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Enhancer of rudimentary homolog (ERH) plays an essential role in the progression of mitosis by promoting mitotic chromosome alignment

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ABSTRACT

The enhancer of rudimentary homolog (ERH) is a small eukaryotic protein that is highly conserved in animals, plants, and protists but not in fungi. ERH has several binding proteins and has been associated with various cellular processes, such as pyrimidine metabolism, cell cycle progression, and transcription control; however, little is known about the exact role of this protein and the underlying molecular mechanisms. We found that ERH has a critical role in the mitotic phase of the cell cycle. ERH depleted-cells showed severe chromosome misalignment and weakened kinetochore-microtubule attachment. ERH depletion also caused dissociation of centromere-associated protein E (CENP-E), a mitotic kinesin that is involved in stabilizing the kinetochore-microtubule attachment, from kinetochores of mitotic chromosomes. We propose that ERH contributes to chromosome alignment at the metaphase plate by localizing CENP-E at kinetochore regions.

1. Introduction

Enhancer of rudimentary (ER) is a gene product of the enhancer of rudimentary gene e (*r*), which was originally cloned as a gene that genetically interacts with the rudimentary gene (*r*) in *Drosophila melanogaster* [1]. *r* encodes the first three enzymatic activities of the pyrimidine biosynthetic pathway, and the *r* mutant causes a characteristic truncation of the wings [2]. The mutation *e(r)* enhances the wing truncation phenotype [3].

The ER homolog (ERH) is widely found in eukaryotic cells among vertebrates, invertebrates, and plants, but not in fungi [1,4–6]. ERH is a small protein (approximately 100 amino acids), and its sequence is highly conserved (e.g., ERH proteins from the human, the mouse, and the frog are completely identical, whereas there are no other similar shared proteins known among these species), and it has not been possible to ascribe any possible function to ERH on the basis of sequence analysis alone. ERH is ubiquitously expressed in both adult and fetal tissues and localizes predominantly in the nucleus [7]. It has been reported that ERH has several binding proteins, some of which are involved in transcriptional regulation, cell growth, or cell cycle progression [5,7–11]. Based on these reports, it is suggested that ERH may be involved in various cellular functions; however, accurate and convincing data is required to determine the precise role of this protein.

Among the possible functions of ERH, a role in cell cycle control was suggested based on the following observations: increased levels of ERH transcript were observed in rapidly dividing mammalian cell lines [6]; levels of ERH transcript were higher in cancer tissue than in the normal tissue [12]; ERH was phosphorylated by CK2, a kinase known to be essential in the G1-S and G2-M transitions [6]; ERH interacted with proteins involved in cell cycle control, such as polymerase δ-interacting protein 46 or S6K1 Aly/REF-like target (PDIP46/SKAR) [7] and Ciz1 [9]. However, there is little evidence as to how ERH regulates cell cycle progression. In this study, we found that ERH is required for the metaphase to anaphase progression of mitosis. We also found that ERH promotes chromosome alignment at the metaphase plate through its effect on the localization of CENP-E, a mitotic kinesin that link kinetochore-microtubule attachment.

2. Materials and methods

2.1. Cell culture and treatments

HeLa cells were grown at 37 °C in Dulbecco's modified Eagle's minimal essential medium with 10% fetal bovine serum and penicillin–streptomycin mixed solution (Nacalai Tesque). Mitotic cells were collected by adding nocodazole at the concentration of 330 nM, followed by mitotic shake off. To observe chromosomes

Abbreviations: ERH, enhancer of rudimentary homolog; CENP-E, centromere-associated protein E.

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alignments, metaphase cells were synchronized by adding MG132 at the concentration of 10 μM for 2 h.

2.2. Antibodies

The following antibodies were used: mouse monoclonal antibodies against ERH (ab54690, Abcam), α-Tubulin (DM1A, Millipore), β-Actin (C4, Santa Cruz Biotechnology), Hec1 (9G3, Abcam), CENP-E (1H12, Abcam), and BubR1 (9/BUBR1, BD Transduction Laboratories); rat monoclonal antibody against HA, (3F10, Roche); and human polyclonal antibody against centromere (NA-8184, MBL).

2.3. Plasmids

cDNAs encoding full-length of ERH was amplified by PCR and subcloned into the pcDNA3 plasmid (Invitrogen) containing sequences coding FLAG/HA sequence.

