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Original

RING1A Regulates RING1B Expression and the Collapse of its Expression Impairs Neuroblastoma Cell Proliferation

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SUMMARY

Neuroblastoma (NB) is the most common pediatric abdominal solid tumor with less than 50% of long-term survival rate in high-risk cases. Ring finger protein 1/2, referred to as RING1A/B respectively, are E3 ubiquitin ligases that compose a catalytic subunit of Polycomb Repressive Complex-1 (PRC1). PRC1 epigenetically down-regulates target gene transcription, especially tumor suppressor genes in tumorigenesis and cancer progression. However, the function of RING1 proteins is still unclear in NB. This study aims to explore the importance of RING1A (gene name, *RING1*) in human NB cells to evaluate its druggability.

According to the Kaplan-Meier analysis based on the public NB patients' transcript data, lower expression of *RING1* showed poor prognosis. As such, we hypothesized the presence of the anti-tumorigenic function of RING1A. First, mouse Ring1A (mRing1A ; gene name, *Ring1*) was transiently expressed in a NB cell line, NGP. The subsequent flat colony formation assay resulted in a decreased number of colonies, and intriguingly, endogenous RING1B expression was dampened in the transfectants. On top of that, we established NGP cells with inducible short hairpin (sh) RNA against *RING1*. Unexpectedly, short hairpin (sh) *RING1* induction repressed cell proliferation. However, consistent with the transient expression of mRing1A, endogenous RING1B expression was elevated up on the sh*RING1* induction.

Here, we showed that both gain and loss of RING1A expression suppressed NGP cell growth, and RING1A negatively controlled RING1B expression. Although the druggability of RING1A is still unclear, the current study suggests that RING1A does not functionally compensate RING1B, and the optimal expression of RING1A is essential for NB cell proliferation.

Key Words : RING1A, RING1B, neuroblastoma

INTRODUCTION

Neuroblastoma (NB) is the most common pediatric abdominal solid tumor derived from the sympathetic nervous system. The overall prognosis of patients with NB has markedly improved with advanced therapies. However, high-risk NB is still one of the most difficult tumors to cure, with only 50% of patients achieving long-term survival despite intensive multimodal therapy[1)]. Multiple genetic abnormalities, such

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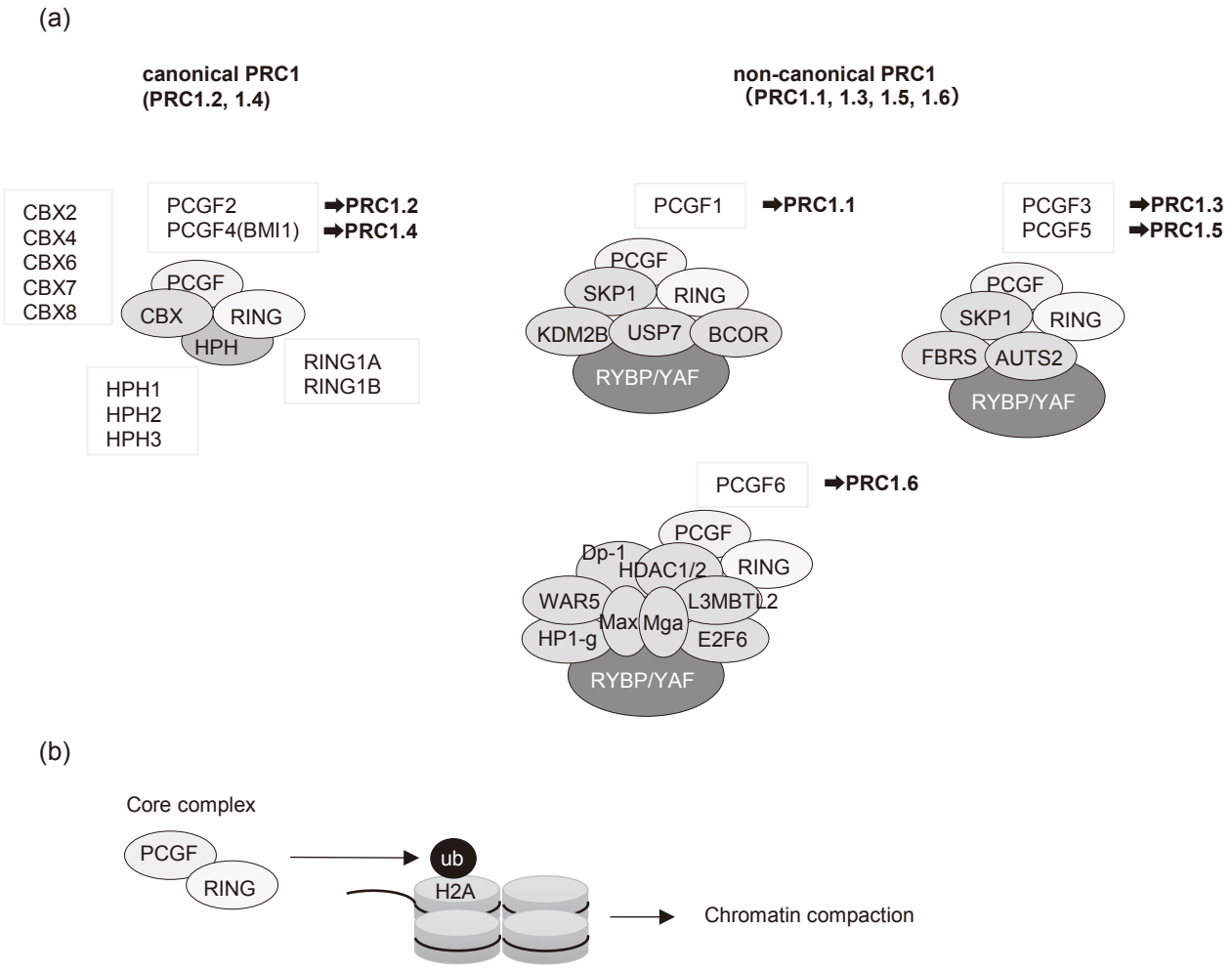

