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The SUMO-targeted ubiquitin ligase RNF4 localizes to etoposide-exposed mitotic chromosomes: Implication for a novel DNA damage response during mitosis

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ABSTRACT

RNF4, a SUMO-targeted ubiquitin ligase (STUbL), localizes to the nucleus and functions in the DNA damage response during interphase of the cell cycle. RNF4 also exists in cells undergoing mitosis, where its regulation and function remain poorly understood. Here we showed that administration of etoposide, an anticancer DNA topoisomerase II poison, to mitotic human cervical cancer HeLa cells induced SUMO-2/3-dependent localization of RNF4 to chromosomes. The FK2 antibody signals, indicative of poly/multi-ubiquitin assembly, were detected on etoposide-exposed mitotic chromosomes, whereas the signals were negligible in cells depleted for RNF4 by RNA interference. This suggests that RNF4 functions as a STUbL in the etoposide-induced damage response during mitosis. Indeed, RNF4-depletion sensitized mitotic HeLa cells to etoposide and increased cells with micronuclei. These results indicate the importance of the RNF4-mediated STUbL pathway during mitosis for the maintenance of chromosome integrity and further implicate RNF4 as a target for topo II poison-based therapy for cancer patients.

1. Introduction

Since administration of drugs that induce DNA damage as well as agents that block mitotic progression is frequently used for the treatment of cancer, it is clinically important to understand how cancer cells deal with DNA damage [1,2]. Current studies revealed that two posttranslational modification systems, SUMOylation and ubiquitinylation, play very important roles in regulating repair factors involved in DNA damage response [3,4]. Thus, clarification of the molecular details underlying the regulations of the SUMO and ubiquitin pathways would be beneficial for cancer therapy.

A wide variety of proteins that contain a really interesting (RING) domain have been demonstrated to function as ubiquitin ligases that promote the transfer of ubiquitin from E2s to lysine residues in target proteins [5]. Among the RING-type E3 ligases, RING finger protein 4 (RNF4 or SNURF) is proposed to ubiquitylated proteins that have been modified by SUMO and therefore is classified into a special category of E3s, termed as the SUMO-targeted ubiquitin ligases (STUbLs) [6,7]. In mammalian cells, RNF4 has a key role in arsenic therapy for acute promyelocytic leukemia [8,9] and in responses against heat shock [10] and hypoxia [11]. RNF4 is also required for the DNA damage response [12–16] as well as base-excision repair (BER)-mediated active DNA demethylation [17], suggesting that STUbL activity mediated by RNF4 is important for the regulation of a wide variety of signaling cascades in vital cellular systems. However, it should be noted that most of these studies described RNF4 regulation and function in interphase cells, leaving a question concerning RNF4 during mitosis.

In this study, we described accumulation of RNF4 on mitotic chromosomes of HeLa cells, a human cervical cancer cell line, when cells undergoing mitosis were exposed to etoposide (VP-16, 4′-dimethylepipodophylloxin-9-[4,6-O-ethylidene-β-D-glucopyranoside]). Etoposide is an anticancer agent that is successfully and extensively used in treatments for various types of cancers in children and adults [18–21]. It is a potent DNA topoisomerase II (topo II)-inhibiting drug that increases the steady-state concentration of topo II covalently attached to DNA, converting topo II into a

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physiological toxin that introduces high levels of DNA damage in the treated cells [22,23]. We demonstrated that SUMO-2/3 accumulation on etoposide-exposed chromosomes was a prerequisite for RNF4 accumulation and poly/multi-ubiquitin chain assembly. We also revealed that RNF4-depleted HeLa cells were more susceptible to etoposide than control cells and were frequently associated with micronuclei, providing the first evidence of RNF4's role in etoposide-induced damage response during mitosis and further implicating RNF4 as a molecular target that enhances the effect of topo II poison on cancer cells.