2.4. siRNA

All siRNAs were purchased from Invitrogen. The sequences of the siRNA duplexes were as follows:

ERH-#1, 5′-AGCUGCCUGGUUUACCGAGCUGAUA-3′;

ERH-#2, 5′-UCCCAACAGUCCCUCUAUCACAUAU-3′.

Stealth™ RNAi negative control Medium GC was used as a negative control.

2.5. Cell proliferation assay

The proliferation of cultured cells was measured by MTT using MTT Cell Count Kit (Nacalai Tesque). The kit used following manufacturer's instructions.

2.6. Cell cycle analysis

For cell cycle analysis, cells were detached with trypsin, collected with supernatant, and washed once with PBS. Then the cells were fixed in 75% (v/v) cold methanol for no less than 12 h and washed with PBS. After washing with PBS, the cells were stained by Guava Cell Cycle Reagent (Guava Technologies, Hayward, CA), and were analyzed by using the GUAVA Cell Cycle Analysis Software (Guava Technologies).

2.7. Mitotic index

Forty-eight hours after siRNA transfection, DNA was stained by incubating the cells for 20 min in medium containing 1 μg/ml Hoechst 33342 (Nacalai Tesque). The mitotic index was generated after counting over 500 cells in each siRNA.

2.8. Immunofluorescence

Cells were grown on chamber slides. For immunostaining using anti-α-tubulin, anti-HA or anti-BubR1 antibodies, the cells were rinsed PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4), fixed in 4% paraformaldehyde (PFA) for 10 min, and were permeabilized in Triton buffer (Hepes at pH 7.9, 150 mM NaCl, 0.5% Triton X-100) for 15 min; for immunofluorescence using anti-Hec1 antibodies, cells were first permeabilized with 0.1% Triton X-100/PBS for 3 min and then fixed with 4% PFA for 10 min; for anti-CENP-E antibodies, the cells were rinsed PBS, fixed in 2% PFA for 2 min and methanol for 10 min, and were permeabilized in 0.2% TritonX-100/PBS for 10 min. Fixed cells were blocked with TBS-T buffer (20 mM Tris–HCl at pH 7.5, 150 mM NaCl and 0.05% Tween-20) containing 3% bovine serum albumin (BSA) for 1 h at room temperature. Then the cells were incubated with indicated antibodies for 2 h, stained with AlexaFluor-conjugated secondary antibodies (Invitrogen) for 1 h, counterstained with 4′6-diamidino-2-phenylindole (DAPI) for 10 min and mounted with Vectashield (Vector Laboratories). Images were captured with LSM 700 (Carl Zeiss) or Biorevo (Keyence, Osaka, Japan), and processed with LSM Software ZEN or BZ-II analyzer, respectively.

2.9. Chromosome spreading

For Giemsa staining, mitotic cells were collected by shake-off and swelled in a hypotonic solution containing 1% Sodium Citrate for 20 min at room temperature. Cells were fixed by dropwise addition of the fixative (methanol:acetic acid at 3:1) for 30 min, washed twice with fixative, and dropped onto glass slides. Then the dried slides were stained with Giemsa staining solution.

3. Results

3.1. ERH is essential for chromosome alignment in mitosis

Previous studies have shown that ERH is implicated in the regulation of cell cycle; however, much remains to be determined about the molecular mechanisms underlying this regulation. Therefore, in order to determine the function of ERH, we depleted HeLa cells of ERH using two different siRNAs (Fig. 1A) and tested cell proliferation by MTT assay (Fig. 1B). Compared with control cells, cell proliferation was markedly inhibited by knocking down ERH, showing that ERH is involved in cell proliferation (Fig. 1B). Next, we examined the cell cycle profile of HeLa cells treated with control siRNA or ERH siRNA in order to determine the essential phase of the cell cycle. As shown in Fig. 1C, ERH knockdown increased the 4N population of the cells 48 h after siRNA transfection, suggesting that ERH is required for the progression of G2 phase or mitosis. Then we tested the mitotic index of HeLa cells treated with control or ERH siRNA for 48 h. Mitotic indexes were approximately 2-fold higher in the ERH-depleted cells than in the control cells (Fig. 1D), suggesting that ERH is required for the progression of mitosis or mitotic exit. Moreover, among the mitotic cells, knocking down ERH expression increased the population of cells from prophase to metaphase (Fig. 1E), showing the inhibition of the metaphase to anaphase transition.

