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ERα regulates chromosome alignment and spindle dynamics during mitosis

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ABSTRACT

Estrogen receptors are activated by the hormone estrogen and they control cell growth by altering gene expression as a transcription factor. So far two estrogen receptors have been found: ERα and ERβ. Estrogen receptors are also implicated in the development and progression of breast cancer. Here, we found that ERα localized on the spindle and spindle poles at the metaphase during mitosis. Depletion of ERα generated unaligned chromosomes in metaphase cells and lagging chromosomes in anaphase cells in a transcription-independent manner. Furthermore, the levels of β-tubulin and γ-tubulin were reduced in ERα-depleted cells. Consistent with this, polymerization of microtubules in ERα-depleted cells and turnover rate of α/β-tubulin were decreased than in control cells. We suggest that ERα regulates chromosome alignment and spindle dynamics by stabilizing microtubules during mitosis.

1. Introduction

The cell ascertains cell cycle processes via checkpoints to prevent the generation of abnormal daughter cells such as cancer cells [1,2]. There are three checkpoints: the G1/S checkpoint, G2/M checkpoint, and M checkpoint. Late in the G1 phase, the G1/S checkpoint determines whether the cell is favorable for entering the following S phase without any problems such as DNA damage [3,4]. The G2/M checkpoint determines whether the cell finishes DNA replication without DNA damage. The M checkpoint is the spindle assembly checkpoint (SAC) which occurs between the metaphase and anaphase and alerts the cell of the proper attachment of microtubules to all chromosomes. Therefore, the SAC is a safety device that ensures the fidelity of chromosome segregation in mitosis. The SAC prevents chromosome mis-segregation and aneuploidy and its dysfunction is implicated in tumorigenesis [5]. Spindle dynamics through depolymerization or polymerization of microtubules (MT) generates the driving force required for chromosome movements in mitosis [6]. Cells contain more than a hundred different MT-binding proteins which regulate assembly or disassembly of α/β-tubulin dimers into the MT polymer. Among them, MT associated proteins (MAPs) such as DDA3, EB1, and HURP stabilize spindle microtubules. In contrast, MT depolymerase such as Kif2a, minus end MT depolymerase, and MCAK, plus end MT depolymerase, destabilize spindle MT [7,8].

The ER is a member of the nuclear hormone receptor family that acts as a transcription factor to active target gene transcription. Thus far, two estrogen receptors, ERα and ERβ, have been identified. Once activated by hormones, estrogen receptors form dimers and enter nuclei to transcribe the target gene. The receptors may form homodimers like ERα/ERα (αα) and ERβ/ERβ (ββ), and also form heterodimers like ERα/ERβ (αβ) [9]. ERα and ERβ are encoded by a separate gene in *ESR1* and *ESR2*, respectively, and have different DNA and hormone binding domains. Estrogen and its receptors are well known to be related to the development and progression of breast cancer, as the overexpression of ERα is frequently identified in the early stage of breast cancer [10]. Although ERα and ERβ are well known transcription factors related to cell growth, a few reports also have been reported that they are involved in mitosis. It has been reported that ERβ interacts with spindle assembly checkpoint protein MAD2 at the G2/M checkpoint [11]. Therefore, ERs might play some roles in mitosis.

It is unusual that ERs, as a transcription factor, are associated with mitosis. However, some proteins that function in the interphase have other functions in mitosis [12–14]. Although the Wnt pathway, for example, controls cell fate in the development of cells, the components of this pathway are localized at mitotic structures such as the centrosome and kinetochore in mitosis. While APC is

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localized at the kinetochore, β-catenin, Axin and GSK3 are localized at the centrosome and play a different role in mitosis [15]. For this reason, we investigated the function of ERs in mitosis. We first found that ERα localized at the spindle and centrosome at metaphase in mitosis. Next, we used siRNA to deplete ERα and discovered that unaligned chromosomes were generated and the density of β-tubulin decreased. Also, the density of γ-tubulin was reduced in ERα-depleted cells. Through an MT repolymerization assay and a fluorescence loss of photobleach (FLIP) experiment, we observed that polymerization of microtubules and turnover rate of α/β-tubulin were decreased in ERα-depleted cells. Thus, we concluded that ERα regulates chromosome alignment and spindle dynamics by stabilizing MTs during mitosis.

2. Materials and methods

2.1. Plasmids and antibodies

Full-length wild-type (WT) ERα was subcloned into pCS2+, which contains a N-terminal GFP tag.