2. Materials and methods

2.1. Hybridoma production

For making the anti-human RNF4 mouse monoclonal antibody 11-3.1, we immunized mice with recombinant GST-tagged human RNF4. Serum titers were monitored by immunoblotting using lysates of HEK293 cells transfected with Flag-RNF4/pcDNA3. After several injections, splenic lymphocytes were fused to the myeloma cell line NS-1. Clonal populations of fusion cells were screened by enzyme-linked immunosorbent assays (ELISAs) for antibody production against bacterially expressed His_6-hRNF4. Productive cells were cloned to monoclonal lines by serial dilution screening. Highly concentrated monoclonal antibodies were isolated from murine ascites after an intraperitoneal injection of hybridoma cells and then IgG was purified by Protein-A affinity chromatography.

2.2. Antibodies

Two rat monoclonal antibodies against SUMO-2/3 and SUMO-1 were described previously [24]. Anti-poly/multi-ubiquitin (FK2), anti-tubulin and anti-β actin antibodies were obtained from MBL. Secondary antibodies used were as follows: anti-rabbit Cy3-conjugated donkey antibody (Jackson ImmunoResearch), anti-mouse Alexa-488 conjugated donkey antibody (Life technologies), anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody (Chemicon), anti-rabbit IgG HRP conjugated secondary antibody (MBL).

2.3. Cell culture, drug treatments and small interfering (si) RNA transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham with 5% fetal calf serum and antibiotics at 37 °C in 5% CO_2 incubator. Etoposide (TCI) was dissolved in dimethyl sulfoxide (DMSO) and added to culture medium as indicated in the text. Hydroxyurea (HU), methyl methanesulfonate (MMS) and camptothecin were obtained from Sigma–Aldrich. To synchronize HeLa cells, double thymidine block was performed as describe previously [25]. For siRNF4 experiments, Hela cells were transfected with siRNA by Lipofectamine RNAiMAX reagent (Life technologies), according to the manufacturer's protocol. The siRNA duplexes designed to target the mRNAs encoding human SUMOs were obtained from Stealth™ siRNA collection (Invitrogen). The sequences were as follows: SUMO-2, 5′-GGCCUACUGCGAGAGGCAGG GCUUG-3′; SUMO-3, 5′-AGCCUAUUGUGAACGACAGGGAUUG-3′. The siRNAs against RNF4 (siGENOME SMARTpool siRNA reagent M-006557-03-0005) were obtained from Thermo Fisher Scientific.

2.4. Indirect-immunofluorescence analysis

Cultured cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 7 min at room temperature. After permeabilization with methanol pre-chilled at −20 °C for 5 min, cells were blocked in PBS containing 0.2% bovine serum albumin, 0.1% Triton X-100 for 20 min and incubated with primary antibody for 1 h, followed by an appropriate secondary antibody. The coverslips were mounted on slide glasses using glycerol-1,4-diazabicyclo[2.2.2] octane (DABCO; Wako Pure Chemical Industries) and samples were analyzed with a DP72 microscope (Olympus). DNA was visualized with 4′, 6-diamidino-2-phenylindole (DAPI).

2.5. Immunoblot analysis

Immunoblot analysis was carried out as described previously [24].

3. Results and discussion

3.1. Development of mouse monoclonal antibody specific to human RNF4

To aid in our study of RNF4 regulation and function in cultured human cells, we generated the mouse monoclonal antibody 11-3.1, which is highly specific to recombinant human RNF4 protein; the antibody did not cross-react with recombinant mouse RNF4 protein (Supplementary Fig. 1). When we probed the immunoblot containing cell lysates from two human cancer cell lines, HEK293 and HeLa cells, a single prominent band at around 30 kDa was detected in both lysates (Fig. 1A). In the lysate prepared from HeLa cells transfected with siRNA against RNF4, the 30-kDa band was barely visualized (Fig. 1B) and indirect immunofluorescence analysis of RNF4 siRNA-transfected HeLa cells failed to detect RNF4 signals (Fig. 1C). These results indicated that the specificity and