The metaphase to anaphase transition is generally caused after every sister kinetochore on a replicated chromosome attaches to microtubules from the opposite spindle poles and every chromosome aligns at the metaphase plate [13]. Failure of this process, which is accompanied by misalignment of chromosomes, inhibits anaphase onset by activating the spindle assembly checkpoint. Because ERH depletion increased prophase to metaphase cell population (Fig. 1E), we tested the alignment of metaphase chromosomes. To test the effects of ERH siRNA on metaphase chromosome alignment, we treated cells with MG132 for 2 h (to accumulate metaphase cells and to prevent premature anaphase onset) (Fig. 2A). As expected, ERH depletion severely compromised chromosome alignment at the metaphase plate (Fig. 2A), which may activate the spindle assembly checkpoint and metaphase arrest. Next, FLAG/HA-ERH was transfected in HeLa cells and the subcellular localization was analyzed. ERH was localized in the nuclei in interphase cells, and in the cytoplasm in mitotic cells (Fig. 2B).

3.2. ERH regulates kinetochore association of CENP-E

There are several possibilities for the alignment defect of metaphase chromosomes in ERH-depleted cells. One possibility is that ERH depletion causes defects in chromosome structures (e.g., de-

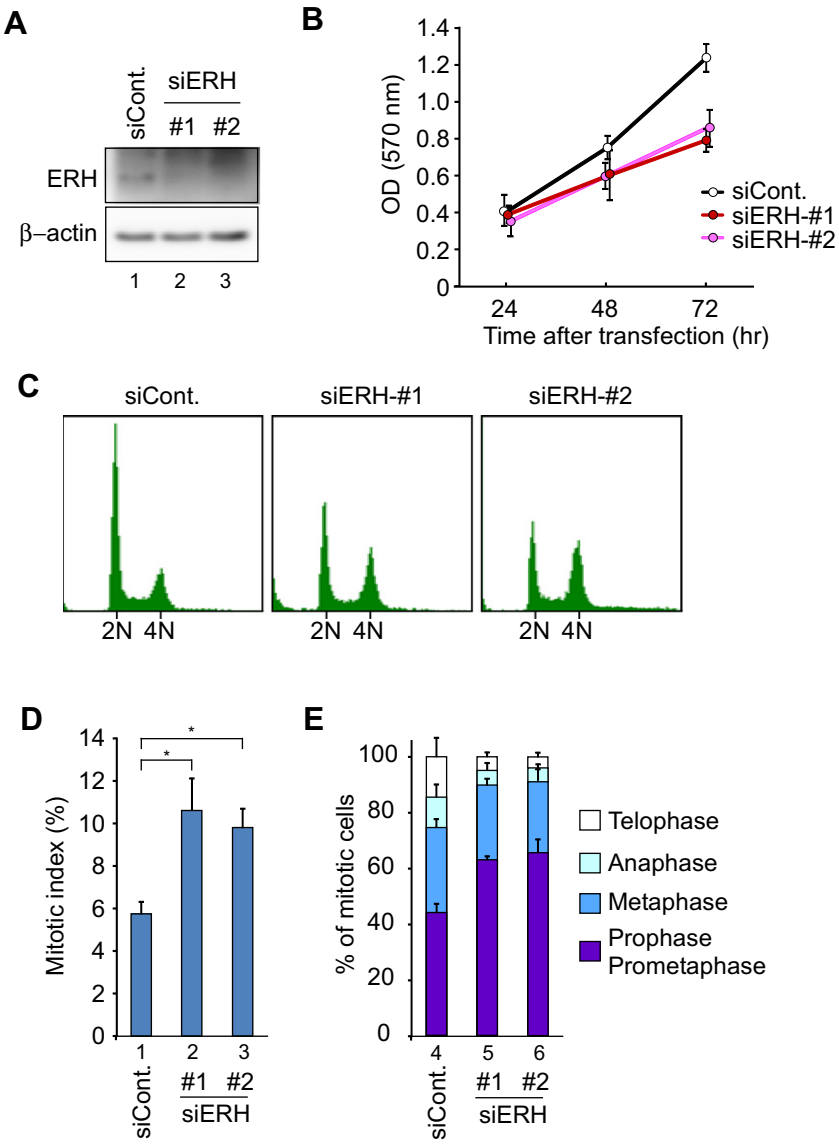

Fig. 1. Depleting cells of ERH disrupts mitotic progression. (A) Efficiency of ERH knockdown. HeLa cells were transfected with control siRNA (siCont.) or two types of ERH-specific siRNA (siERH, #1 or #2). Forty-eight hours after transfection, whole cell extracts were immunoblotted with the indicated antibodies. (B) Reduced cell proliferation by ERH depletion. ERH was knocked down using two different siRNAs, and cell proliferation was detected by MTT assay 24, 48, and 72 h after transfection of siRNA. (C) Increase in the ratio of G2/M phase cells by ERH depletion. HeLa cells were treated with control siRNA (siCont.) or ERH-specific siRNA (siERH) for 72 h. Fixed cells were subjected to flow cytometry. (D and E) Mototic arrest in cells with knocked down ERH expression. We examined the mitotic index (D) and classified mitotic phase (E) in control (siCont.) or ERH-depleted (siERH) cells. $N = 3 > 1000$ cells were examined.