Fig. 1

Schematic of canonical PRC1 component and mechanism of chromatin repression. (**a**) Canonical PRC1 consists of 4 components : PCGF2/4, CBX, HPH and RING. Non-canonical PRC1 is composed of RING proteins, PCGF1/3/5/6, RYBP/YAF2, and other co-factors. (**b**) PCGF and RING form a heterodimer and function as an E3 ubiquitin ligase. H2AK119Ub causes chromatin compaction leading target gene silencing.

as aneuploidy, chromosomal gains (extra copies of 17q) and losses (allelic loss of 1p and/or 11q), amplification of chromosomal material (*MYCN*), reconstruction of telomerase reverse transcriptase (TERT) promoter, and specific mutations in anaplastic lymphoma kinase (*ALK*) and alpha-thalassemia/mental retardation, X-linked (*ATRX*) genes appear to reflect the different clinical outcomes[2~4].

Polycomb group (PcG) proteins are considered to act as transcriptional repressors that are required for maintaining the correct spatial and temporal expression of homeotic genes during embryological development[5]. PcG proteins form multiprotein complexes, known as Polycomb Repressive Complex 1 (PRC1) and PRC2. PRC1 mono-ubiquitinates histone H2A at lysine 119 (H2AK119Ub), whereas PRC2 methylates histone H3 at lysine 27 (H3K27me3) in the target gene silencing. Canonical PRC1 consists of four proteins including polycomb group ring finger proteins 2 and 4 (PCGF2/4), chromobox homolog (CBX), polyhomeotic homolog (HPH) and RING proteins, whereas non-canonical PRC1 encompasses RING proteins, PCGF1/3/5/6, RING1 and YY1 binding protein (RYBP)/YY1 associated factor 2 (YAF2), and other co-factors[6] (Fig. 1a). Among them, RING proteins (RING1A/B) interact with PCGF family (PCGF1-6) to form a heterodimer and function as an E3 ubiquitin ligase (Fig. 1b).