Anti-ERα and anti-p38MAPK antibodies were purchased from Santa Cruz Biotechnology (USA). The anti-GFP sera were raised against the full-length recombinant GFP and affinity-purified. Anti-γ-tubulin antibody was purchased from Sigma (USA). Anti-β-tubulin E7 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank (USA). ERα inhibitor (ICI 182,780) was purchased from TOCRIS Bioscience (UK).

2.2. Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc.) supplemented with 10% fetal bovine serum (FBS, Invitrogen), penicillin (100 units/mL) and 100 μg/mL streptomycin (Invitrogen). For ERα inhibition experiment, charcoal/dextran treated FBS was used instead of normal FBS. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. siRNAs were synthesized by Bioneer, Inc. (South Korea). The sequence targeting ERα were 5′-CUGUCUUCUGUUGGGAACA-3′ (si ERα-1) and 5′-GGAGAAUGUUGAAACACAAUU-3′ (si ERα-2). The control siRNA (siGL2) was 5′-CGTACGCGGAATACTTCGATT-3′. siRNAs were transfected into HeLa cells using DharmaFect 1 (Dharmacon, Inc.). DNA transfection was performed using Lipofectamine 2000 (Invitrogen, USA) as instructed by the manufacturer.

2.3. Immunofluorescence

HeLa cells on coverglasses were fixed with methanol at −20 °C for 30 min. Alternatively, cells were extracted with the BRB80-T buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 5 mM EGTA and 0.5% Triton X-100) and then fixed with 4% paraformaldehyde for 15 min at room temperature (Fig. 1B). The fixed cells were then permeabilized and blocked with PBS-BT (1× PBS, 3% BSA, and 0.1% Triton X-100) for 30 min at room temperature. Coverslips were then incubated in primary and secondary antibodies diluted in PBS-BT. Images were acquired with AxioVision 4.8.2 (Carl Zeiss) under a Zeiss Axiovert 200 M microscope using a 1.4 NA plan-Apo 100× oil immersion lens and a HRm CCD camera. Deconvolved images were obtained using AutoDeblur v9.1 and AutoVisualizer v9.1 (AutoQuant Imaging).

2.4. Live cell image

For time-lapse microscopy, HeLa cells stably expressing GFP-H2B were cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). Cells were placed into a sealed growth chamber heated to 37 °C and observed on a Zeiss Axiovert 200 M microscope with a 20× lens. Images were acquired every 3 min for 5 h with AxioVision 4.8.2 (Carl Zeiss).

For FLIP, HeLa cells stably expressing GFP–α-tubulin were transfected with a control or siSirt3 and placed in a sealed growth chamber heated to 37 °C. Cytoplasmic GFP–α-tubulin was photobleached with a laser and images were acquired at 0.632 s intervals for 337.459 s with ZEN (Carl Zeiss) under a LSM 700 confocal microscope (Carl Zeiss) with a 40× lens. 10 half-spindles from 10 metaphase cells in each transfection were analyzed by measuring the absolute GFP–α-tubulin fluorescence intensity in a defined circular area contained entirely within each half-spindle. Fluorescence intensities for each half-spindle were normalized to their maximum intensity at the beginning of the time lapse and the 10 normalized datasets were averaged to generate a single trace for each transfection.

3. Results and discussion

3.1. ERα localizes onto the spindle at metaphase in mitosis

It has been reported that ERβ interacts with the spindle assembly checkpoint protein MAD2 at the G2/M checkpoint [11]. This report prompted us to examine the function of the other ER protein, ERα, in mitosis. At first we determined the cellular localization of ERα across mitosis. Interestingly, ERα localized at mitotic structures, spindle and centrosome, in the metaphase (Fig. 1A). The cell cycle-dependent localization of ERα is specific, as GFP-ERα also localized at the spindle in the metaphase (Fig. 1B).

Next, we examined the levels of ERα protein during the cell cycle. To examine the ERα protein profile during mitosis and the mitotic exit, Hela S3 cells were synchronized at the prometaphase by thymidine–nocodazole (TN) treatment and then released from the prometaphase into G1 [16,17]. The protein level was analyzed by Western blotting. The level of ERα was not changed in early mitosis and gradually increased during mitosis and mitotic exit (Fig. 1C), suggesting that ERα plays as a mitotic regulator from the metaphase to the cytokinesis.

