이학석사학위논문

마우스에서 아세칠화 결핍 **BubR1**에 대한 심도 연구

In-depth Investigation of BubR1 Acetylation-deficiency in Mice

2020년 8월

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In-depth Investigation of BubR1 Acetylation-deficiency in Mice

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College of Natural Sciences
at
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By
Taerim Lee

Date Approved

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ABSTRACT

In-depth Investigation of BubR1 Acetylation-deficiency in Mice

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To ensure equal distribution of genetic information during mitosis, the spindle assembly checkpoint (SAC) works to stop cells from passing on to anaphase by generating mitotic checkpoint complex (MCC). MCC binds to anaphase-promoting factor/cyclosome (APC/C) and inhibits the ubiquitination of APC/C substrates.

BubR1 is a key regulator of SAC as a component of MCC along with error correction of kinetochore-microtubule (KT-MT) attachment by ensuring all kinetochores are attached to each microtubule. Previous works showed that BubR1 acetylation at lysine243(in human, K250) is essential in both functions. We suggested that acetylation insufficient mouse (K243R/+)

showed the signs of chromosomal instability (CIN) such as chromosome aberration and heightened aneuploidy rate, weakened SAC, and destabilization of KT-MT attachments. Meanwhile embryos with homozygous acetylation-deficient alleles (K243R/K243R) were lethal approximately in E6.5 primarily due to apoptosis and same incidence was observed from BubR1-deficient (-/-) mice. Interestingly, mice with one acetylation-deficient BubR1 allele (K243R/-) was born without showing abnormal phenotypes and was able to reproduce.

By establishing MEFs, here I showed that K243R/- MEFs exhibit chromosomal instability, weakened SAC and erroneous KT-MT attachment along with instable presence of BubR1 even though APC/C was completely inhibited when compared to WT MEFs.

As further research, detailed molecular interaction must be tested and the key question that how K243R/- MEFs live out through cell cycle and showed comparable results to K243R/+ without WT allele should be enlightened. I expect this study will introduce new insights into the action of acetylation-deficient BubR1.

Keywords : Spindle assembly checkpoint (SAC), BubR1, Mitotic checkpoint complex (MCC), Chromosomal instability (CIN), Kinetochore-Microtubule (KT-MT) attachment

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I. Introduction

Genetic stability is essential in mitosis for the maintenance of genetic information. Spindle assembly checkpoint (SAC) acts as an observer. SAC monitors whether each kinetochore (KT) of chromosome is attached to microtubule (MT) stretching from two spindle poles, which indicates amphitelic interaction, and whether tension is satisfied. If kinetochore-microtubule (KT-MT) interaction is not satisfied or tension is weakened, ‘wait anaphase signal’ is made and SAC is activated sequentially (Lara-Gonzalez et al., 2012).

SAC signal starts by Mad1 and Mad2 interaction with unattached KT and Mad2 constitutes mitotic checkpoint complex (MCC) with BubR1, Bub3, and Cdc20 (Sudakin et al., 2001). MCC binds to anaphase-promoting factor/cyclosome (APC/C) and prevents the activation of Cdc20, a coactivator of APC/C, which provides time to correct attachments (Kim and Yu., 2011). As all KTs bind to MTs and tension is formed, MCC is separated from APC/C. APC/C acts as E3 ubiquitin ligase and degrades cyclin B and securin, which leads to anaphase onset and mitotic exit.

BubR1 mouse models

Among MCC proteins, BubR1 is known to play a major role in the inhibition of APC/C along with Cdc20. As further research of the role of BubR1, various mouse models were designed. BubR1 knockout model was generated in 2004, by using gene-trapping method by insertion of neomycin cassette in the middle of the exon 1 and 2 of *mBubR1*, which leads to the disruption of the allele. When both allele was destroyed, $BubR1^{-/-}$, embryos showed lethality due to extensive apoptosis in E6.5 (Wang et al., 2004). In the case of $BubR1^{+/-}$ MEFs showed lower expression of protein and showed increased rate of micronuclei and polyploidy which proves compromised SAC activity. Also haploinsufficient $BubR1^{+/-}$ mice showed the phenotypes of splenomegaly and megakaryopoiesis (Dai et al., 2004).

Unlike disastrous phenotype of $BubR1^{+/-}$ mice, mice with phased amount of BubR1 showed roles of BubR1 in regulating the aging process. Expression of BubR1 is regulated from wild type (WT) to hypomorphic allels (H) to knock out (-). When BubR1 level was low as 10% of that of WT, the mice died due to respiraotry problems within several hours after birth. However when both alleles were hypomorphic, $BubR1^{H/H}$, the mice survived but had short life span of 6 months, and showed aging phenotypes (Baker et al.,

2004).