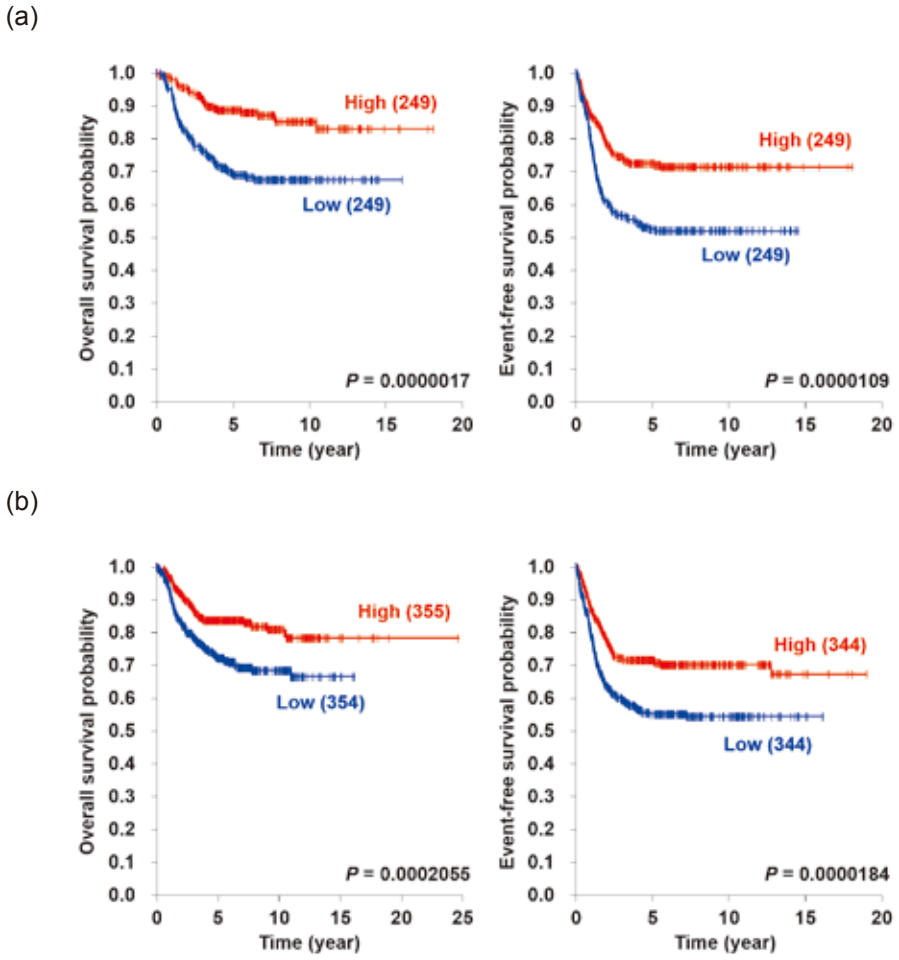

Fig. 2

Relationship between clinical outcome and *RING1* expression on messenger RNA (mRNA) level. (**a**) Overall and event-free Kaplan-Meier curves for 498 of NB patients with low versus high *RING1* mRNA expression (GE62564). (**b**) Overall and event-free Kaplan-Meier curves for 709 of NB patients with low versus high *RING1* mRNA expression (E-MTAB-1781).

The association of PRC1 with cancer development was first revealed in 1991 by Haupt et al. and Lohuizen et al.[7,8]. They identified mouse *Pcgf4*, also referred to as *Bmi-1*, as a proto-oncogene that cooperates with MYC to promote the generation of B- and T-cell lymphomas. Later studies demonstrated that BMI-1 inhibits MYC-induced apoptosis through suppression of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus[9,10]. *CDKN2A* encodes two structurally distinct tumor suppressors, INK4A and ARF, that restrict cell growth in response to abnormal mitogenic signals. Afterwards, overexpression of BMI-1 has been reported in several types of human cancers, including mantle cell lymphoma[11], colorectal carcinoma[12], liver carcinoma[13], non-small cell lung cancer[14] and small cell lung cancer[15]. In NB, Ochiai et al. previously showed that MYCN directly induced BMI-1 and regulated tumorigenesis through repression of tumor suppressor genes, tumor suppressor in lung cancer1 (*TSLC1*) and kinesin family member1Bβ (*KIF1B*β)[16]. Yi et al. also revealed that MYCN recruited RING1B, inhibited fatty acid elongase 2 (*ELOVL2*) transcription leading docosahexaenoic acid repression, and promoted NB cell growth[17]. Previously, RING1A was presumed to play an adjunct role to RING1B and other PRC1 components, but very recently, a few papers suggested that RING1A acts as an oncogene[18~20]. However, the specific roles

