NUDC. By immune-precipitation analysis, we found that WD40 domains were necessary for the association with NUDC (Fig.5A,B,C). And C-terminal NUDC was necessary for the interaction (Fig.5D,E).

We tried to examine physiological function of EML4 and NUDC. Firstly, we checked the expression of EML4 or NUDC in NUDC depleted cells or EML4 depleted cells. We couldn't find significant changes in control cell and siRNAs transfected cells (Fig.6A,B).

Later, we checked the localization of NUDC in EML4 depleted cells, by immunostaining with anti-NUDC antibody and anti-tubulin antibody. In control cells, we can see NUDC localized at tubulin, spindle, midzone and both sides of midbody. In contrast, NUDC was dis-localized in EML4 depleted cells (Fig.6C,D).

<Discussion>

In this report, we showed that EML4 was required for mitotic spindle organization and for microtubule-kinetochore attachment. The depletion of EML4 by siRNAs induced cells with disorganized mitotic spindles and uncongressed chromosomes during metaphase. Uncongressed chromosomes are often observed when microtubule-kinetochore attachment is disrupted. Consistently, the stable organization of k-fibers decreased, and the side-on attachment of kinetochores to microtubules increased. These results show that EML4 has a crucial role in mitosis by contributing to mitotic spindle organization and to microtubule-kinetochore attachment.

We found that EML4 associating with NUDC is required for the localization of NUDC to the mitotic spindle. NUDC is a highly conserved gene in a wide range of species, including filamentous fungi, plants, invertebrates and vertebrates. NUDC depletion induces multinuclear cells and defects in spindle organization. The phosphorylation of NUDC by PLK1 is essential for the end-on attachment of microtubules and kinetochores. NUDC is also required for the accumulation of PLK1 at the kinetochore to promote chromosome congression. These results clearly show that NUDC is critical for spindle formation and for microtubule-kinetochore attachment. EML4 is required for proper spindle organization and for microtubule-kinetochore attachment; therefore, one of the crucial functions of EML4 in mitosis may be the recruitment of NUDC to the mitotic spindle.

<Conclusion>

Time-lapse microscopy analysis demonstrated that EML4 depletion induced chromosome misalignment during metaphase and delayed anaphase initiation. Further analysis by immunofluorescence showed that EML4 was required for the organization of the mitotic spindle and for the proper attachment of kinetochores to microtubules. We found that the nuclear distribution gene C (NUDC) protein was associated with EML4. This interaction was mediated by the WD40 repeat of EML4 and by the C-terminus of NUDC. In the absence of EML4, NUDC was no longer able to localize to the mitotic spindle, whereas NUDC was dispensable for EML4 localization. Our results show that EML4 is critical for the loading of NUDC onto the mitotic spindle for mitotic progression.

Abbreviations:

EML4, NUDC, cytokinesis, mitosis, spindle, kinetochore.