In our lab, through previous studies, we generated acetylation-deficient Bubr1 and introduced into mice by knock-in process. We enlightened that BubR1 is not only highly phosphorylated but also acetylated at lysine250 (in mice lysine243) by PCAF and deacetylated by HDAC2/3 when SAC is satisfied (Choi et al., 2009; Part et al., 2017). When lysine is substituted to arginine as K243R and introduced to mice, the rate of spontaneous tumor formation increased. Chromosomal instability (CIN), which is characterized by alteration in chromosome number and structural aberration, increased and mitotic timing decreased. Moreover KT-MT attachment is disrupted.

In comparison, when acetylation-mimetic form (K243Q) is introduced instead of K243R, mitotic timing increased and anaphase onset was delayed as a result. These result in the conclusion that acetylation of BubR1 is critical in maintaining genetic integrity. Of note, because BubR1 acetylation requires BRCA2 which acts as a scaffold, we proved BubR1 acetylation possess tumor-suppressive role (Park et al., 2013).

II. MATERIALS AND METHODS

II-1. Genotyping

BubR1 mutant mice were kept in pathogen-free system. DNA of each mouse were isolated from the tail by Tail lysis reagent (VIAGEN) according to the protocol suggested by the manufacturer and went through PCR. The nucleotide sequences of the PCR primers were designed in two groups; one to distinguish K243R and Wild type (WT), the other WT and -. The sequences were as follows: forward primer of K243R and WT BubR1, 5'-ACC CAG TCG TGC TGT TTC TTT AT – 3'; reverse primer of K243R and WT BubR1, 5' - CAT CTC ACC AGC CCA GAA GA – 3'; forward primer A of WT and – BubR1, 5' - GGG AGG ATC GAG GAG GTC G – 3'; forward primer B (mutant) of WT and – BubR1, 5' - AAA TGG CGT TAC TTA AGC TAG CTT GC – 3'; reverse primer of WT and – BubR1, 5' - CTG TTC GCC TTC AGT GCT CAA AAT GGT AGT CG – 3'. The amplification protocol for both BubR1 genotyping protocols consist of an initial denaturation at 94℃ for 8 minutes; 30 cycles of 94℃ for 45 seconds, 55℃ for 30 seconds, and 72℃ for 30 seconds; and a final extension at 72℃ for 10 minuates. PCR products were loaded on a 1.2% agarose gel with ethidium bromide (EtBr) staining. The PCR product of

BubR1 WT allele in first set is 188-bp, K243R allele is 258-bp. In the second set BubR1 WT allele is 468-bp, - allele is 100-200-bp.

II-2. Cell culture

For the establishment of mouse embryonic fibroblasts (MEFs), haploinsufficient mouse (BubR1+/-) was crossed with acetylation-insufficient mouse(K243R/+). Female mouse was sacrificed in E13.. MEFs were cultured under 37℃, 5% CO2 with Dulbecco's modified Eagle's medium(DMEM) supplemented with 16% of fetal bovine serum, 100units/mL penicillin and 100㎍/mL of streptomycin, 0.1% 2-mercaptoethanol.

II-3. Immunoflourescence assay

For better yield of mitotic cells, cell cycle synchronization was performed. MEFs were treated with 0.1% FBS/DMEM for 26 h and released into fresh DMEM supplemented with 20% FBS. After 23 h, drugs were treated for indicated time points. To observe chromosome alignment, MG132 was treated for 2 h and incubated in serum-free DMEM with 20mM HEPES, pH 7.3, 10μM MG132 on ice for 10min. For monastrol washout assay, cells were treated with 100μM monastrol for 6 h and 10μM MG132 was added in final 2 h. To completely washout monastrol, cells were washed for more

than 5 times with fresh 16% FBS/DMEM containing 5μM MG132. After undergoing cold-stable microtubule assay as described earlier, cells were fixed with 4% paraformaldehyde for 10min and permeabilized with 0.5% Triton X-100/PBS (0.5% PBS-T) for 30min. For blocking agent and antibody dilution 20% goat serum in 0.1% PBS-T was used. Cells were incubated in blocking solution for 1 h and incubated overnight in 4℃ with indicated primary antibodies followed by incubation with proper secondary antibodies.

For imaging, fixed cells were acquired by a microscope (DeltaVision; Applied Precision, GE healthcare) equipped with a 100X objective lens. The images were taken in 0.2㎛ distance in z-axis.