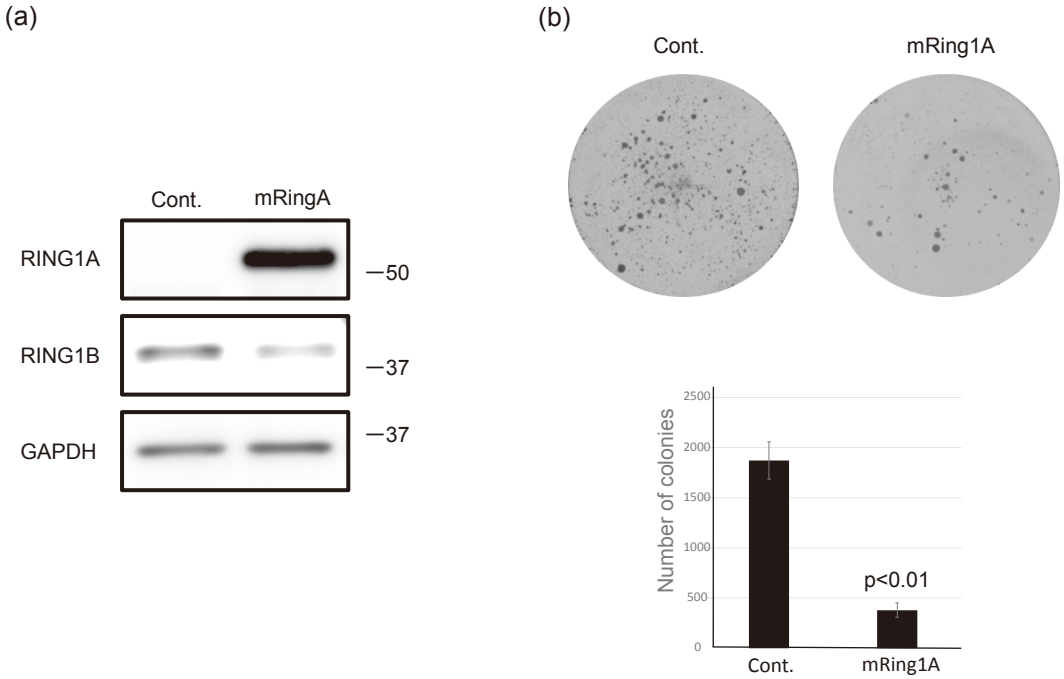

Fig. 3

Over expression of mRing1A on NGP. Results were representative of three independent experiments. (**a**) Expression of RING1A and RING1B after mRing1A over expression was confirmed by Western blot analysis. (**b**) Cell viability was investigated by flat colony assay on the 19th day after transfection.

and druggability of RING1A have not been examined in NB. In this study, we demonstrated the gain and loss of the RING1A expression in a NB cell line, NGP, to verify the importance of RING1A in NB.

MATERIALS AND METHODS

1. Kaplan-Meier survival curve analysis

We obtained RNA sequence data of 498 human NB specimens from NCBI Gene Expression Omnibus GSE62564, and 709 human NB specimens from the European Bioinformatics Institutes E-MTAB-1781. Samples were classified into two groups according to the median value of *RING1* expression. The difference of the high/low groups was analyzed by Log-rank test.

2. Cell culture

NGP and HEK293T cells were cultured in RPMI-1640 and D-MEM culture medium (Fuji Film Wako, Osaka, Japan), respectively, supplemented with 10% fetal bovine serum (Gibco/Thermo Fisher Scientific, MA, USA) and 1% Penicillin/Streptomycin, (Fuji Film Wako, Osaka, Japan), at 37℃ in 5% CO_2, moisture condition.

3. Transient expression of mRing1A

RIKEN kindly provided p-CAG-mouse *Ring1* construct. The plasmid was transfected in NGP using Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA), following the manufacture's protocol. pCDH-EGFP plasmid was used as a negative control. One day after the transfection, the cells were subjected to protein extraction and flat colony formation assay.

4. Flat colony assay

To perform a flat colony assay, 1×10^4 cells were seeded into 6-well plates with 1 μg/ml of puromycin. Colonies were visualized by Giemsa staining on the 19th day after the transfection. The number of colonies was automatically counted by ImageQuant LAS 4000 (GE Healthcare Life Sciences, Buckinghamshire, England).