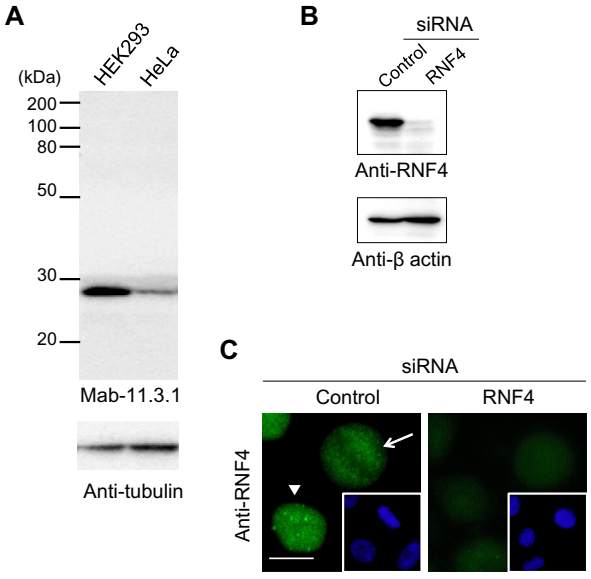

Fig. 1. Generation of human RNF4-specific antibodies. (A) Protein extracts from HEK293 (lane) or HeLa (lane 2) cells (1×10^5) were separated by 12% SDS–PAGE and subjected to immunoblot analysis using mouse monoclonal antibody 11-3.1 (upper panel) or anti-tubulin antibody (lower panel). Protein molecular mass standards are shown on the left. (B) Exponentially growing HeLa cells were transfected with control siRNA or siRNA against RNF4, followed by incubation for 24 h. Cells were suspended in SDS sample buffer and proteins were subjected to immunoblot analysis using anti-RNF4 antibody (upper panel) or anti-β actin antibody (lower panel). (C) Exponentially growing HeLa cells were transfected with control siRNA (left panel) or siRNA against RNF4 (right panel), followed by 24 h incubation. Cells were subjected to indirect immunofluorescence analysis using anti-RNF4 antibody. DNA was visualized with DAPI staining (inset). The arrowhead indicates an interphase cell. The arrow shows a cell undergoing mitosis. Bars indicate 20 μm.

sensitivity of our newly developed 11-3.1 mouse monoclonal antibody were sufficient to detect endogenously expressed RNF4 in cultured human cells. This monoclonal antibody is referred to as anti-RNF4 antibody in this study.

3.2. RNF4 localization to etoposide-exposed mitotic chromosomes

We used the newly generated antibody to analyze the subcellular localization of endogenous RNF4 in HeLa cells cultured in either the presence or absence of etoposide. In the absence of etoposide (DMSO-treated cells), the antibody mainly stained the nucleus of interphase cells, whereas it barely visualized mitotic chromosomes in cells undergoing mitosis (Fig. 1C and Fig. 2A). In contrast, when cells were exposed to etoposide for 20 min, prominent staining was observed in mitotic cells (Fig. 2A and Supplementary Fig. 2A). The antibody staining overlapped DAPI-positive objects, suggesting that RNF4 accumulated on mitotic chromosomes upon etoposide exposure in cells undergoing mitosis. The nuclear localization of RNF4 during interphase appeared not to alter significantly upon etoposide-exposure under our experimental conditions, which would be due to either a subtle change of RNF4 localization or a limitation of our experimental resolution. We reproducibly detected that 80–90% of etoposide-exposed mitotic cells contained chromosomes which were stained heavily with anti-RNF4 antibody (Fig. 2B). It should be noted that etoposide induced RNF4 localization to chromosomes at prophase, metaphase, anaphase and telophase during mitosis (Supplementary Fig. 3), indicating that RNF4 accumulation was induced at all phases of mitosis. We also tested whether other DNA/chromatin damaging agents, including hydroxyurea (HU), methyl methanesulfonate (MMS) and camptothecin, facilitated accumulation of RNF4 to mitotic chromosomes. Notably, when asynchronously cultured HeLa cells were exposed to 5 μM camptothecin for 20 min, anti-RNF4 antibody detected accumulation of RNF4 on mitotic chromosomes in most cells undergoing mitosis (Supplementary Fig. 4). In contrast, exposure of cells to HU or MMS appeared not to facilitate