II-4. Cytogenetics

For metaphase spreads, MEFs were treated with 500ng/ml colcemid for 6 h and were collected along with supernatant. Cells were incubated with 0.075M KCl for 25min in 37℃ and were centrifuged for 950rpm for 3min. After removing supernatant, cells were incubated with fixative (methanol: acetic acid= 3:1). The cells were fixed in -27℃ overnight and dropped onto humidified glass slides while placed on a 65℃ water bath. The slides were dried and stained in DAPI and went through mounting with vectashield.

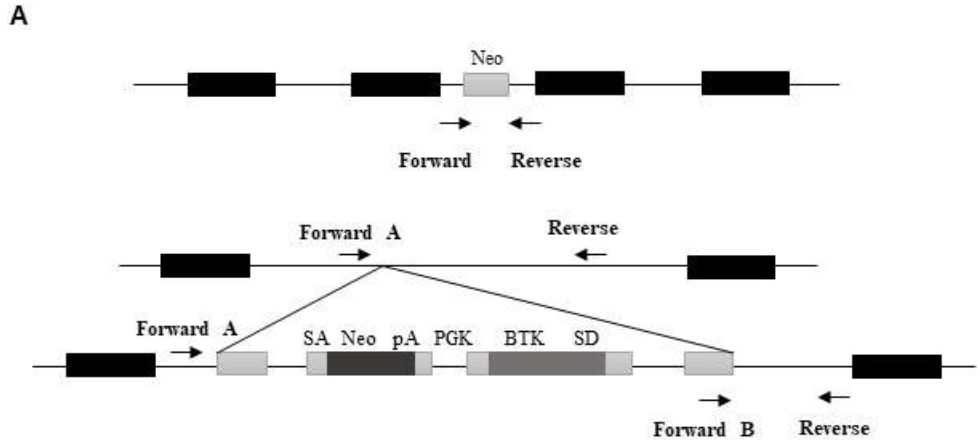
II-5. Western blot analysis

Cell lysates were obtained by mitotic shake-off after treatment of 200ng/ml nocodazole for 6 h. For the group treated with both nocodazole and MG132, 10μ MG132 was added for last 2 h. After centrifugation, NETN buffer (150mM NaCl, 1mM EDTA, 20mM Tris, pH 8.0, and 0.5% NP-40) with protease inhibitors were used for cell lysis step.

III. RESULTS

III-1. Validation of genotypes of MEFs

Prior to analysis of the phenotypes of K243R/- mice, genotyping of each MEFs obtained by the sacrifice of female K243R/+ mice was performed through PCR. MEFs were obtained by mating male BubR1 +/- mouse and female K243R/+ mouse. The genotype was confirmed through two sets of primers and they are indicated in Figure 1. In first mating, the ratio of each BubR1 genotype was measured; +/+: +/-: K243R/+: K243R/- = 1:3:4:1. None of the genotypes showed embryonic lethality. Whether the mating result satisfies Mendelian ratio should be monitored continuously.


A
Neo
Forward
Reverse
Forward A
Reverse
Forward A
SA Neo pA PGK BTK SD
Forward B
Reverse

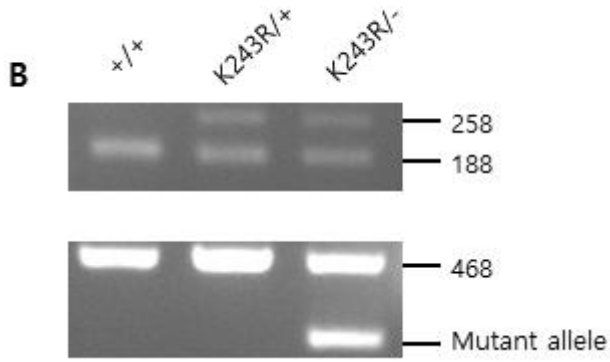

B
+/+
K243R/+
K243R/-
258
188
468
Mutant allele

Figure 1. Generation of BubR1 mutant mice

(A) Structure of BubR1 K243R knock-in allele and disruption of the BubR1 locus by gene-trapping method. The position of primers were indicated.