3.2. ERα is involved in chromosome movement and spindle formation

To further investigate the function of ERα in mitosis, we took advantage of siRNA to deplete the protein of ERα and examined the mitotic phenotype (Fig. 2A and B). As complete depletion induced cell death, we only partially knocked down ERα. The depletion of ERα generated unaligned chromosome in metaphase cells (Fig. 2C and D). Most ERα-depleted cells displayed a bipolar spindle but failed to align the chromosomes at the metaphase plate. Notably, expression of the GFP-ERα in cells depleted for endogenous ERα by a siERα-1 targeting to the 3′ noncoding sequence of the endogenous ERα gene successfully rescued chromosome alignment defects (Fig. 2E and F), indicating that ERα is involved in chromosome congression in mitosis. To test whether this mitotic defect was caused by transcriptional activity of ERα, we took advantage of ERα inhibitor. Even-though ERα inhibitor induced apoptosis, mitotic progression was normal (Fig. 2G and H). This indicates that the mitotic defects caused by ERα-depletion is not because the problem of target gene expression but because the mitotic function of ERα protein. To further confirm the mitotic function of ERα, the intercellular function of ERα was analyzed by time-laps imaging of Hela cells that stably express GFP-Histone H2B. As shown in Fig. 3, depletion of ERα caused unaligned chromosomes in the metaphase and lagging chromosomes in the anaphase (Fig. 3A). Also, the duration of mitotic progression was