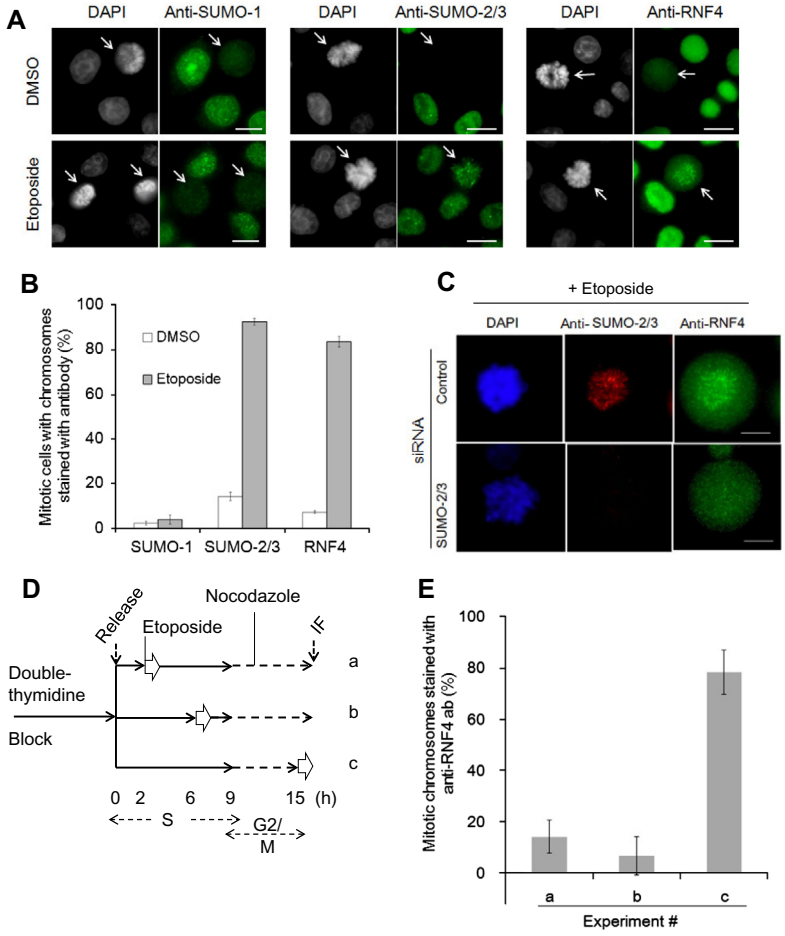

Fig. 2. SUMO-2/3-dependent RNF4 localization to etoposide-exposed mitotic chromosomes. (A) Exponentially growing HeLa cells were incubated in the absence (DMSO: upper panel) or presence of 250 μM etoposide (lower panel) for 20 min, followed by indirect immunofluorescence analysis using anti-SUMO-1, anti-SUMO-2/3 or anti-RNF4 antibody. DNA was visualized with DAPI staining. Arrows indicate cells undergoing mitosis. Bars indicate 20 μm. (B) The numbers of cells in which mitotic chromosomes were stained with anti-SUMO-1, anti-SUMO-2/3 or anti-RNF4 antibody were counted. The values shown represent the ratio of the stained cells versus cells undergoing mitosis ±SE of three independent experiments. White bars represent results obtained from control (DMSO-treated) cells. Gray bars indicate results of etoposide-treated cells. (C) Exponentially growing HeLa cells were transfected with control siRNA (upper panel) or siRNA against SUMO-2/3 (lower panel), followed by 24 h incubation. Cells were then exposed to 250 μM etoposide for 20 min, followed by indirect immunofluorescence analysis using anti-SUMO-2/3 (middle panel) and anti-RNF4 (right panel) antibody. DNA was visualized with DAPI staining (left panel). Bars indicate 20 μm. (D) Timeline of the experiment to assess the 'sensitive' period during which etoposide can exert its effect. HeLa cells were synchronized in early S phase by double-thymidine block. During incubation, 250 μM etoposide was added into the culture and cells were incubated for 20 min as indicated by the white arrows (a, b and c). After washing with culture medium, cells were continuously cultured in medium with or without 75 ng/ml nocodazole (indicated by the black or dotted arrows, respectively), followed by indirect immunofluorescence analysis using anti-RNF4 antibody. Predicted phase of cell cycle along the experimental timeline is shown at the bottom. (E) The numbers of cells in which mitotic chromosomes were stained with anti-RNF4 antibody were counted. The experiment was performed six times. Mitotic cells were detected by DAPI staining and at least 24 mitotic cells were investigated in each experiment. The values shown represent the ratio of the stained cells among cells undergoing mitosis ±SE of six independent experiments.

