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Plk1 is negatively regulated by RNF8

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ABSTRACT

RNF8 is a nuclear protein having an N-terminal forkhead-associated (FHA) domain and a C-terminal RING-finger (RF) domain. Depletion of RNF8 caused cell growth inhibition and cell cycle arrest at not only S but also G2/M phases. In addition, cell death was frequently observed in RNF8-depleted cells. Analyses of time-lapse microscopy revealed that the cells died in mitosis and interphase. To elucidate the RNF8 function in M phase, the Plk1 content in RNF8-depleted cells was examined. The amount of RNF8 decreased time-dependently, whereas Plk1 reciprocally increased by transfection of RNF8 siRNA. Protein contents of RNF8 and Plk1 among various cell lines were also compared. RNF8 in normal cell lines was much higher than that in many cancer cell lines. Conversely, Plk1 in normal cell lines was lower than in cancer cell lines. These results suggest that RNF8 is downregulated in many cancer cells and inversely correlated with Plk1.

1. Introduction

RNF8 is a 485-residue nuclear protein having an N-terminal forkhead-associated (FHA) domain and a C-terminal RING-finger (RF) domain [1,2]. FHA recognizes and interacts with specific phosphopeptides, and RF has ubiquitin E3 ligase activity. RNF8 is shown to participate in DNA damage response including ionizing radiation (IR), and also interacts with ATM-mediated phosphorylated MDC1 which is bound to γ-H2AX at IR-induced subnuclear foci [3–6]. Subsequently, other important DNA damage response proteins including 53BP1 and BRCA1 are recruited to DNA damaged sites. In this DNA repair process, histone H2AX ubiquitination was catalyzed by Ubc13-RNF8. In addition to its function during DNA damage response, RNF8 has been reported to play a role in cell cycle progression especially in M phase [7,8]. Overexpression of RNF8 led to a delay in cytokinesis and the frequent occurrence of aberrant mitotic apparatus formation, while its depletion caused a delay in the exit from the mitotic arrest induced by nocodazole, associated with a weaker degradation of cyclin B1. Human RNF8 localized at kinetochores, centrosomes, and the midbody at M phase [8,9]. Furthermore, other functions of RNF8 were discovered by analysis of RNF8 mutant mice. Rnf8-deficient mice are viable but have multiple phenotypes including a growth retardation phenotype, increased sensitivity to IR, and impaired spermatogenesis [10–12]. *Rnf8* mutant mice are immunodeficient, because IgH class switch recombination is impaired in a gene dose-dependent manner in these mice. *Rnf8*$^{-/-}$ mice exhibit increased genomic instability and elevated risks for tumorigenesis, which indicates that Rnf8 is a tumor suppressor [10].

Four members of the polo-like kinase have been identified in humans (Plk1–4) [13]. Plks have a highly conserved polo-box domain (PBD) in the carboxy-terminal part of the protein. By phosphorylating different substrates, Plk1 controls a number of processes throughout cell division in vertebrate cells [13,14]. In the absence of Plk1, animal cells fail to establish a bipolar spindle and to properly attach kinetochores to the microtubules. Instead, a monopolar spindle surrounded by a characteristic circular arrangement of chromosomes is induced by Plk1 inhibition. Plk1 localizes to centrosomes and kinetochores in almost all stages of mitosis, and it also accumulates at the spindle midzone and the midbody at the onset of anaphase. This striking localization of Plk1 is mediated by PBD, which is thought to be an important mechanism to both temporally and spatially regulate Plk1 activity toward specific substrates. Consistent with its multiple functions in promoting mitosis, the overexpression of Plk1 has been observed in many types of tumors, including breast cancer, colorectal cancer and ovarian cancer [15,16].

Here, we report that RNF8-depleted cells were arrested at S and G2/M phases. Cell death was also observed in both interphase and

Abbreviations: FHA, forkhead-associated; RF, RING-finger; IR, ionizing radiation; FCS, fetal calf serum; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; BSA, bovine serum albumin.

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M phase. Plk1 content was upregulated by depleting RNF8. Expression levels of RNF8 in normal cell lines were much higher than in cancer cell lines, showing a negative correlation with Plk1.