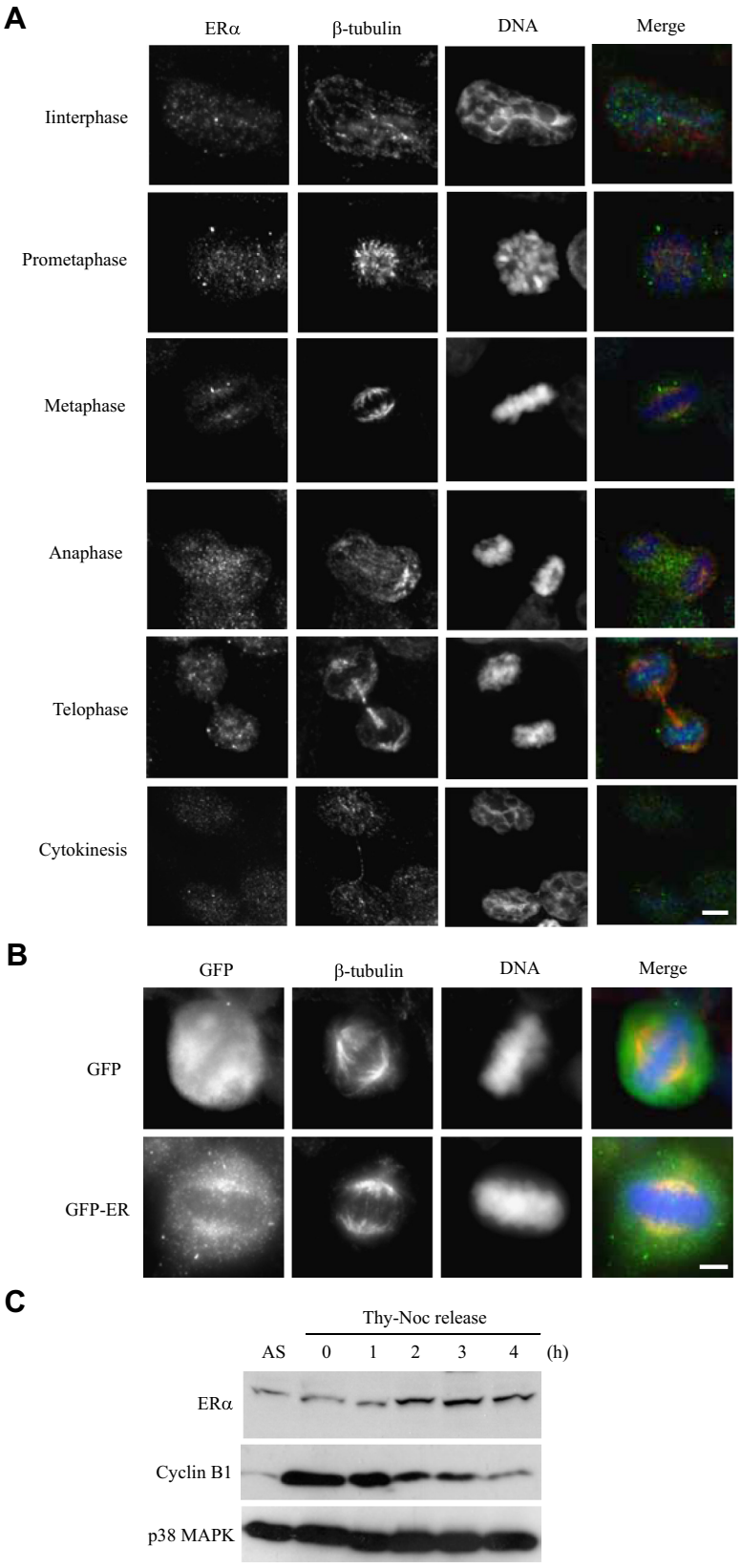

Fig. 1. *ERα localizes on the mitotic spindle at the metaphase.* (A) HeLa cells were fixed with MeOH and stained with antibodies as indicated. Images are maximum projections from z stacks of representative cells stained for ERα (green), β-tubulin (red), and DNA (blue). (B) HeLa cells were transfected with GFP-ERα and fixed with MeOH. Images are maximum projections from z stacks of representative cells that were stained for GFP (green), tubulin (red), and DNA (blue). Scale bars, 5 μm. (C) HeLa S3 cells were synchronized by thymidine–nocodazole treatment and then released into fresh media. Cells were harvested at the indicated time. Cells were analyzed by Western blotting. p38MAPK served as a loading control. AS, unsynchronized cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