RNF4 accumulation on mitotic chromosomes. These observations implied that a specific type of DNA/chromatin stress, besides etoposide exposure, in cells undergoing mitosis could trigger RNF4 accumulation to mitotic chromosomes.

3.3. Negligible RNF4 localization to etoposide-exposed mitotic chromosomes in SUMO-2/3-depleted cells

Since RNF4 is a member of the STUbLs, we assumed that RNF4 localization was dependent on SUMO-2/3 accumulation on etoposide-exposed mitotic chromosomes, and thus SUMOylation preceded RNF4 loading on chromosomes. As shown in Fig. 2A, B and Supplementary Fig. 2B, we detected accumulation of SUMO-2/3, but not SUMO-1, on etoposide-exposed mitotic chromosomes. We also found that in many cells transfected with siRNA against SUMO-2/3, RNF4 accumulation on etoposide-exposed mitotic chromosomes was negligible (Fig. 2C). These results supported the idea that RNF4 localization to mitotic chromosomes followed by SUMO-2/3 modification. Given that previous report by Agostinho et al. described that beside etoposide, the topo II poison ICRF-187 induced the accumulation of SUMO-2/3, but not SUMO-1, on mitotic chromosomes [26], it seems feasible that topo II poisons in general are able to induce SUMOylation and/or SUMO-2/3 accumulation, followed by RNF4 recruitment, to etoposide-exposed mitotic chromosomes.

Our observations thus far suggested that damage/stress on mitotic chromosomes that was introduced directly by etoposide or topo II inhibition possibly during mitosis could facilitate SUMOylation followed by RNF4 localization on mitotic chromosomes. However, it is also possible that damage/stress on genomic DNA introduced by etoposide during the previous S-phase would be manifested in the following mitosis. To rule out this possibility, we treated synchronized HeLa cells with etoposide at various times and examined whether RNF4 accumulated on mitotic chromosomes in the subsequent mitosis. As shown in Fig. 2D, HeLa cells synchronized in early S phase by double-thymidine block were released to drug-free medium. After 2, 6 or 15 h incubation, etoposide was administered for 20 min, followed by washing with drug-free culture medium (the white arrow in experiment #a, b or c). Cells were continuously cultured in medium with or without nocodazole (the dotted and lined arrows indicated in the timeline). We then performed indirect immunofluorescence analysis using anti-RNF4 antibody and detected substantial accumulation of RNF4 on mitotic chromosomes when cells were treated with etoposide at 15 h after release (c), but not at 2 (a) or 6 h (b) after release (Fig. 2E). Since we assumed that based on the cell cycle of