2. Materials and methods

2.1. Cell culture, transfection, and cell count

HeLa (cervical cancer), WI-38 (normal fibroblasts), and IMR-90 (normal fibroblasts), HEK293T (renal cell carcinoma), RKO (colon cancer), Saos-2 (osteosarcoma), U2OS (osteosarcoma), HT1080 (fibrosarcoma), TC-135 (Ewing's sarcoma), and Caco-2 (colon cancer) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). NHDF (normal human dermal fibroblasts), astrocytes, and HAEC (human aortic endothelial cells) were purchased from Lonza and cultured in fibroblast growth medium (FGM-2 BulletKit), astrocyte growth medium (AGM BulletKit), and endothelial growth medium (EGM-2 BulletKit), respectively. NB69 (neuroblastoma) were cultured with RPMI-1640 medium supplemented with 15% FCS. SK-N-SH (neuroblastoma) and SH-SY5Y were cultured in α-minimum Eagle's medium supplemented with 10% FCS. HepG2 (hepatocellular carcinoma), MCF-7 (breast cancer), and various colon cancer cell lines (LoVo, SW480, Colo201, Colo320DM, LS174T, H630, HT29, HCT116, HCT15, DLD-1) were cultured in RPMI-1640 medium with 10% FCS. Transfection of siRNA was performed using Lipofectamin RNAiMAX (Invitrogen) according to the manufacturer's protocol. Two siRNAs for RNF8 (siRNF8-1: GGACAAUUAUGGACAACAAdTdT, siRNF-2: GGAGAUAGCCCAAGGAGAAdTdT) and that of luciferase (siGL3: CUUACGCUGAGUACUUCGAdTdT) as a control were used.

2.2. Flowcytometry

For cell-cycle analyses, the cultured cells were labeled with 20 μM BrdU (Roche) for 1 h before harvesting, then were harvested and fixed with 70% ethanol for overnight. They were then denatured, incubated with in FITC-conjugated anti-BrdU antibody for 1 h at room temperature, followed by incubation with 5 μg/ml propidium iodide in PBS. Subsequent flowcytometric analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA).

For analysis of Plk1 expression, the cells fixed in 70% ethanol overnight were extracted on ice with 0.25% Triton X-100/PBS for 5 min, washed with phosphate buffered saline (PBS), and then incubated with an antibody against Plk1 (Cell Signaling #4513) diluted in 1% bovine serum albumin (BSA)/PBS for 1 h. The cells were then washed with PBS, incubated for 30 min with Alexa-Fluor 488-conjugated anti-rabbit antibody (Molecular Probes) diluted in 1% BSA/PBS, washed again, and resuspended in PBS supplemented with 25 μg/ml RNase (Roche) and propidium iodide. After 20 min incubation, the cells were analyzed by flowcytometry (Becton Dickinson).

2.3. Western blotting

The cells were lysed in a Nonidet P-40 extraction buffer (0.5% Nonidet P-40, 50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM DTT, 5 mM EGTA, 1 mM EDTA) supplemented with complete protease inhibitor (Rosche). The lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was recovered. Proteins (100 μg/lane) were subjected to SDS–polyacrylamide gel electrophoresis and transferred to Immobilon (Millipore) by standard methods. Membranes were blocked for 2 h in TBST (0.1% Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.6) with 3% nonfat dry milk (blocking buffer). Probing with the specific antibody against Plk1 and RNF8 (Abcam #4183) and β-actin (Sigma A-2066) (1:1000) was carried out for 1 h at room temperature in the blocking buffer containing 0.02% sodium azide. After washing four times in TBST, the membranes were probed with 200 ng/ml peroxidase-labeled goat anti-rabbit or anti-mouse IgG for 1 h in TBST. After further extensive washing in TBST, the membranes were processed for enhanced chemiluminescence using Chemi-Lumi One L reagents according to the manufacturer's instructions (Nacalai Tesque, Kyoto). Exposures were carried out using LAS-4000 IR multicolor (Fujifilm, Tokyo).