5. Inducible shRNA construction, and transduction

pLKO-Tet-On vector was obtained from Addgene, MA, US. Synthesized sh*RING1* oligo DNAs were

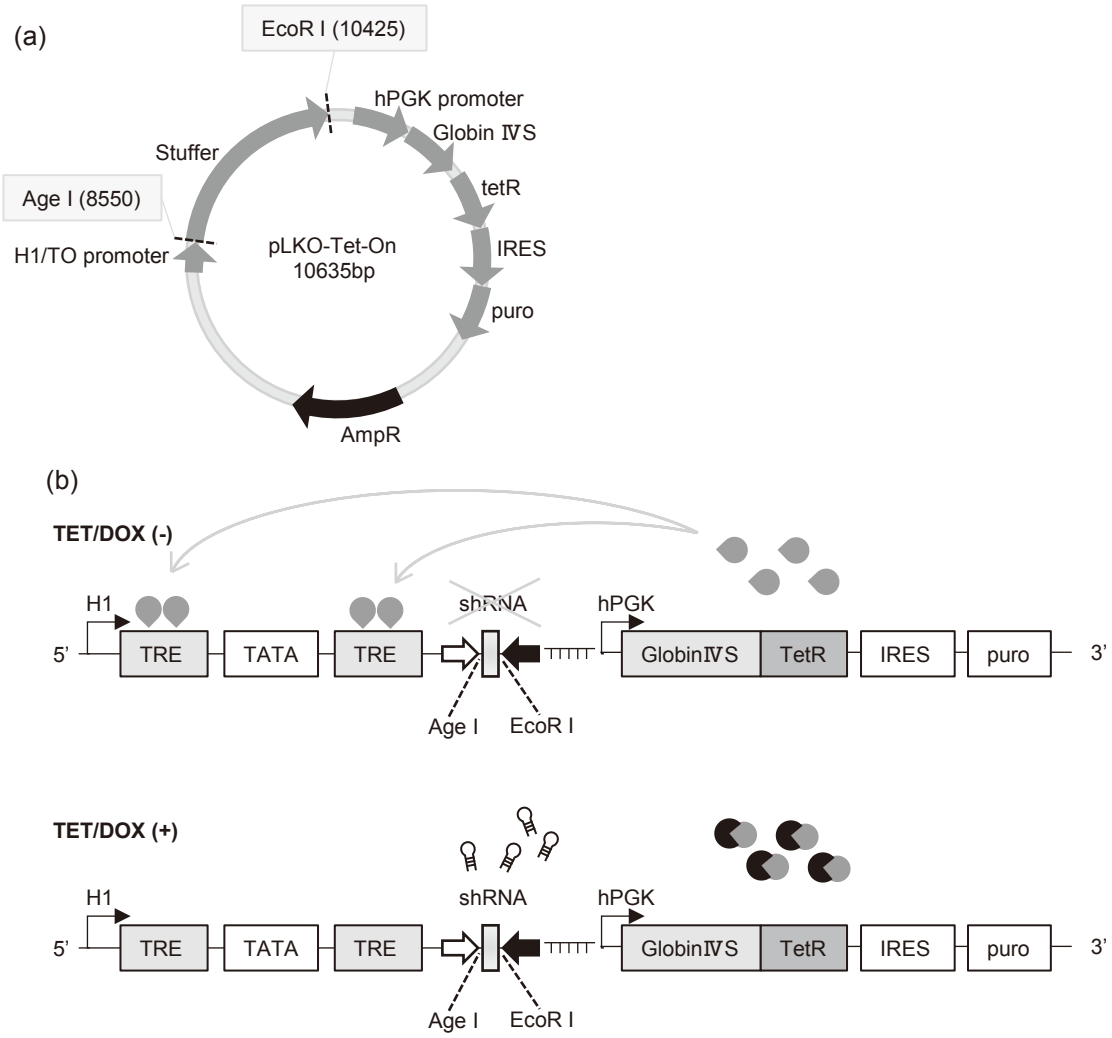

Fig. 4

Structure of pLKO-Tet-On vector and mechanism of inducible knockdown. (**a**) pLKO-Tet-On vector has 2 restriction enzymes, EcoR I and Age I, into which any shRNA can be inserted. (**b**) In the absence of tetracycline (TET)/doxycycline (DOX), shRNA expression is repressed by constitutively-expressed TetR protein. Upon the addition of TET or DOX to the growth media, shRNA expression is triggered resulting in a target gene knockdown.