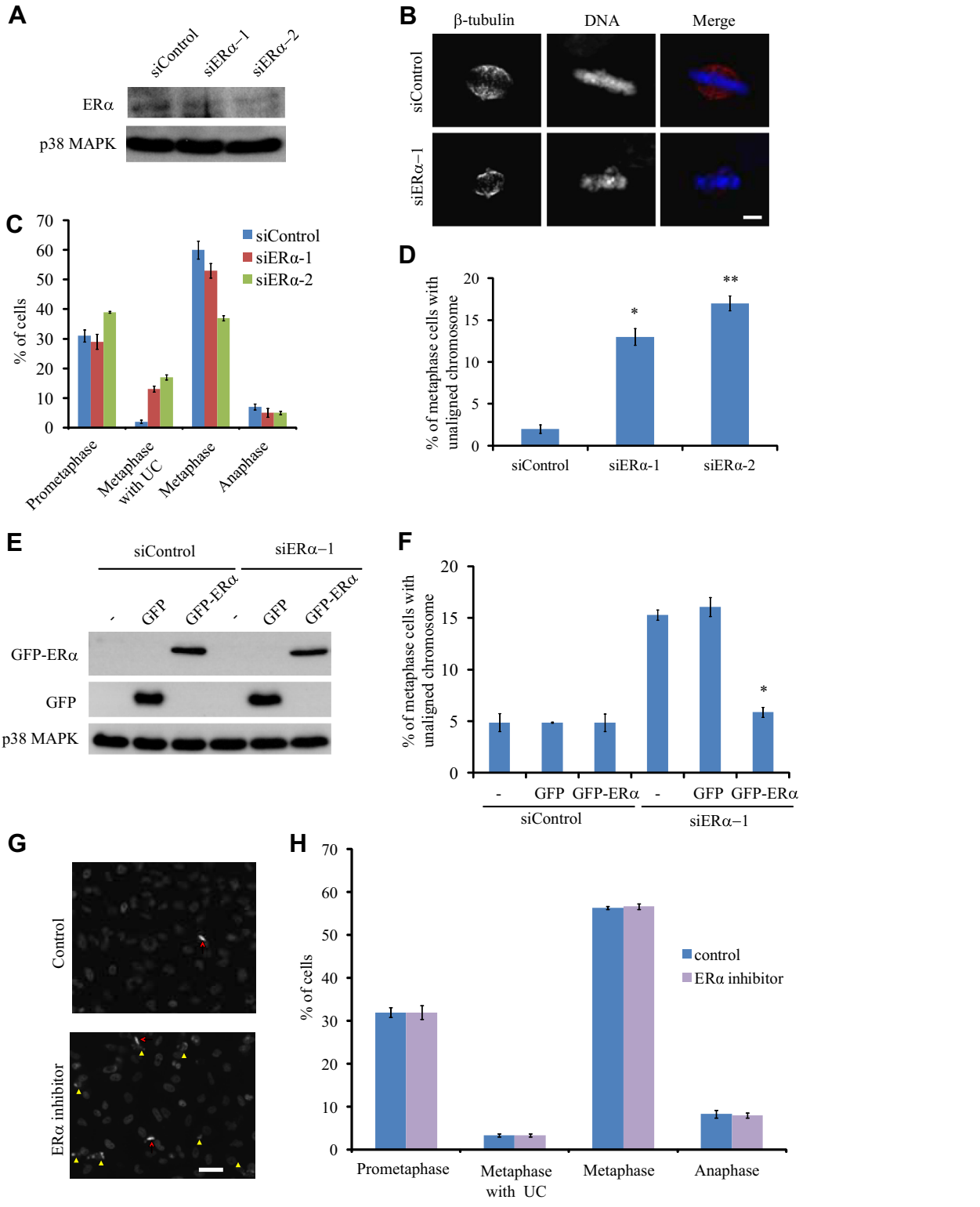

Fig. 2. *Depletion of ERα generates unaligned chromosomes in a transcription-independent manner.* (A) HeLa cells were transfected with control (siControl) or ERα-specific siRNAs. Cells were harvested at 72 h post-transfection and lysates were analyzed by Western blotting against the indicated antibodies. (B) Shown are the maximum projections from deconvolved z stacks of representative cells stained for β-tubulin (red) and DNA (blue). Scale bar, 5 μm. (C) The percentage of mitotic cells with the indicated phases were quantified and plotted ($n = 300$ cells for each quantification). UC, unaligned chromosome. (D) The percentage of metaphase cells with unaligned chromosomes over total metaphase cells were quantified and plotted. $^{*}p < 0.00015$; $^{**}p < 8.36 \times 10^{-5}$ (two-tailed t test relative to control cells). Error bars, SEM. (E and F) 48 h after siRNA transfection, HeLa cells were transfected with GFP or GFP-ERα and analyzed by Western blotting with p38 MAPK serving as a loading control (E). The percentage of metaphase cells with unaligned chromosomes over total metaphase cells in GFP-positive cells were quantified and plotted ($n = 100$ cells from three independent experiments). Error bars, SEM. $^{*}p < 5.93 \times 10^{-4}$ (two-tailed t-test relative to ERα depleted and GFP transfected cells) (F). (G) 5 h after incubation with charcoal/dextran treated FBS containing media, cells were treated with ERα inhibitor (50 μM ICI 182,780) for 10 h. Shown are images stained for DNA. Arrows point to normal mitotic cells. Arrowheads point to apoptotic cells. Scale bar, 20 μm. (H) The percentage of mitotic cells with the indicated phases were quantified and plotted ($n = 300$ cells for each quantification). UC, unaligned chromosome. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