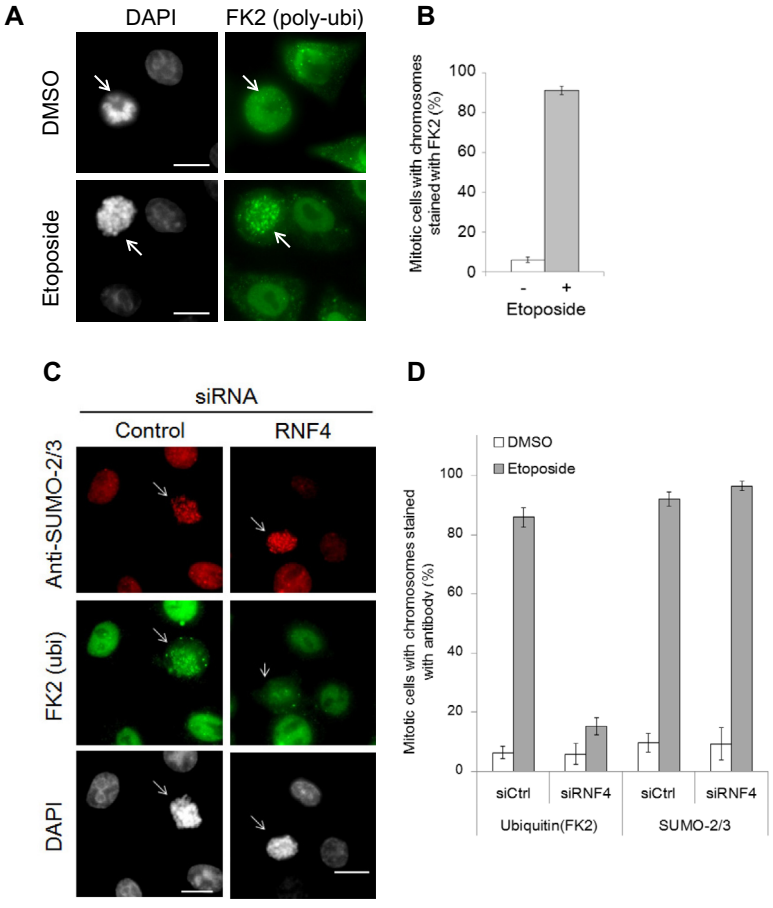

Fig. 3. RNF4-dependent FK2 staining of etoposide-exposed mitotic chromosomes. (A) Exponentially growing HeLa cells were incubated in the absence (DMSO: upper panel) or presence of 250 μM etoposide (lower panel) for 20 min, followed by indirect immunofluorescence analysis using anti-poly/multi-ubiquitin antibody (FK2). DNA was visualized with DAPI staining (left panel). Arrows indicate cells undergoing mitosis. Bars indicate 20 μm. (B) The numbers of cells in which mitotic chromosomes were stained with FK2 were counted. The values shown represent the ratio of the stained cells versus cells undergoing mitosis ±SE of three independent experiments. The white bar represents the result obtained from control (DMSO-treated) cells. The gray bar indicates the result from etoposide-treated cells. (C) Exponentially growing HeLa cells were transfected with control siRNA (left column) or siRNA against RNF4 (right column), followed by 24 h incubation. Cells were then subjected to indirect immunofluorescence analysis using anti-SUMO-2/3 (upper) and FK2 (middle) antibodies. DNA was visualized with DAPI staining (bottom). Bars indicate 20 μm. (D) The numbers of cells in which mitotic chromosomes were stained with anti-SUMO-2/3 or FK2 antibody were counted. The values shown represent the ratio of the stained cells versus cells undergoing mitosis ±SE of three independent experiments. White bars represent results of control (DMSO-treated) cells. Gray bars indicate results of etoposide-treated cells.

synchronized HeLa cells, the later point (c) and the earlier points (a and b) might represent G2/M and S/G2 phases, respectively [25], these results supported that etoposide exerted its effect on RNF4 localization to chromosomes in cells in M phase, but not in cells in the preceding S phase. We cannot completely rule out the possibility that RNF4 localization to mitotic chromosomes might be 'sensitive' to etoposide during G2 phase. To precisely determine the sensitive period during the cell cycle, time-lapse observation of living cells will be performed in future studies.