fects in chromosome cohesion or kinetochore structure), which leads to chromosome misalignment. Another possibility is that ERH does not affect the chromosome structure but affects kinetochore-microtubule attachment.

To test the first possibility, chromosome spreads were prepared 48 h after siRNA transfection and stained with Giemsa. However, ERH depletion had little effect on the overall chromosome morphology; more than 80% of chromosomes from both the control and the ERH-depleted cells displayed normal morphology (Fig. 3A). To test the second possibility, control or ERH-depleted HeLa cells were treated with MG132 to accumulate metaphase cells and examined by immunostaining with anti-α-tubulin antibodies and anti-centromere antibodies (ACA) (Fig. 3B). In ERH-depleted cells, many chromosomes were not aligned at the metaphase plate. Notably these misaligned sister chromatid pairs often exhibited the mono-attachment or no-attachment to the microtubules (Fig. 3B), showing that ERH was required for the proper kinetochore-microtubule attachment. It is known that several proteins; such as the Ndc80 complex [14,15], BubR1 [16,17], and CENP-E [18,19] are involved in stable kinetochore-microtubule attachment. Therefore, we then tested whether ERH depletion affected kinetochore association of these proteins by immunofluorescence staining (Fig. 3C). Control or ERH-depleted cells were stained with anti-Hec1 (a component of the Ndc80 complex), anti-BubR1, or anti-CENP-E antibodies. Kinetochore association of Hec1 or BubR1 was not so markedly affected by the depletion of ERH (Fig. 3C). On the other hand, ERH depletion attenuated the kinetochore association of CENP-E (Fig. 3C). In the light of these results, ERH promotes chromosome alignment by localizing CENP-E to kinetochores, thereby stabilizing kinetochore-microtubule attachment.

4. Discussion

ERH, a very highly conserved protein in eukaryotes, is thought to be implicated in several cellular functions [1,4–6]. However, only limited information about its molecular basis (e.g., evidence