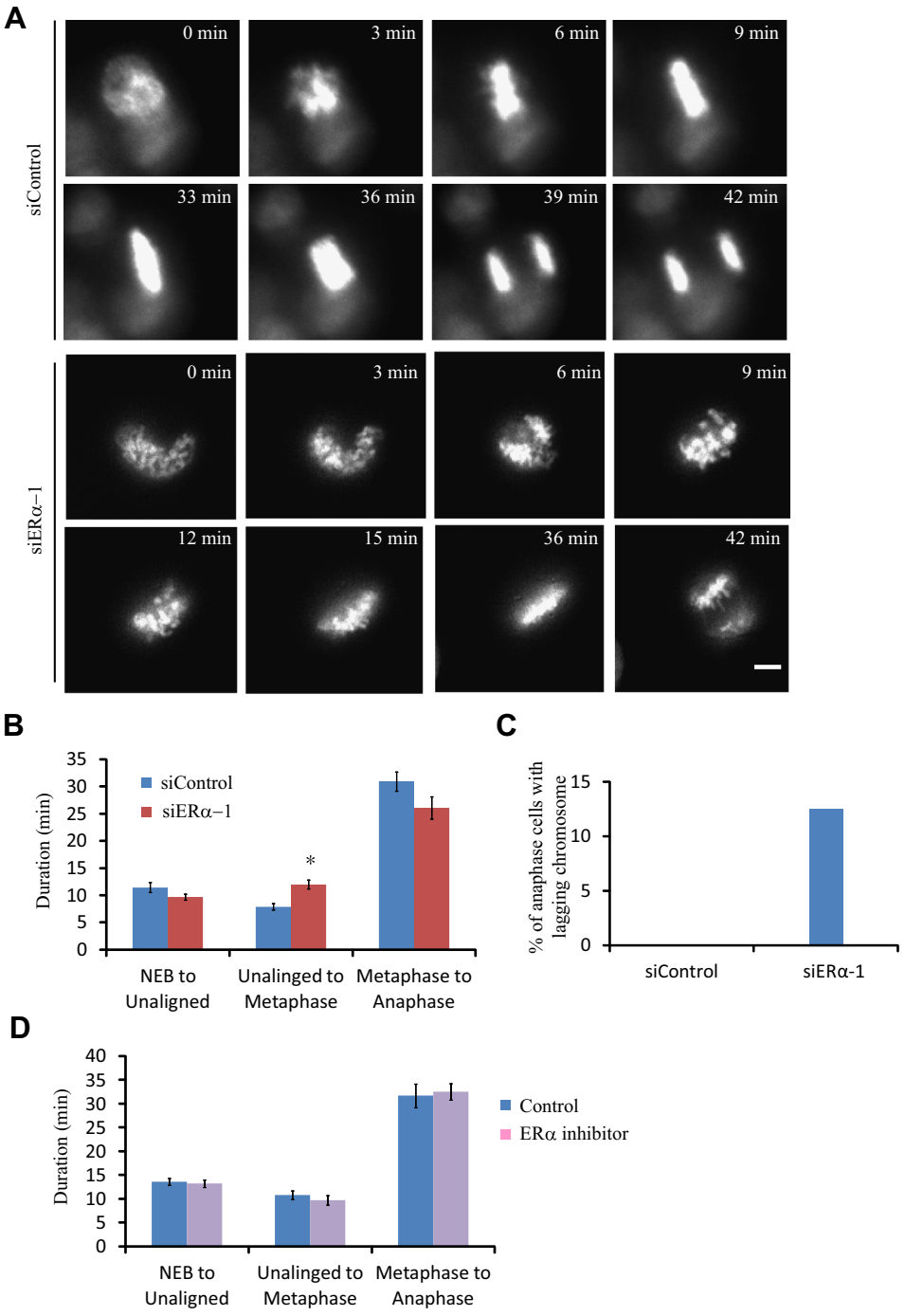

Fig. 3. *ERα regulates chromosome congression and segregation.* (A) HeLa/GFP-Histon H2B cells were transfected with siRNAs and imaged for GFP-H2B by time laps starting from 72 h after transfection. Images were captured every 3 min to monitor mitotic progression. Still frames from time-lapse movies of representative cells. Scale bar, 10 μm. (B) The duration from nuclear envelope breakdown (NEB) to the formation of a bipolar spindle/metaphase plate with some unaligned chromosomes (NEB to unaligned), from the unaligned state to metaphase without unaligned chromosomes (unaligned to metaphase), and from metaphase without unaligned chromosomes to anaphase (metaphase to anaphase) were determined for control and ERα-depleted cells ($n = 57$ cells). $^{*}p < 4.85 \times 10^{-4}$ (two-tailed t test relative to control cells). Error bar, SEM. (C) The percentage of anaphase cells with the lagging chromosome were quantified and plotted ($n = 57$ cells for each quantification). (D) Cells were incubated with charcoal/dextran treated FBS containing media for 5 h. 5 h after treatment of ERα inhibitor (50 μM ICI 182,780), the duration of mitotic progression was determined as in (B) in the presence of ERα inhibitor.

altered by ERα-depletion. Quantitative analysis of mitotic progression indicated that depletion of ERα prolonged the duration of the prometaphase (from the initial formation of the metaphase plate to the perfect formation of metaphase plate) to some extent. In contrast, the duration of the metaphase (from the perfect formation of the metaphase plate to the onset of anaphase) was slightly decreased (Fig. 3B). Based on a previous report [11], this decrease of duration of the metaphase might be caused by abnormality of the spindle assembly checkpoint. Consistent with this, lagging chromosomes were generated at the anaphase in ERα-depleted cells (Fig. 3A and C). In contrast, the duration of mitotic progression was also not affected by ERα inhibitor (Fig. 3D).