3.4. Detection of etoposide-exposed mitotic chromosomes by FK2, an anti-poly/multi-ubiquitin antibody

Based on the notion that RNF4 is a member of the STUbLs, we expected that SUMO-2/3-dependent RNF4 recruitment to etoposide-exposed mitotic chromosomes might be followed by assembly of poly/multi-ubiquitin. To detect poly/multi-ubiquitin assembly on etoposide-exposed mitotic chromosomes, we used FK2, a mouse monoclonal antibody that preferentially recognizes poly/multi-ubiquitin chains over the monomeric free form of ubiquitin [27]. As shown in Fig. 3A, FK2 clearly stained etoposide-exposed mitotic chromosomes, but not non-treated mitotic chromosomes (Fig. 3A and Supplementary Fig. 2C). Approximately 90% of cells undergoing mitosis were stained with FK2 when exposed to etoposide, while non-treated chromosomes showed negligible staining of the antibody (Fig. 3B). Localization of poly/multi-ubiquitin to etoposide-exposed mitotic chromosomes appeared to be dependent on RNF4, as more than 80% of RNF4-depleted cells undergoing mitosis exhibited FK2-negative staining on etoposide-exposed mitotic chromosomes (Fig. 3C and D). These results implied that RNF4 might function as STUbL to promote poly/multi-ubiquitin assembly on etoposide-exposed mitotic chromosomes. Indeed, cells depleted for SUMO-2/3 by RNA interference also exhibited reduced accumulation of FK2 signals on etoposide-exposed chromosomes (Supplementary Fig. 5), supporting the idea that poly/multi-ubiquitin assembly on the chromosomes was a consequence of STUbL activity mediated by RNF4. Although the target(s) of RNF4 remains unidentified, the RNF4 and SUMO-2/3 signals as well as FK2 signal appeared to distribute throughout chromosome axes without any specific concentration on chromosomal domains, such as centromeres and telomeres (Supplementary Fig. 2), suggesting that the target(s) might be a protein(s) in basic chromosome structure and/or scaffolds of chromosomes.

3.5. RNF4 is part of a signaling cascade governing resistance to etoposide-induced damage during mitosis

Regardless of the target(s) of RNF4 on mitotic chromosomes, accumulation of SUMO-2/3 followed by poly/multiple-ubiquitin assembly on etoposide-exposed mitotic chromosomes implied the presence of the SUMO-2/3-RNF4-ubiquitin-mediated signaling cascade, which responds to etoposide exposure during mitosis. To investigate the significance of this signaling pathway, we compared the effect of etoposide exposure on cell survival in RNF4-depleted versus non-depleted HeLa cells. As shown in Fig. 4A, cells were transfected either with control siRNA or RNF4-siRNA followed by incubation with nocodazole for 14 h to arrest cells at mitosis. After washing with drug-free medium, cells were incubated in the presence or absence of etoposide for 30 min, followed by incubation under standard (drug-free) culture conditions for 24 h. When cells were not exposed to etoposide, both control- and RNF4 siRNA-transfected cells doubled cell numbers, suggesting that RNF4-depletion might not impair cell division, at least under these experimental conditions (white bars in Fig. 4B). In contrast, we found a decrease of cell numbers in control siRNA-transfected cells as well as RNF4 siRNA-transfected cells upon exposure to etoposide. Importantly, the growth inhibiting effect was enhanced in RNF4 siRNA-transfected cells, indicating that