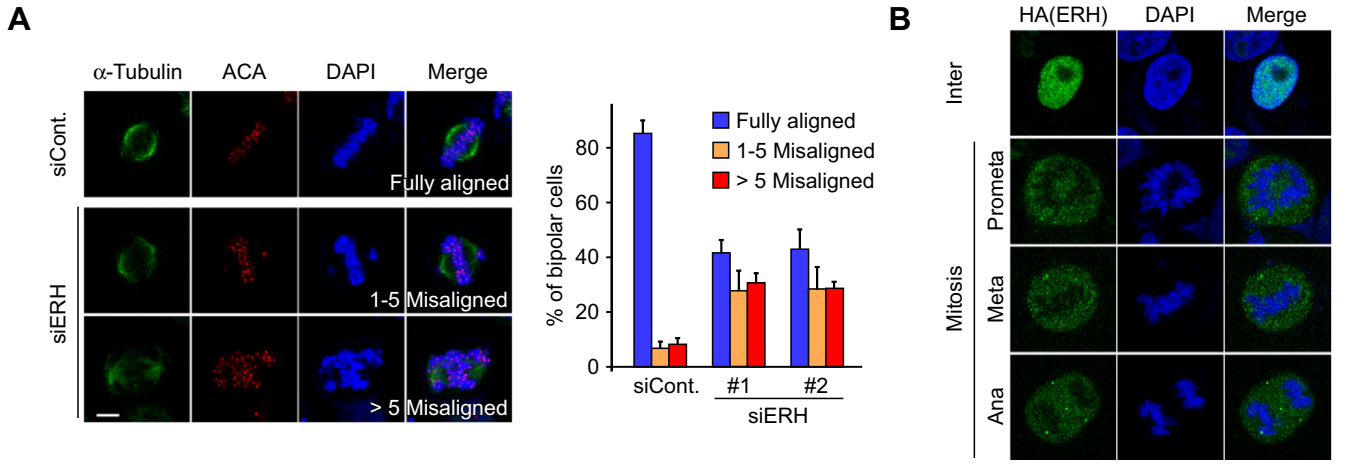

Fig. 2. ERH depletion disrupts chromosome alignment. (A) Defective chromosome alignment in cells depleted of ERH. Metaphase chromosome spreads representing control or ERH-depleted cells were stained with anti-α-tubulin antibodies (green), anti-centromere antibodies (ACA; red), and DAPI (blue). The percentages of mitotic cells with different degrees of chromosome alignment are presented in the right graph. (B) Localization of ERH. HeLa cells were transfected with expression plasmids encoding FLAG/HA-ERH. Thirty-six hour after transfection, cells were stained with anti-HA antibodies (green) and DAPI (blue).