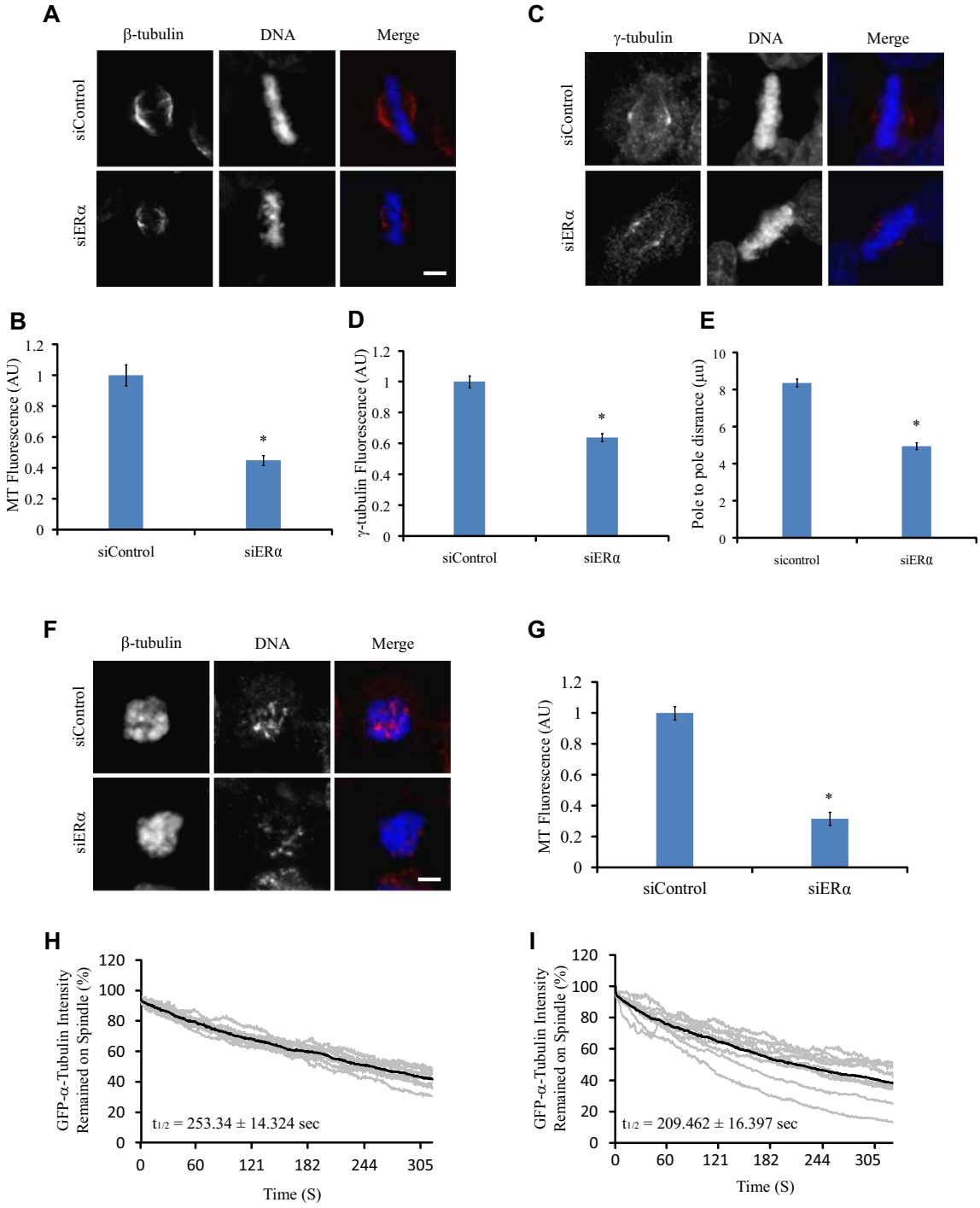

Fig. 4. *ERα stabilizes mitotic spindle*. (A) Shown are maximum projections from the deconvolved z stacks of representative cells stained for β-tubulin (red) and DNA (blue). Images were acquired under a constant exposure time for the β-tubulin channel. (B) MT fluorescence intensity in metaphase cells was quantified and plotted ($n = 10$ cells) $^{*}p < 1.8 \times 10^{-6}$ (two-tailed t test relative to control cells). Images were acquired under a constant exposure time for the β-tubulin channel. (C) Shown are maximum projections from the deconvolved z stacks of representative cells stained for γ-tubulin (red), and DNA (blue). Images were acquired under a constant exposure time for the γ-tubulin channel. (D) Total immunofluorescence intensity for γ-tubulin on the metaphase spindle ($n = 10$ cells for each quantification) was quantified and plotted. $^{*}p < 9.5 \times 10^{-7}$ (two-tailed t test relative to control cells). (E) Pole-to-pole distances in metaphase cells were quantified by γ-tubulin immunofluorescence in (C) and plotted ($n = 10$ cells each). $^{*}p < 0.0001$ (two-tailed t test). (F) HeLa cells were transfected with a siControl or siERα. Control or ERα-depleted cells were treated with 1 μg/ml nocodazole for 10 min at 37 °C to completely depolymerize the mitotic spindle. Nocodazole treated cells were washed, released into fresh media, and fixed with MeOH at 6 min after release. Cells were analyzed by immunofluorescence staining of β-tubulin. (G) Images for β-tubulin in (F) were acquired under a constant exposure time. β-tubulin immunofluorescence intensity on spindles was quantified and normalized to the control sample ($n = 10$ cells for each quantification). $^{*}p < 7.2 \times 10^{-10}$ (two-tailed t test relative to control cells). Error bar, SEM. Scale bars, 5 μm. (H and I) HeLa cells stably expressing GFP-α-tubulin were transfected with control (H) or ERα-specific (I) siRNAs. GFP fluorescence intensity was acquired every 0.632 s while a photobleaching laser was focused to a diffraction-limited spot in the cytoplasm away from the spindle. 10 half-spindles from 10 metaphase cells were quantified and fluorescence signals for each half spindle were normalized to their intensity at 0 s and averaged across the 10 half-spindles at each time point (thick traces). Turnover half-lives for GFP-α-tubulin on the spindle were calculated from the averaged fluorescence signal traces. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. ERα stabilizes mitotic spindle in mitosis