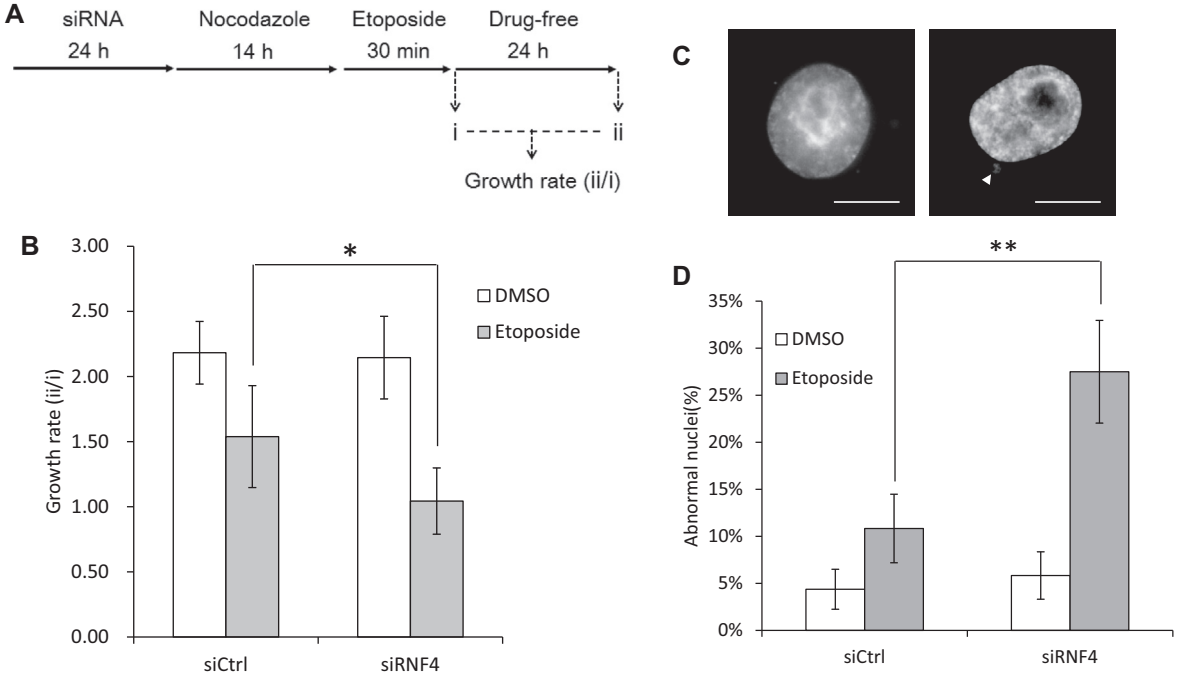

Fig. 4. Effect of RNF4-depletion on cell division after etoposide exposure. (A) Timeline of siRNA transfection and drug treatments. Control siRNA or RNF4-siRNA transfected HeLa cells were arrested at mitosis by 75 ng/ml nocodazole for 14 h, followed by exposure to 50 μM etoposide for 30 min. After washing cells with drug-free medium, cells were cultured under standard conditions for 24 h. Cell numbers were counted at two points, i and ii. (B) The values of growth rate (ii/i) were calculated. The values shown represent means ± SE of three independent experiments. $^{*}P < 0.05$ by *t*-test. (C) Typical nuclear morphologies observed in RNF4-siRNA treated cells after etoposide exposure. DNA was stained with DAPI. The arrowhead indicates the micronucleus. Bars indicate 20 μm. (D) Numbers of cells with/without micronuclei were counted. Cells with micronuclei versus cells without micronuclei were counted. The values shown represent means ± SE of three independent experiments. $^{**}P < 0.01$ by *t*-test.

RNF4-depletion sensitized mitotic HeLa cells to etoposide exposure (gray bars in Fig. 4B). It should be mentioned that we often observed cells with micronuclei in RNF4 siRNA-transfected cells, indicating that abnormal chromosomal segregation and/or unwanted reassembly of the nuclear membrane around the chromosomes were induced during progression and after completion of mitosis in RNF4-depleted cells (Fig. 4C and D). Together, these results indicated that RNF4-depletion might increase the sensitivity of mitotic HeLa cells to etoposide exposure, leading to a reduction of cell survival rate upon etoposide treatment. Since this DNA topoisomerase II poison is successfully and extensively used in chemotherapy for various types of cancers in children and adults [18–21], depletion and/or inhibition of RNF4 activity could be an effective protocol that could sensitize etoposide-based chemotherapy for cancer patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.106.

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