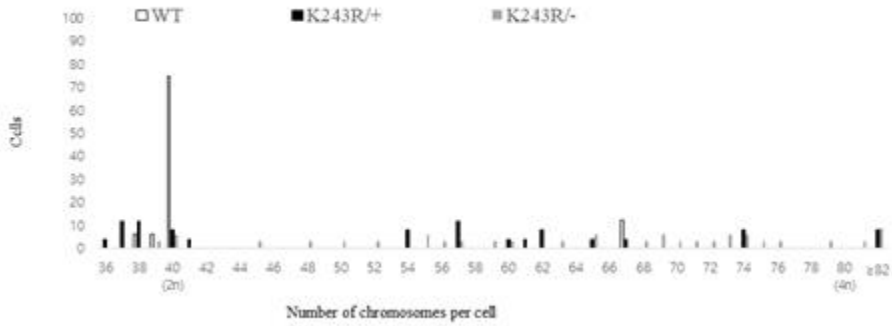
(B) PCR result of BubR1.

III-2. Chromosomal instability in acetylation-deficient mice

Through previous studies it has been suggested that acetylation-deficient BubR1 (K243R-BubR1) contributes on chromosomal instability (CIN). CIN is characterized by two aspects; numerical and structural aberrations. By observing chromosome spread result it was able to detect the hallmark of defective mitotic checkpoint. To detect the consequence of depleted WT allele, K243R/+ MEFs were included in the experiment along with WT MEFs.

The level of aneuploidy was revealed by metaphase chromosome spreads in each genotype. While WT MEFs showed high percentage of diploid cells, K243R/+ and K243R/- MEFs showed rather distributed tendency comparable to WT MEFs (Figure 1A). Along with the numerical difference, structural difference was spotted by the rate of PMSCs (Figure 1B). PMSCs occurs due to premature onset of anaphase which leads to degradation of cohesion by APC/C. To deter the degradation of cohesion proteasome inhibitor MG132 was added and compared to the nontreated group (Figure 1C). Because MG132 is proteasome inhibitor and blocks the degradation of securing and cyclin B, it can be assumed that PMSCs was due to acetylation deficiency which resulted in premature activation of APC/C.

A


WT
K243R/+
K243R/-
Cells
Number of chromosomes per cell
(2n)
(4n)

B


WT
K243R/+
K243R/-

C

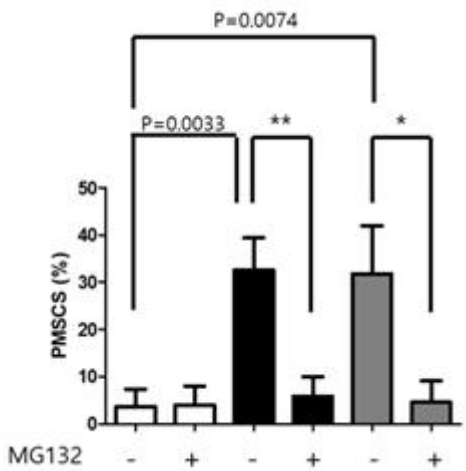

P=0.0074
P=0.0033
**
*
PMSCS (%)
MG132
- + - + - +

Figure 2. K243R-BubR1 induces aneuploidy and CIN

(A) Number of chromosomes per cell was counted for each genotype.

(B) Representative images of the metaphase chromosome spreads.

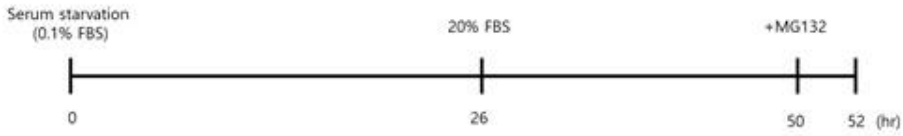
(C) Occurrence of PMSCs with or without treatment of MG132 for 2 h. (WT, n= 27; WT treated with MG132, n=25; K243R/+, n=49; K243R/+ treated with MG132, n=34; K243R/-, n=22; K243R/- treated with MG132, n=22; *, P=0.0186, **, P=0.0032)