2.4. Immunofluorescence microscopy

HeLa cells, plated on coverslips in 35-mm dishes, were transfected with siRNA for RNF8 or GL3 as described above. After 60 h the cells were washed in PBS, pH 7.4, fixed for 10 min in 4% paraformaldehyde in PBS, washed twice in PBS, and permeabilized for 3 min with 0.2% Triton X-100 in PBS. After blocking with 0.1% BSA in PBS, the coverslips were incubated for 2 h with a rabbit polyclonal antibody against Plk1, washed with PBS, and then incubated for 2 h with Alexa-546-conjugated goat anti-rabbit antibody. All procedures were performed at room temperature.

2.5. Time-lapse imaging

HeLa cells were transfected with siRNA for RNF8 or GL3. Images of RNF8 or GL3 siRNA transfected cells were monitored by a time-lapse microcopy system using a BIOREVO BZ-9000 microscope (Keyence, Osaka).

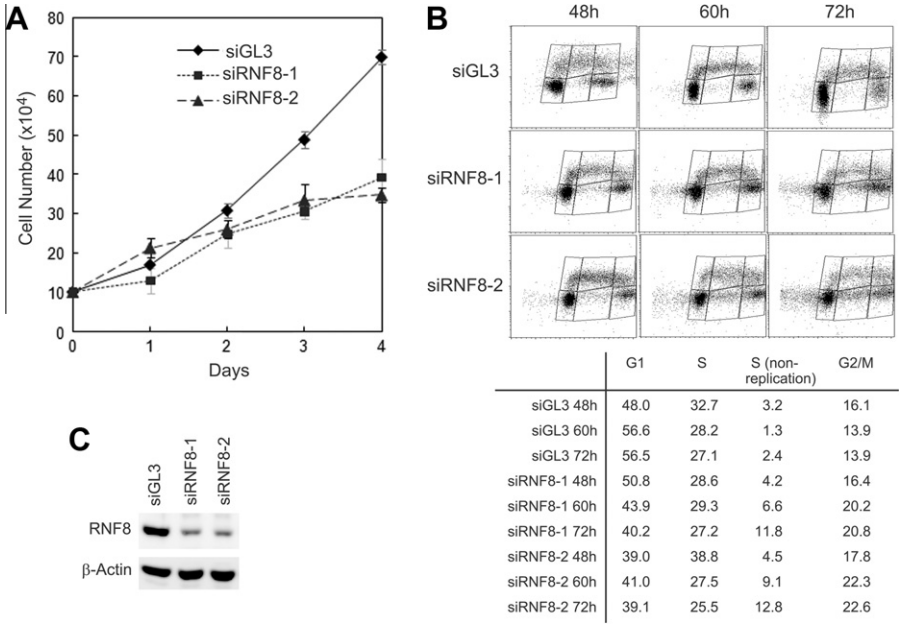
3. Results

3.1. Inhibition of cell growth by RNF8 knockdown

In order to investigate the function of RNF8 in cell growth, two siRNAs termed siRNF8-1 and siRNF8-2 were transfected to HeLa cells and cell numbers were counted for 4 days. Cell growth was inhibited in RNF8 siRNA-transfected cells (Fig. 1A). To clarify whether these cells were dead or just arrested, flowcytometrical analyses were performed. The RNF8 siRNA transfected cells were pulse labeled with BrdU and its incorporation was studied with anti-BrdU antibody. The percentages of S phase without DNA replication in siRNF8-1, siRNF8-2, or siGL3 at 72 h were 11.8, 12.8, or 2.4, respectively. These results suggest that RNF8 siRNA transfected cells were arrested at the S phase without BrdU incorporation (non-replicating). At the same time point, the percentage of cells at the G2/M phase of siRNF8-1 or siRNF8-2 transfected cells was high (150% or 163%) compared with control. These results indicate that RNF8 siRNA transfected cells were also arrested at G2/M phase. In addition, a part of the cells showed a lower intensity of propidium iodide staining, indicating that RNF8 siRNA caused cell death. Control GL3 siRNA transfected cells showed normal distribution of cell cycle stages (Fig. 1B). Under this condition, the lower RNF8 protein content was confirmed in RNF8 siRNA-transfected cells compared with control siRNA-transfected cells (Fig. 1C). These results suggest that RNF8 has functions in S phase and G2/M phase progression and in the checkpoint mechanism.