inserted into the vector following the manufacturer's protocol. The sequences of sh*RING1* #1, #2 are described as below.

sh*RING1*#1 : CCGGGCCCTGATCTCTAAGATCTA
TCTCGAGATAGATCTTAGAGATCAGGGCTTTTT
sh*RING1*#2 : CGGCTGGAGCTGGTGAATGAGAAA
CTCGAGTTTCTCATTCACCAGCTCCAGTTTTT

For virus packaging, HEK293T cells, or human embryonic kidney cells, were transfected with the pLKO-Tet-On vectors and 2nd generation's lentivirus packaging vectors with FuGENE HD reagent (Promega, WI, US). The conditioned medium was centrifuged with Lenti-X concentrator (TAKARA Bio, Shiga, Japan), and concentrated lentiviruses were resuspended in 10% RPMI-1640. NGP cells were then infected in the medium supplemented with 8 μg/ml polybrene. The infected cells were selected with 1 μg/ml of puromycin for 48 hours.

6. RING1A knockdown

To induce sh*RING1*, 100 ng/ml of doxycycline (DOX) was added in growth medium. DOX was sup-

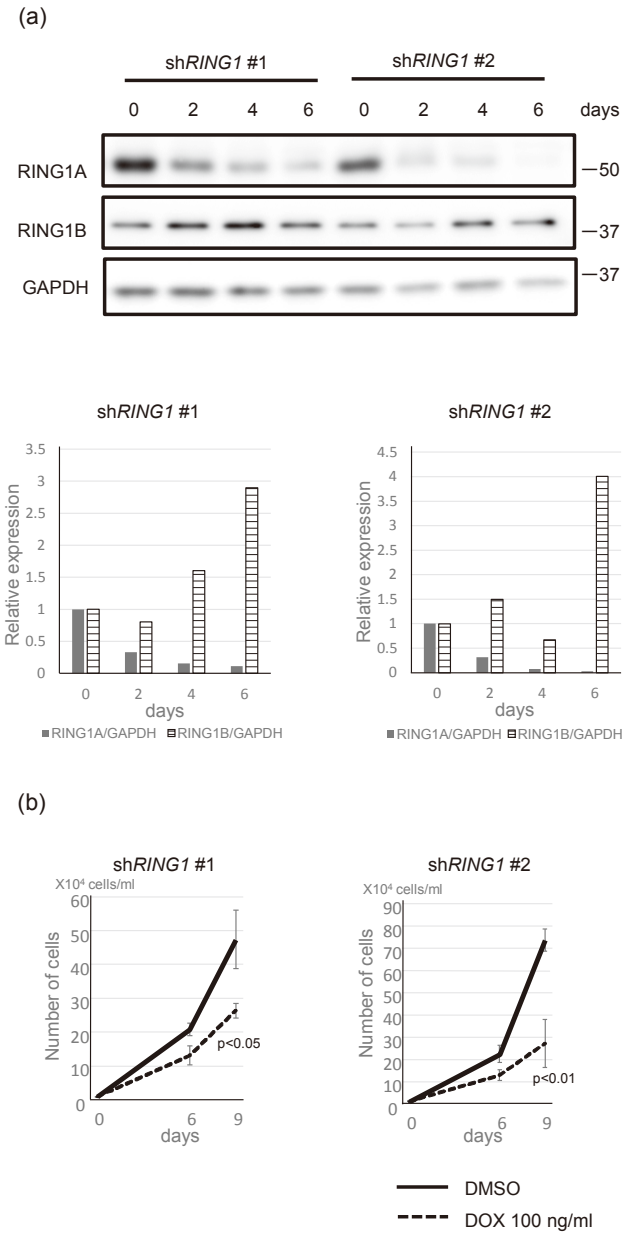

Fig. 5

RING1A knockdown by inducible sh*RING1* #1, #2 in NGP. (**a**) Expression of RING1A and RING1B after RING1A knockdown was confirmed by Western blot analysis. 100 ng/ml of doxycycline (DOX) was added every other day to induce RING1A knockdown. Signals were scanned and analyzed by ImageJ software. RING signals were normalized by GAPDH signals and indicated as bar graphs. (**b**) Cell viability was investigated by counting the number of cells on 6 and 9 days after seeding 1×10^4 cells/5 ml each on the first day. 100 ng/ml of DOX or equal amount of dimethyl sulfoxide (DMSO) was added every other day to induce RING1A knockdown or as a control. Results were representative of three independent experiments.