III-3. K243R-BubR1 causes chromosome alignment defect

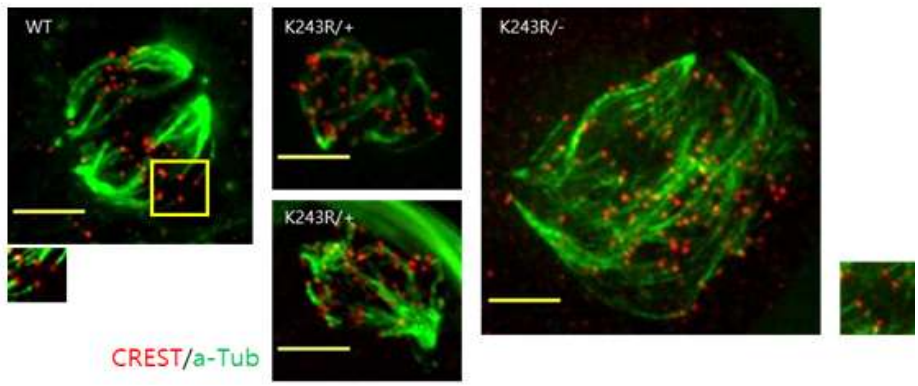
As shown before, MEFs carrying K243R-BubR1 harbored chromosomal instability which is represented in various forms (Fig. 1) and were prone to early degradation by APC/C (Fig. 2). In this vein, we previously reported K243R/+ MEFs show lagging chromosomes and chromosome bridges in mitosis. Because K243R/- MEFs lack WT allele compared to K243R/+ MEFs, it is plausible that chromosome missegregations will prevail. Chromosome missegregation is reported to arise from bi-orientation inability or absence of attachment to mitotic spindles. (E.A Foley and Kapoor, 2013). BubR1 is known to play critical role in managing kinetochore-microtubule (KT-MT) attachment. To directly observe the action of K243R-BubR1 at kinetochore, cold-stable microtubule assay was performed. In low degrees such as 4℃, only kinetochore-fiber (K-fiber) is intact. To prevent premature onset of anaphase due to early degradation of K243R-BubR1, MG132 treatment is included. As reported in previous studies, K243R/+ MEFs showed severe defects in congression compared to WT MEFs. In the case of K243R/- MEFs, congression defect rate appeared to be like K243R/+ MEFs (Figure 3). However, in K243R/+ MEFs, servere congression error were shown with monotellic attachments of microtubules to kinetochores. On

the other hand in the K243R/- MEFs, kinetochores were attached amphitellically but they still showed congression defects.

A


Serum starvation
(0.1% FBS)
20% FBS
+MG132
0
26
50
52 (hr)

B


WT
K243R/+
K243R/+
K243R/-
CREST/a-Tub

C

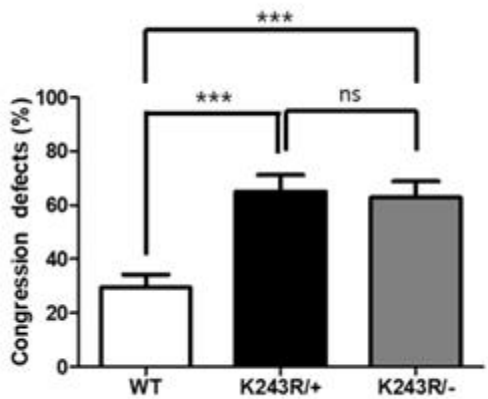

***
***
ns
Congression defects (%)
100
80
60
40
20
0
WT
K243R/+
K243R/-

Figure 3. Weakened SAC and congression failure in WT, K243R/+, K243R/- MEFs.

(A) Schematic graph of cold-stable microtubule assay and representative images from each genotype.

WT, K243R/+ and K243R/- MEFs were serum starved for 26 h and release into fresh DMEM medium containing 20% FBS. After 23 h, MG132 was treated for 2 h, followed by cold-stable microtubule assay and stained with CREST for kinetochore marker and anti-α -tubulin antibodies for K-fiber. Green, α-tubulin; red, CREST immunostaining. Bars: (yellow) 5㎛.

(B, C) Congression defects were scored.

Results are shown as bar graphs (mean ± SEM). Number of cells scored: WT, n= 64; K243R/+, n= 46; K243R/-, n=53. ***: $P < 0.0001$.

III-4. Error correction ability of WT, K243R/+ and K243R/-

When KT-MT attachment is not accomplished, Aurora B senses and phosphorylates KMN network to destabilize the attachment. This reaction makes error correction activity to happen. In previous studies, we proved error correction system is compromised in K243R/+ MEFs compared to WT MEFs. To test the difference of K243R/- MEFs with K243R/+ MEFs, monastrol washout assay was performed. Monastrol, kinesin-5 (Eg5) inhibitor, hinders bipolar spindle formation and this results in monopolar arrangement of chromosomes. MG132 treatment was included following monastrol washout step. When compared with WT MEFs, K243R/+ and K243R/- showed lower rate of correction ability. After monastrol washout assay, congression rate were scored (Figure 4).

As it is noticeable in both cold-stable microtubule assays, K243R/- MEFs display more signals of CREST staining. This can be in line with increased rate of polyploidy (Figure 1).