3.2. Cell death induced by RNF8 depletion

To study the cell death induced by RNF8 depletion, time-lapse images of RNF8-depleted HeLa cells were captured from 60 to 66 h after siRNA transfection. The representative images of the cells transfected with control or RNF8 siRNA are shown in Fig. 2. Many of the cells transfected with siRNF8-2 showed a rounded shape; 23% and 42% of the siRNF8-1 and siRNF8-2 transfected cells



	G1	S	S (non-replication)	G2/M
siGL3 48h	48.0	32.7	3.2	16.1
siGL3 60h	56.6	28.2	1.3	13.9
siGL3 72h	56.5	27.1	2.4	13.9
siRNF8-1 48h	50.8	28.6	4.2	16.4
siRNF8-1 60h	43.9	29.3	6.6	20.2
siRNF8-1 72h	40.2	27.2	11.8	20.8
siRNF8-2 48h	39.0	38.8	4.5	17.8
siRNF8-2 60h	41.0	27.5	9.1	22.3
siRNF8-2 72h	39.1	25.5	12.8	22.6

Fig. 1. Inhibition of cell growth by RNF8 knockdown. (A) HeLa cells were transfected with either siRNAs for RNF8 (siRNF8-1and siRNF8-2) or luciferase (siGL3) and the cell number was counted at indicated timepoints. The experiment was repeated three times and similar results obtained. (B) HeLa cells were transfected with RNF8 or GL3 siRNA and labeled with BrdU for 1, 48, 60, or 72 h after transfection. Then, the cells were fixed, incubated with anti-BrdU antibody and propidium iodide, and analyzed by flowcytometry. A representative result is shown. The distribution of cells corresponding to the boxed areas is presented in the table beneath the graph. (C) The cells transfected with RNF8 or GL3 siRNA were subjected to Western blotting with antibodies against RNF8 and β-actin after 72 h.