plemented every other day to sustain sh*RING1* expression. 1×10^4 of NGP transfectants were seeded into 6-well plates, and the number of cells were counted on 6 and 9 days after the cell introduction. Negative control cells were treated with dimethyl sulfoxide (DMSO).

7. Western blotting

For protein extraction, cells were lysed in RIPA buffer (1% NP-40 Substitute (Fuji Film Wako, Osaka, Japan), 0.5% Sodium deoxycholate (Sigma-Aldrich, MO, US), 0.1% SDS (Nacalai Tesque, Kyoto, Japan) in phosphate-buffered saline). Protease inhibitor cocktail (Sigma-Aldrich, MO, US) was supplemented into the lysis buffer just before the usage. 20 μg of lysates were subjected to SDS-PAGE and transferred to PolyVinylidene DiFluoride membranes. The membranes were immersed in blocking buffer (5% skim milk in 0.1% tris-buffered saline with Tween 20) for 1 hour at room temperature. The membranes were then incubated overnight at 4℃ with the following primary antibodies : anti-RING1A (1 : 1000 ; D2P4D, Cell Signaling Technology, MA, USA), anti-RING1B (1 : 500 ; Homemade[21]), and anti-GAPDH (1 : 1000 ; 14C10, Cell Signaling Technology, MA, USA). Afterwards, the membranes were immersed in secondary HRP-conjugated anti-rabbit or mouse IgG antibodies for 1 hour at room temperature. Chemiluminescent signal was detected in ImageQuant LAS 4000 (GE Healthcare Life Sciences, Buckinghamshire, England).

8. Statistical analysis

Means of two experimental groups and three experimental groups were analyzed by two-tailed Student's T test and Tukey's test, respectively.

RESULTS

To shed light on the clinical significance of *RING1* in human NB, we obtained two independent transcriptome data sets of human NB samples (GSE62564 and E-MTAB-1781) and classified into *RING1* high (higher expression level) and low (lower expression level) groups to compare their overall/event-free survival rates. As shown in Fig. 2 (a, b), Kaplan-Meier survival curves of both overall and event-free survival show