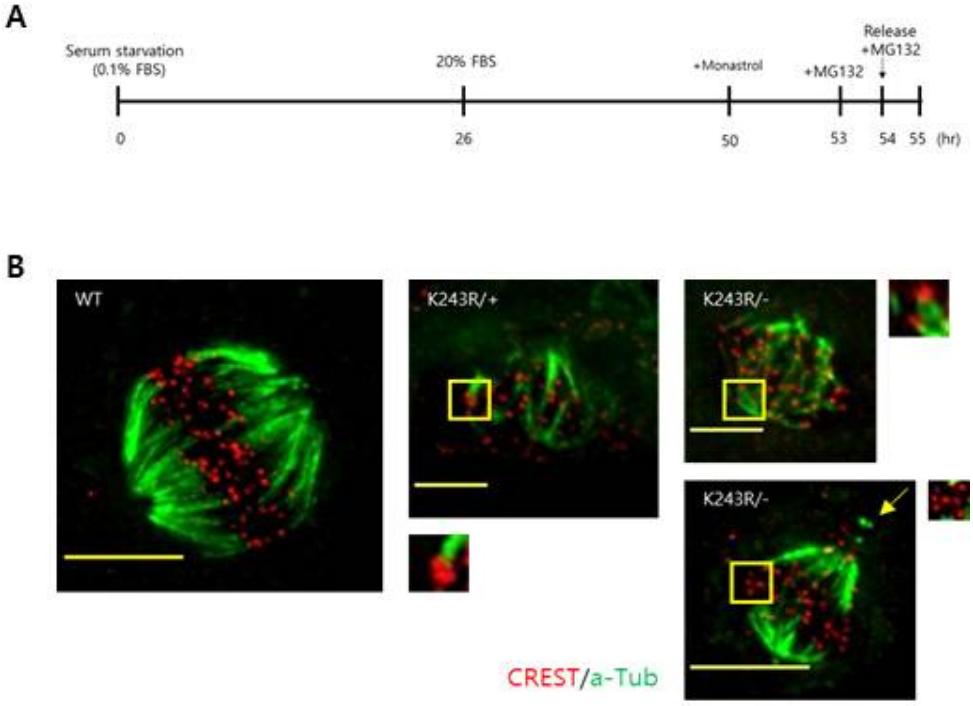

A
Serum starvation
(0.1% FBS)
20% FBS
+Monastrol
+MG132
Release
+MG132
0
26
50
53
54
55 (hr)
B
WT
K243R/+
K243R/-
K243R/-
CREST/a-Tub
C
***
ns
ns
Congression rate (%)
100
80
60
40
20
0
WT
K243R/+
K243R/-

Figure 4. Acetylation-deficient BubR1 severly impairs error correction ability.

(A) Schematic graph of experiment.

After serum starvation for 26 h, MEFs were released into 20% FBS/DMEM media. Monastrol was treated for 4 h for both groups. In first group monastrol was not washed out for control while MG132 was added for last 2 h, followed by cold-stable microtubule assay. In second group, monastrol was treated for 4 h and MG132 treatment was involved for last 1 h. Then monastrol was washed out with fresh media containing MG132 for at least 5 times. MEFs were treated with MG132 for 1 h and went through cold-stable microtubule assay.

(B) Represetative images of each genotype.

Congression rate was scored. Bars: (yellow) 5㎛.

(C) Bar graph for congression defects after release from monastrol.

***: P=0.0090.

III-5. Detection of BubR1 in WT, K243R/+ and K243R/- MEFs

To assess the stability of K243R-BubR1 in each MEF genotype, western blot was performed with or without the existence of drug nocodazole and MG132.

WT blot shows clear bands of BubR1 in nocodazole-treated group and nocodazole-MG132-treated group compared to asynchronous group which showed only non-phosphorylated BubR1 band. NT group shows fade band because BubR1 is mainly synthesized in mitosis rather than interphase. WT MEFs showed strong phospho-BubR1 band in both +Noc group and +Noc, +MG132 group. In the case of K243R/+, phopho-BubR1 band of +Noc showed similar signal to nonphospno-BubR1. However treatment of MG132 retained the level of phospho-BubR1. In K243R/- MEFs, BubR1 was nearly undetected in the presence of MG132 when compared to K243R/+. This leads to the conclusion that K243R/+ and K243R/- shares problem in maintaining spindle assembly checkpoint (SAC). Also this result might drop a hint for the sequence of acetylation and phosphorylation of BubR1 (Part et al., 2017). Due to complex phosphorylation code of BubR1, overall sequence of acetylation and phosphorylation is unknown . Fade

phospho-BubR1 band might indicate acetylation must precede majority of phosphorylation code.