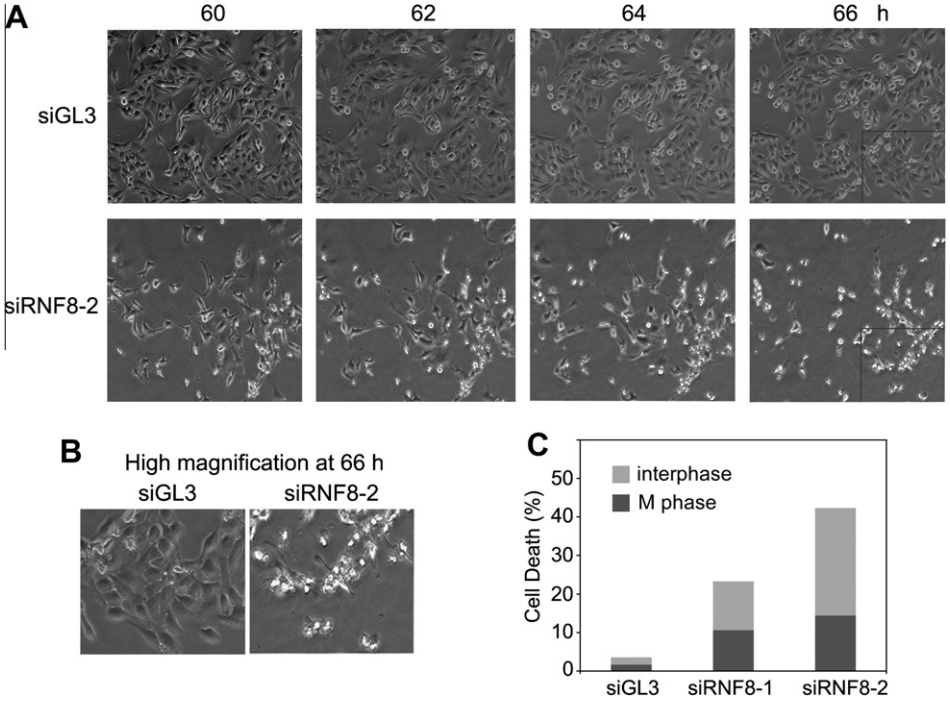
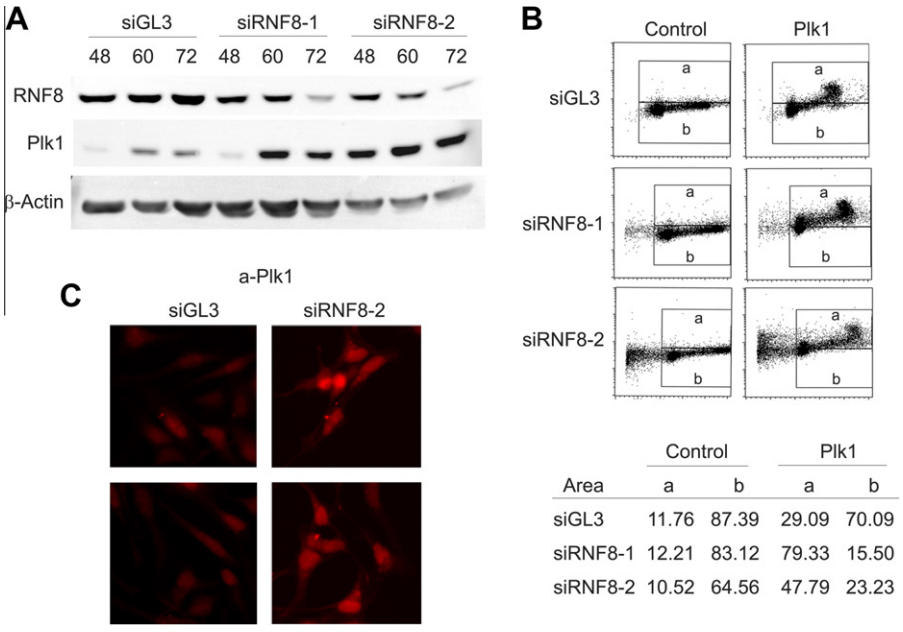


Fig. 2. Time-lapse imaging of RNF8-depleted HeLa cells. HeLa cells were transfected with either GL3 or RNF8 siRNA. (A) The cells were monitored by time-lapse microcopy from 60 to 66 h after transfection. (B) Magnified images are also shown. (C) Percentage of cells which died at M phase (dark gray) and interphase (light gray) during the 60 to 66 h were counted. Averages of two independent experiments are shown.

died, respectively (Fig. 2). Control GL3 siRNA transfected cells have normal morphology and the percentage of their cell death was 4%. In the siRNF8-transfected cells, death occurred in both interphase (light gray) and M phase (dark gray), suggesting that RNF8 has functions at multiple points during the cell cycle (Fig. 2C).

3.3. Increase in Plk1 content by RNF8 depletion

CHFR and RNF8 are related proteins having FHA and RF. RNF8 was reported to play a role in mitotic exit, whereas CHFR has a role in the antephase checkpoint early in M phase and induces destruc-



	Control		Plk1	
Area	a	b	a	b
siGL3	11.76	87.39	29.09	70.09
siRNF8-1	12.21	83.12	79.33	15.50
siRNF8-2	10.52	64.56	47.79	23.23

Fig. 3. Effects of RNF8 knockdown on Plk1 protein level. (A) HeLa cells were transfected with either RNF8 or GL3 siRNA and total cell extracts were subjected to Western blotting with antibodies against RNF8, Plk1, and β-actin. (B) HeLa cells were transfected with either GL3 or RNF8 siRNA for 60 h. The cells were fixed with 70% ethanol, stained with propidium iodide and anti-Plk1 antibody and then subjected to flowcytometry. The experiments were repeated three times and a representative is shown. (C) HeLa cells were transfected with either GL3 or RNF8 siRNA for 48 h and stained with anti-Plk1 antibody and DAPI. They were observed by immunofluorescence microscopy and representative images are shown.