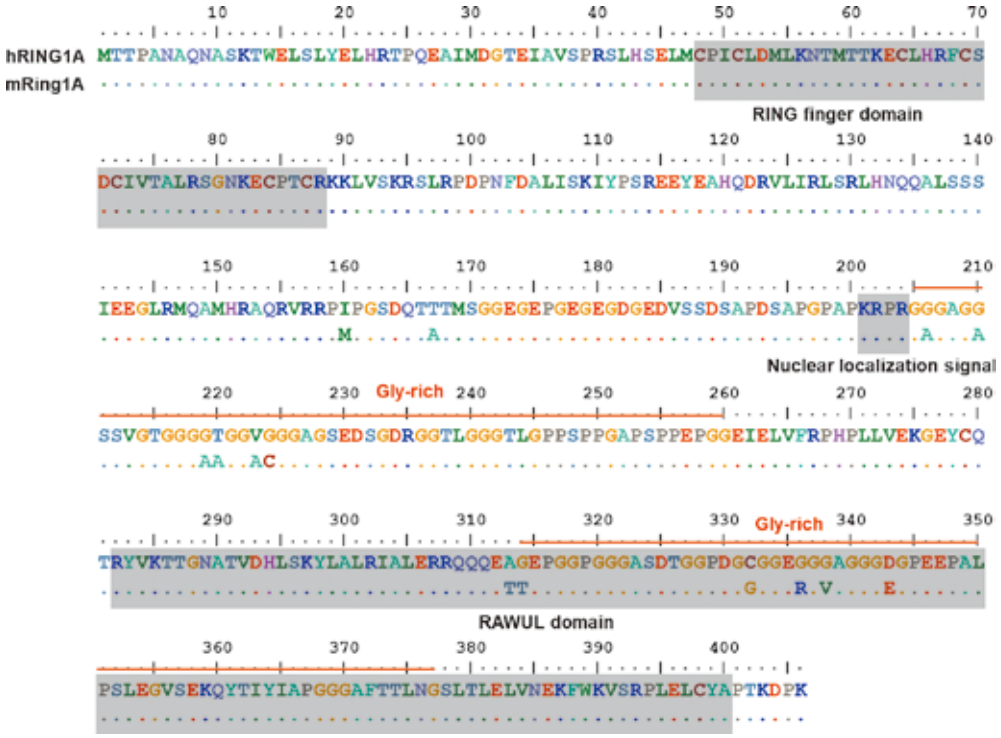

Fig. 6

Amino acid sequences of mouse Ring1A (mRing1A) and human RING1A (hRING1A). Sequences are obtained from NCBI, National Center for Biotechnology Information with accession number NP_033092.3 (mRing1A) and NP_002922.2 (hRING1A).

that the lower expression level of *RING1* indicated worse prognosis.

Hypothesizing the negative effect of RING1A on NB malignancy, we transiently expressed mRing1A in NGP cells (Fig. 3a). As expected, the cell proliferation was impaired in exogenous mRing1A expression (Fig. 3b). Intriguingly, endogenous RING1B expression was depressed in mRing1A overexpression (Fig. 3a).

Next, to obtain the opposite phenotype, we established NGP with two independent inducible sh*RING1* (Fig. 4a, b) and perturb endogenous RING1A expression. To evaluate knockdown efficiency, we treated NGP transfectants with DOX in a time-dependent manner. Western blot analysis resulted in corresponding reduction of RING1A upon the exposure to DOX in a time-dependent manner. Along with the *RING1* reduction, the RING1B expression was elevated in the RING1A knocked down (sh*RING1* #1 : >2.5 folds ; sh*RING1* #2 : >4 folds) (Fig. 5a). However, unexpectedly, sh*RING1* induction also repressed the cell proliferation (Fig. 5b) as well as shown in mRing1A overexpression.

DISCUSSION

In the current study, we focused on RING1A function and its relationship with RING1B expression in NB. Kaplan-Meier survival curves indicated poorer outcome in the *RING1* lower expression group. However, the clinical importance of RING1A is still vague, because the difference between the median value of the *RING1* high/low groups is less than 1.5-fold, suggesting that RING1A is ubiquitously expressed in the samples. Despite the survival analysis in clinical data, both gain and loss of RING1A expression suppressed NGP cell proliferation. This implies that NB cells require adequate RING1A expression for their survivals. In the transient expression, we utilized mouse Ring1A, RAWUL domain of which has mismatches in amino acid sequence, compared to human RING1A (Fig. 6). Since the domain is known to be important for protein-protein interactions, thus it is possible that exogenous human RING1A might cause a unique phenotype in NB cells. Besides, the gain and loss of RING1A expression depressed and elevated RING1B

expression, respectively. Endoh et al. previously reported that RING1A was up-regulated in RING1B-deficient mouse ES cells[22]. Therefore, similar complementarity between RING1A and RING1B proteins possibly occurred in our experiments. On the other hand, RING1B seemed to not functionally compensate RING1A, because, despite the RING1B up-regulation, the RING1A knockdown repressed NB cell proliferation. In embryonic development, RING1B mainly function as an E3 ligase of PRC1 and RING1A substitutes the counterpart, whereas in breast cancer cell lines, RING1B rather promotes transcription through its interaction with enhancer and RING1A is responsible for H2AK119ub[18]. Further studies are required to clarify the functional substitution between RING1A and RING1B in NB.

In this study we did not reveal clinical significance and druggability of RING1A in NB. However, we shed light on the importance of optimal RING1A expression for NB cell viability and expressional substitution of RING1A/B. Further genome-wide association study will uncover its target genes and association with other PcG proteins, that will make us re-evaluate RING1A function in progression and pathogenesis of NB.

CONCLUSION

We declave the importance of optimal RING1A expression for NB cell viability, and expressional substitution of RING1A/B.

Conflicts of Interest and Source of Funding

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