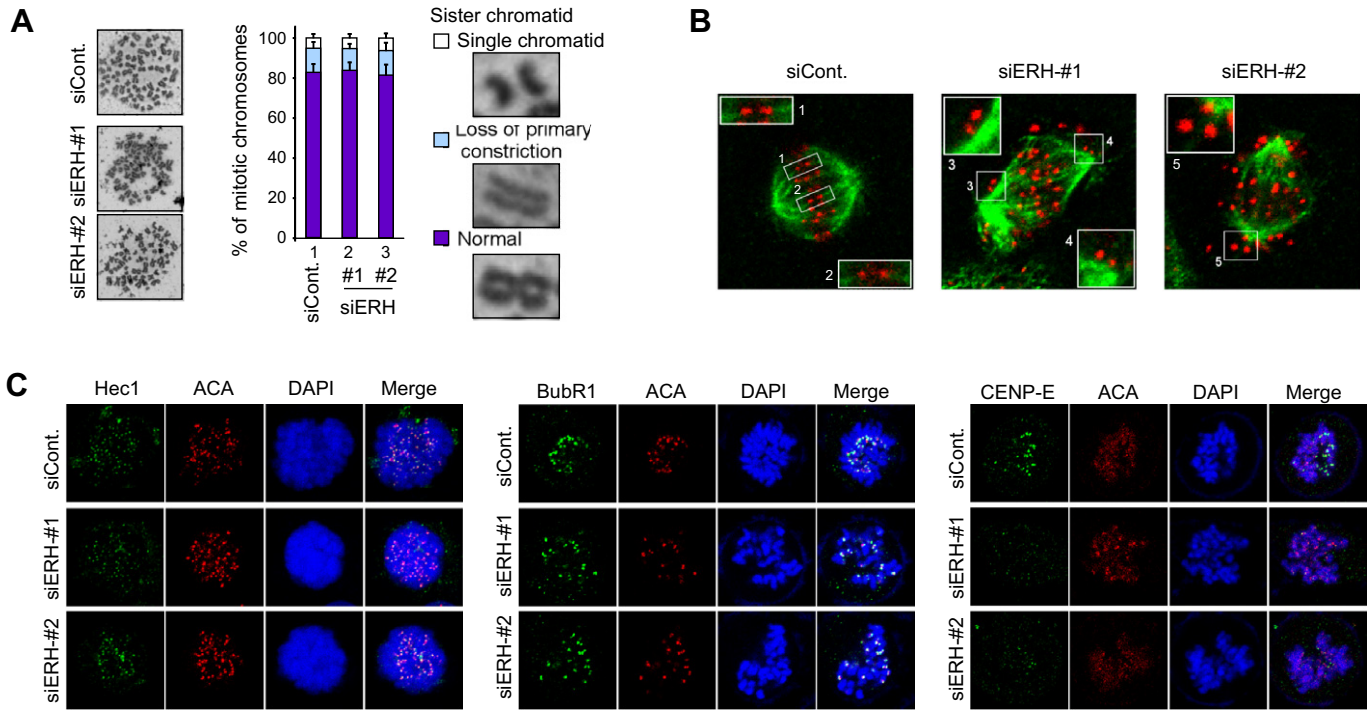

Fig. 3. ERH regulates centromeric association of CENP-E. (A) Mitotic chromosome spreads were prepared and stained with Giemsa (Left panel). The graph shows the percentages of normal and aberrant chromosomes (Center). The images on the right are the typical examples of normal chromatid, chromatid with loss of primary constriction, or single chromatid. More than 100 chromosomes were examined. (B) Defective kinetochore-microtubule attachment in ERH-depleted cells. HeLa cells were treated with control siRNA (siCont.) or ERH-specific siRNA (siERH) for 48 h, and with MG132 for the final 2 h. Cells were stained with anti-α-tubulin antibodies (green), anti-centromere antibodies (ACA; red), and DAPI (blue). Insets 1 and 2 show bi-oriented chromosome pairs and insets 3–5 show kinetochore pairs without stable microtubule attachment. (C) The reduction of CENP-E signal at kinetochores in ERH-depleted cells. HeLa cells were stained with anti-Hec1, anti-BubR1, anti-CENP-E antibodies (green), anti-centromere antibodies (ACA; red), and DAPI (blue).

connected with interacting proteins) is available. For example, it is suggested that ERH is involved in cell cycle regulation because it is associated with cell cycle-regulating proteins and its expression is higher in proliferating cells [7,9]; however, the phase in the cell cycle at which ERH functions and how ERH contributes to the progression of the cell cycle are poorly understood. Through this study, we have clearly demonstrated that ERH is essential for the progression of mitotic phase of the cell cycle (Fig. 1D) by establishing the alignment of mitotic chromosomes (Fig. 3A). Importantly, depletion of ERH compromised kinetochore-microtubule attachment (Fig. 3B), a process which is essential for chromosome alignment at the metaphase plate [20].

Our study also revealed that depletion of ERH impaired CENP-E localization at kinetochores (Fig. 3C). Thus, ERH may achieve metaphase chromosome alignment by locating CENP-E at kinetochores, considering that CENP-E is essential for stable

kinetochore-microtubule attachment. It is reported that Septin 7, a binding partner of CENP-E, is required for CENP-E localization at kinetochores [21]. It is also reported that chromosome alignment-maintaining phosphoprotein (CAMP), which colocalizes with CENP-E at mitotic kinetochores through its FPE region, is involved in the kinetochore localization of CENP-E [22]. Thus, these proteins may recruit CENP-E to kinetochores, thereby regulating the chromosome alignment at the metaphase plate. On the other hand, ERH may not be the direct recruiter of CENP-E because ERH was excluded from the mitotic chromosomes (Fig. 2B). Other paper reports that Aurora kinase and protein phosphatase 1 mediate chromosome alignment through regulation of CENP-E, showing presence of the regulating mechanism by phosphorylation [23]. However, ERH is not likely to regulate phosphorylation status of CENP-E because ERH possesses neither kinase motif nor phosphatase motif [1]. Nevertheless, considering that ERH have many binding partners, we cannot still rule out the possibility that ERH may indirectly regulate CENP-E phosphorylation. In conclusion, we reveal that ERH regulates mitotic chromosome alignment by promoting the kinetochore localization of CENP-E; however, the underlying molecular mechanism remains to be determined.

Moreover, it has been reported that ERH was more abundantly expressed in tumors than in normal tissue samples [12]. Curiously, it has been also reported that CENP-E was more abundant in tumors and CENP-E expression significantly correlated with cancer progression [24–27]. Moreover, CENP-E inhibitor showed inhibition of proliferation of cancer cell lines [26]. Thus, it is possible that ERH overexpression promotes tumor initiation and/or progression via regulating CENP-E. However, we cannot exclude the other possibility because ERH has several binding partners and cellular functions. Neither can we exclude the possibility that ERH may regulate tumor by affecting the progression of phases of the cell cycle other than mitosis.

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