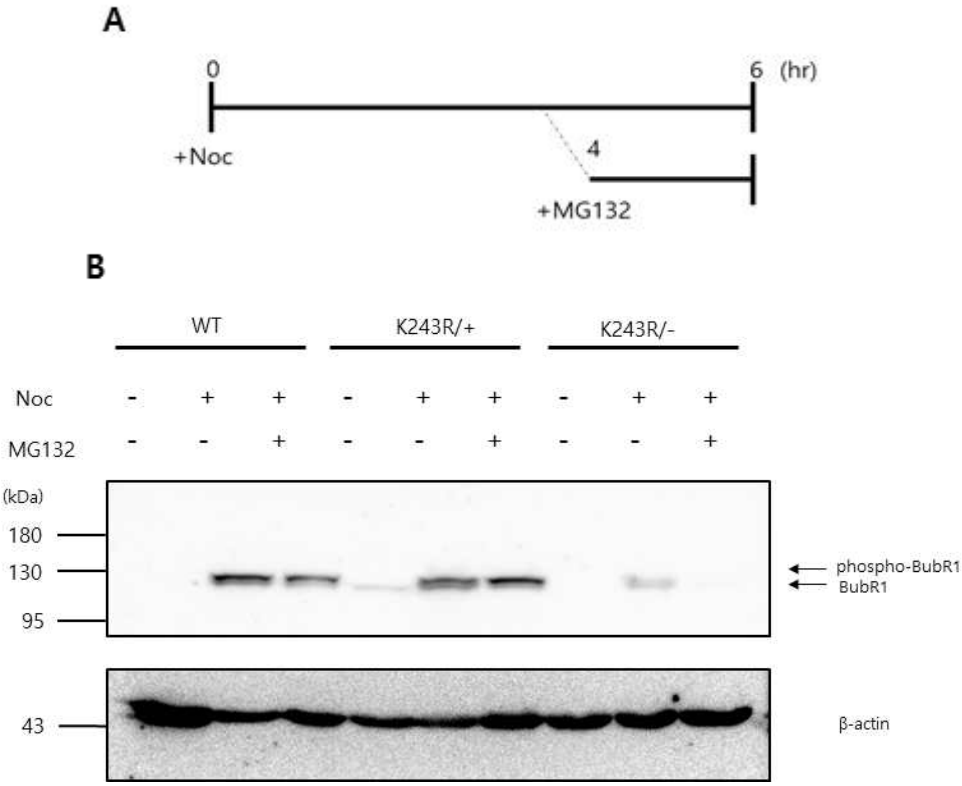

A
0
6 (hr)
+Noc
4
+MG132
B
WT
K243R/+
K243R/-
Noc
MG132
(kDa)
180
130
95
43
phospho-BubR1
BubR1
β-actin

Figure 5. Acetylation-deficient BubR1 shows instability compared to WT

(A) Schematic illustration of the experiment. The MEFs were treated with nocodazole (+MG132) and subjected to mitotic shake-off for only mitotic cells. Asynchronous cells were attained by scraping.

(B) Assessment of BubR1 levels in WT, K243R/+ and K243R/- MEFs in asynchronous condition and drug-treated conditions.

IV. DISCUSSION

Deacetylation of BubR1 acts as a cue for anaphase onset which decides the transition from metaphase to anaphase. Through previous studies, we were able to observe shortened mitotic timing, which indicates weakened SAC, and heightened cancer incidence in K243R/+ mice. We might have implicated that K243R-BubR1 alone would not be enough to handle the dual role of BubR1; maintenance of SAC and monitoring KT-MT attachment because of lethality found in K243R/K243R and -/- embryos, which means acetylation of BubR1 is critical (Wang Q et al., 2004). However, the fact that K243R/- mice does not show embryonic lethality imposes new question to this field.

Here I showed increased aneuploidy and PMSCs rate in K243R/- MEFs which are indicators of compromised SAC (Figure 2). Moreover, it can be supported by the level of BubR1 in the presence of microtubule drug nocodazole and proteasome inhibitor MG132 (Figure 3). In this vein, congression error was captured in K243R/- MEFs and cells failed to correct the attachment after released into Eg5-inhibitor- free condition for 2 h which is long enough to correct it (Figure 4). Also K243R-BubR1 showed extensive instability in K243R/- compared to K243R/+ (Figure 5). Although

it is not presented by the figure, when attached cells were collected in +Noc group and +Noc, +MG132 group of K243R/-, they showed only nonphosphorylated BubR1 band. This might indicate that acetylation is critical in the phophorylation process of BubR1 and acetylation status can be followed by phophorylation of BubR1.