tion of Plk1 by ubiquitination [17,18]. To study the RNF8 function in M phase, it is intriguing to examine the protein content of Plk1 in RNF8-depleted cells, so time sequential changes in RNF8 and Plk1 contents were examined. Protein content of RNF8 in its siRNA transfected cells decreased gradually from 48 to 72 h, whereas Plk1 content increased from 48 to 72 h reciprocally with RNF8 depletion (Fig. 3A). Both of the RNF8 siRNAs have strong effects, but siRNF8-2 more greatly affects the depletion of the protein. Few changes were observed in β-actin, indicating that almost identical protein amounts were loaded on the lanes. Flowcytometrical analysis was performed to determine the cell cycle stages when Plk1 content increased. The RNF8 siRNA-transfected cells were stained with anti-Plk1 antibody and propidium iodide, and content of Plk1 at each cell cycle stage was analyzed. Percentages of area a (stained with anti-Plk1 antibody) increased in RNF8 siRNA-transfected cells compared with control siRNA transfected cells, but area b (unstained with anti-Plk1 antibody) decreased. As shown in Fig. 3B, Plk1 appeared to increase independently of cell cycle stage. Subcellular localization and intensity of Plk1 in RNF8 or GL3 siRNA transfected cells were studied. Localization of Plk1 in nuclei was observed in siRNF8-2 transfected cells, but only a faint signal was detected in GL3 siRNA transfected cells in interphase (Fig. 3C). The decreased RNF8 content correlated with the increase in Plk1 protein amount, suggesting that RNF8 regulated the Plk1 expression level.

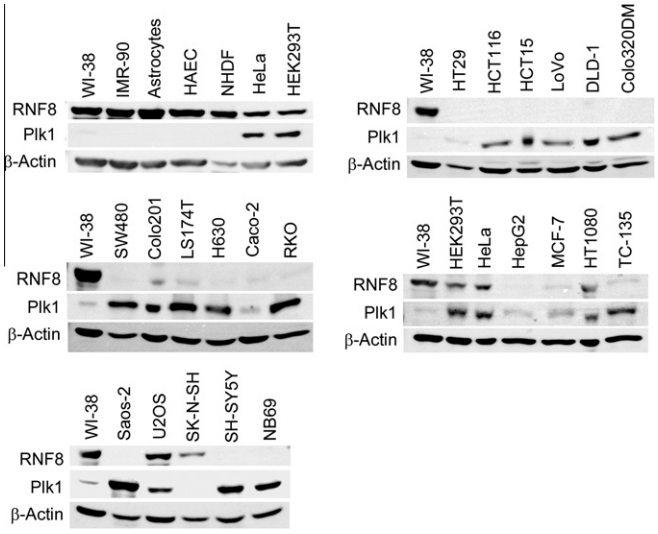


Fig. 4. Protein expression profiles of RNF8 and Plk1 in various cell lines. Total proteins from 5 normal and 23 cancer cell lines were subjected to Western blotting with antibodies against RNF8, Plk1, and β-actin. WI-38, IMR-90, Astrocytes, HAEC, and NHDF are normal cell lines and the others are cancer cell lines. WI-38 was examined in each panel to confirm that experiments were done under equivalent conditions.

3.4. Protein content of RNF8 is lower in cancer cells, showing a negative correlation with Plk1 amount

RNF8 knockout mice have a higher tumorigenicity and RNF8 is thought to be a tumor suppressor gene. In order to study the expression of RNF8 in human cancer cells, Western blotting was performed using total proteins from 5 normal and 23 cancer cell lines. Decreased expression of RNF8 was observed in some of these lines including HeLa, U2OS, and HEK293T compared with normal cell lines (WI-38, IMR-90, Astrocytes, HAEC, and NHDF, and in most of the cancer cell lines it was hardly detectable (Fig. 4). Conversely, the Plk1 protein amount in cancer cell lines was higher than in normal cell lines. For comparison, WI-38 (normal fibroblast) is shown in each panel. The amount of β-actin from these cell lines is shown to indicate that the identical amount of proteins was loaded.