To investigate how spindle-associated ERα controls chromosome congression and segregation, we tested the role of ERα in spindle assembly and spindle dynamics. Because ERα localized at the spindle and centrosome at the metaphase, we determined the levels of β-tubulin and γ-tubulin in ERα-depleted cells (Fig. 4A and C). The levels of β-tubulin decreased by 50% and γ-tubulin by 40% in ERα-depleted cells compared with control cells (Fig. 4B and D), suggesting that ERα plays a role in spindle formation and centrosome maturation. To further demonstrate the role of ERα on spindle formation, we determined the kinetics of microtubule polymerization (Fig. 4F and G). Cells were transfected with siControl or siERα for 72 h and treated with 1 μg/ml nocodazole to depolymerize the microtubules. Cells were then released into fresh media and the rate of microtubule polymerization was analyzed kinetically. The polymerization of spindle MT in ERα-depleted mitotic cells was slower than in control cells (Fig. 4G). Thus, ERα stabilizes the mitotic spindle and contributes to MT growth.

Furthermore, spindle dynamics was changed in ERα knockdown cells, because depletion of ERα led to a decrease in the pole-to-pole distance (4.95 ± 0.18) more than in control cells (8.36 ± 0.2) (Fig. 4E). To determine whether ERα directly regulates spindle dynamics, we measured the assembly/disassembly rate of α/β-tubulin heterodimers on the mitotic spindle through a fluorescence loss in photobleaching (FLIP) experiment. Consistent with Fig. 4E, the half-life of GFP-α-tubulin on the metaphase spindle in ERα-depleted cells was decreased (Fig. 4H and I), indicating that ERα controls spindle dynamics in mitosis. Taken together, we concluded that ERα stabilizes the mitotic spindle and controls the dynamics of the mitotic spindle and chromosome movement.

We described here a new function of ERα in mitosis. Even-though ERα acts as a transcription factor in the interphase, it regulates chromosome movement and spindle dynamics by stabilizing microtubules during mitosis. At the cellular level, ERα associated with the mitotic spindle and centrosome. Depletion of ERα increased unaligned chromosomes and lagging chromosomes to certain levels (Figs. 2D and 3A), indicating that ERα is involved in spindle dynamics for chromosome alignment and segregation. Consistent with this, not only the levels of β-tubulins and γ-tubulins but also the kinetics of MT polymerization decreased in ERα-depleted cells. Thus, ERα is responsible for centrosome maturation, the function of the centrosome, and spindle nucleation from the γ-tubulin organizing center in the centrosome.

Dynamic turnover of the mitotic spindle is essential for the congression of chromosomes at the prometaphase, establishment of tension at the metaphase, and segregation of sister chromatids at the anaphase [7]. Spindle dynamics is regulated by MT nucleators like γ-tubulin, MT depolymerase such as the kinesin-13 family of proteins, and MT-associate proteins [18,19]. MAPs can be divided into MT-stabilizing factors, such as TPX2, and destabilizing factors, such as katanin [6–8]. We showed that ERα regulates spindle dynamics. Depletion of ERα decreased levels of MTs and polymerization of mitotic spindle MTs (Fig. 4A and F), indicating that ERα has a MT stabilizing function in mitosis. But the mechanism remains to be elucidated.

Depletion of ERα led to the generation of lagging chromosomes in the anaphase and the alteration of mitotic progression. Quantitative analysis of mitotic progression indicated that depletion of ERα prolonged the duration of chromosome congression but slightly decreased the duration of the metaphase (Fig. 3B). The decrease of the duration of the metaphase may be because of an abnormality of the spindle assembly checkpoint. However, it has been previously reported that ERα did not interact with the MAD2 protein [11]. Thus, it will be determined whether ERα associates with other mitotic checkpoint complex (MCC) in the spindle assembly checkpoint in further experiments.

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