Of note, it is worthy to concentrate on the result that although K243R/- MEFs lack WT allele compared to K243R/+ MEFs, K243R/- MEFs still show comparable result to K243R/+ MEFs. As mentioned earlier, in the case of congression status, while K243R/+ MEFs displayed severe conditions, such as monotellic attachments, K243R/- MEFs rather showed amphitellic attachments of kinetochore and microtubule spindles.

In correcting KT-MT attachment, Aurora B kinase is critical. Aurora B kinase phosphorylates the KMN network to destabilize the incorrect attachments. Apart from this, after phosphorylation of KARD domain in BubR1, PP2AB56a is recruited by recognizing the phosphorylated site and counteracts the activity of Aurora B (Suijkerbuijk et al., 2012). In K243R/+ MEFs recruitment of PP2AB56a was decreased compared to WT MEFs (Park et al., 2013). Thus it would be worthy to check out the mutual interaction of interacting proteins for the further analysis.

Moreover, *BubR1*$^{+/-}$ and *BubR1*$^{H/H}$ mice did not show the sign of tumorigenesis while *BubR1*$^{K243R/+}$ mice showed spontaneous tumorigenesis. It can be inferred that the amount of BubR1 might be a cue for the arise of phenotype in mice. Because K243R/+ mice showed higher level of BubR1 compared to +/- mice while showed lower expression than WT mice. K243R/- mice lacks WT allele which means it will show lower expression of BubR1 than K243R/+ and it is proved by western blot analysis in my research. Therefore it can be one of the possibilities that K243R-BubR1 leads to detrimental phenotype in dose-dependent manner or K243R-BubR1 might affect normal BubR1 and brings out synergetic effects. Further analysis whether acetylation mutation of BubR1 occurs in human and if the interaction among MCC complex along with APC/C changes or not should be carried out.

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국문 초록

세포 분열기에서 유전 정보가 고르게 분배되도록 하기 위해 세포에서는 spindle assembly checkpoint (SAC) 이 작동하여 mitotic checkpoint complex (MCC)를 구성하는 방법을 통해 세포 분열기 중 후기로 넘어가는 것을 막는다. MCC는 anaphase-promoting factor/cyclosome (APC/C)와 결합하며 APC/C의 기질의 ubiquitination을 저해한다.

BubR1은 MCC를 구성하여 SAC의 작용에 주요한 역할을 담당하며 동시에 모든 동원체가 미세소관과 결합할 수 있도록 조절하는 데에도 관여한다고 알려져 있다. 앞선 연구들을 통해 우리는 BubR1의 250번째 라이신 잔기의 아세틸화가 두 작용에 필수적임을 밝힌 바 있다. 아세틸화가 불가능한 아르기닌으로 치환되었을 때 아세틸화 결핍 마우스 (K243R/+)는 염색체의 수와 형태 이상을 통해 염색체 불안정성이 나타남을 보였고, SAC이 약해지며 동원체-미세소관의 결합도 불안정해지는 것을 증명하였다. 한편 모든 allele이 아세틸화 결핍인 경우 (K243R/K243R), E6.5에 세포 사멸이 일어나며 embryonic lethal함을 관찰하였고 BubR1 결핍 마우스 (-/-) 에서도 동일한 현상이 관찰되었다. 흥미롭게도, 하나의 아세틸화 결핍 allele만을 가지는 마우스 (K243R/-)가 태어났고 비정상적인 표현형을 보이지 않았으며 번식이 가능한 것을 관찰하였다.

Mouse embryonic fibroblasts (MEFs)를 얻어 실험을 하였고 본 연구를 통해 K243R/- MEFs가 염색체 불안정성을 보이고, SAC이 약해진 상태임을 관찰하였다. 또한 동원체-미세소관의 결합도 오류를 보였으며 APC/C가 저해된 상태임에도 불구하고 BubR1의 양이 현저히 감소한 것을 관찰할 수 있었다.

후속 연구로 상세한 분자적 상호작용에 대한 추가적인 실험을 진행하고 있으며 WT allele을 가지지 않음에도 불구하고 K243R/-가 K243R/+와 비교하였을 때 큰 차이를 보이지 않고 세포 분열 과정을 넘길 수 있는지에 대한 원인 분석이 이루어져야 할 것이다. 이러한 시도들을 통해 아세틸화 결핍 BubR1의 작용에 대한 이해를 넓힐 수 있을 것으로 기대한다.