4. Discussion

Only five proteins have both FHA and RF: CHFR and RNF8 in metazoans and SpDma1, and ScDma1 and ScDma2 in yeasts

[1,17–21]. In budding yeast, ScDma1 and ScDma2 have a role in proper spindle positioning and ScDma2 overexpression induces defects in septin ring disassembly at the end of mitosis and in cytokinesis [19]. SpDma1, a multicopy suppressor of cdc16 mutation, is required if the assembly of the spindle is to be prevented. SpDma1 is thought to be a component of the spindle assembly checkpoint, which is required to prevent premature septum formation and premature exit from mitosis if spindle function is impaired [20,21]. In addition, Dma1 localizes to spindle pole bodies and it antagonizes Plo1p localization. CHFR, another FHA-RF containing protein in mammals, has ubiquitin ligase (E3) activity to create ubiquitin chains on target proteins including Plk1 [17,18]. In addition to DNA damage response, RNF8 has a function in M phase like other FHA-RF containing proteins. Overexpression of RNF8 leads to a delay in cytokinesis and the frequent occurrence of aberrant mitotic apparatus formation. Depletion of RNF8 caused a delay in the exit from the mitotic arrest induced by nocodazole, associated with lower degradation of cyclin B1. Human RNF8 localized at kinetochores, centrosomes, and the midbody at M phase [8,9]. In the present investigation, RNF8-depleted cells were shown to arrest at G2/M phase as well as S phase. RNF8-depleted cells frequently died in M phase in addition to interphase. These results suggest that RNF8 plays an important role(s) in M phase like other FHA-RF containing proteins. Transfection of RNF8 siRNA caused a significant reduction of RNF8 and increase in Plk1. The RNF8 levels in normal cell lines were much higher than in cancer cell lines, whereas Plk1 protein amounts in normal cells were low. Subcellular localization of RNF8 at kinetochores, centrosomes, and midbody is exactly the same as that of Plk1. It is tempting to speculate that one of the target proteins of RNF8 at M phase is Plk1. It is reported that CHFR ubiquitinates Plk1 for its breakdown [17,18]. These results suggest that CHFR and RNF8 may have overlapping targets and/or functions in mitosis.

Plk1 controls a number of processes throughout cell division in vertebrate cells [13,14]. Consistent with its multiple functions in promoting mitosis, overexpression of Plk1 has been observed in many tumor types, including breast cancer, colorectal cancer and ovarian cancer [15,16]. CHFR, a highly related protein with RNF8, is downregulated in a number of human cancer cell lines and primary tumors [22,23]. CpG methylation and deacetylation of histones H3 and H4 in the CpG-rich regulatory region of CHFR gene were observed. RNF8 was also downregulated in many cancer cell lines, but its expression profiles in human cancer specimen have not yet been investigated. The mechanism of RNF8 protein downregulation remains to be determined.

RNF8 has been shown to participate in the DNA damage response including ionizing radiation (IR). RNF8 interacts with phosphorylated MDC1 which binds to γ-H2AX at IR-induced subnuclear foci. Other DNA damage response proteins including 53BP1 and BRCA1 are recruited to DNA damaged sites depending on histone H2AX ubiquitination catalyzed by Ubc13-RNF8. Rnf8-deficient mice are viable but have phenotypes including a growth retardation phenotype, increased sensitivity to IR, and impaired spermatogenesis. *Rnf8* mutant mice are immunodeficient, because IgH class switch recombination is impaired in a gene dose-dependent manner in these mice. These biological processes include DNA strand break and joining. In addition, RNF8 also plays an important role in spermatogenesis through histone ubiquitination, resulting in trans-histone acetylation and global nucleosome removal. Cell growth inhibition and cell cycle arrest at S and G2/M phases were induced by depletion of RNF8, suggesting that RNF8 has a function in S phase in addition to G2/M phase and checkpoint control.

In conclusion, we have shown that cell growth inhibition and cell cycle arrest at both S and G2/M phases were induced by depletion of RNF8. Plk1 content in RNF8-depleted cells increased. Protein content of RNF8 in normal cells was much higher than in cancer cells, which has a negative correlation of Plk1 protein amount.

Acknowledgments

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