이학석사 학위논문

Investigation of change in localization of centromeric proteins due to replicative stress and DNA damage

복제 스트레스 및 유전자 손상으로 인한 중심절 단백질들의 위치 변화 연구

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유 호 성

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이 논문을 이학석사 학위논문으로 제출함

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유 호 성

유호성의 석사 학위논문을 인준함

2023년 01월

위 원 장 정종경 (인)

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ABSTRACT

Centromere specification and maintenance is key in protecting chromosomal integrity as it serves as the platform for inner and outer kinetochore assembly. A successfully assembled kinetochore allows the onset of events which ensure chromosomal stability such as kinetochore-microtubule attachment and the activation of the spindle assembly checkpoint. One of the pathways which allows centromere specification is the incorporation and maintenance of centromeric protein A (CENP-A). CENP-A is a histone 3 variant which is incorporated at centromeric regions of the chromosome in place of histone 3. Current studies show that the incorporation of CENP-A occurs throughout the cell cycle. CENP-A dilution and equal distribution of pre-existing CENP-A to the template and the newly synthesized DNA occurs in S-phase. Later in late M-phase and early G1 phase the stabilization and incorporation of CENP-A occurs. This active incorporation is done by the CENP-A incorporation machinery composed of the holiday junction recognizing protein (HJURP) and the Mis18 complex. Furthermore, the epigenetic status of lysine 9 of histone 3 (H3K9) at centromeres has been suggested to regulate CENP-A incorporation by a tri-methylation: acetylation switch.

Despite the fact that CENP-A phenotype aberrations are often observed in cancers, the potential physiological cause for

the abrogated CENP-A incorporation is not clear. Interestingly, not only is CENP-A incorporation a partially replication coupled event, but CENP-A have also been previously suggested to be recruited to double stranded break regions. However, the potential effect of disrupted replication, by factors such as replication stress and DNA damage, on CENP-A incorporation have not been explored. Therefore, through this research, we aimed to explore the potential effect prolonged replicative stress and DNA damage may have on CENP-A incorporation and further downstream kinetochore assembly.

Here, I show that, cells which undergo replicative stress and or DNA damage and enter mitosis, shows aberrations in CENP-A incorporation as well aberrations of CENP-C, which is directly recruited to centromeres by CENP-A. Furthermore, I show that upon replicative stress induction and DNA damage induction, the previously mentioned epigenetic status of H3K9 show distinct phenotypes where the tri-methylation of H3K9 shows a relatively diffused phenotype across the chromosome. On the other hand there is a significant drop in global H3K9 acetylation upon replicative stress and DNA damage induction. Therefore, it is deducible that these epigenetic changes of centromeric histone 3 caused by replicative stress and DNA damage leads to abrogated CENP-A incorporation. Further direct link between the replicative stress or DNA damage caused epigenetic changes of H3K9 and CENP-A incorporation is under

investigation. Additionally, I show that despite the observed CENP-A and CENP-C aberration, the localization of outer kinetochore components KNL1 and NDC80 remains unchanged. This raises the question for the existence of a potential pathway which is able to compensate for the abrogation of CENP-A incorporation upon replicative stress and or DNA damage.

Collectively, I show that replicative stress and DNA damage causes abrogated CENP-A incorporation and could be the cause of chromosomal instability. The study further provides the question of the potential effect of the replicative stress and or DNA damage caused epigenetic changes of H3K9 on CENP-A incorporation. The study also hints at the potential existence of a pathway which is able to successfully recruit the outer kinetochore despite the observed CENP-A incorporation aberration.

주요어 : CENP-A, Epigenetics, Chromosomal instability, Replicative Stress, DNA damage

학 번 : 2021-26101

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I. Introduction

Chromosomal instability and cancer

Chromosomal instability, a phenomenon where chromosomes are frequently mis-segregated, is known to be one of the hallmarks of cancer onset with 60-80% of human cancers exhibiting chromosomal abnormalities. (Bakhoum & Cantley., 2018) It is widely understood that chromosomal instability is caused by functional aberrations during chromosomal segregation during mitosis. Chromosomal instability causes various deleterious phenotypes such as aneuploidy, micronuclei formation, translocations and various deleterious mutations. (Bochtler et al., 2018; Thompson et al., 2010) One of the key events which is required to maintain chromosomal stability is proper kinetochore-microtubule attachment. This proper attachment is ensured by various mechanisms such as the spindle assembly checkpoint where improper kinetochore-microtubule attachment leads to the recruitment of the mitotic checkpoint complex. (MCC) The MCC then inhibits the anaphase promoting complex/cyclosome (APC/C) activity, halting mitosis through preventing the degradation of proteins such as cyclin B and securin. (Musacchio and Salmon., 2007)

Previously we have discovered one of the underlying

molecular mechanism which causes abrogation of this spindle assembly checkpoint and leads to chromosomal instability. We have shown that in humans, acetylation deficiency of BubR1, a core component of the spindle assembly checkpoint, leads to premature ubiquitin-dependent BubR1 degradation. This premature degradation of BubR1 leads to premature onset of anaphase which ultimately leads to chromosomal instability. (Choi et al., 2009; Choi et al., 2012; Park et al., 2013)

Interestingly, we have also observed chromosomal instability in cells which experience prolonged DNA damage during interphase and enters mitosis. Cells which experienced DNA damage during interphase, showed increased rate of whole chromosome mis-segregations. (Jiho Mo., unpublished) If so, we questioned how interphase specific DNA damage would transfer into the observed defective mitosis and chromosomal instability.

One of the S-phase specific cellular events which have been shown to be also crucial in maintaining chromosomal stability and extends from S-phase into mitosis is the replication coupled distribution of pre-existing CENP-A. (Pan et al., 2019)

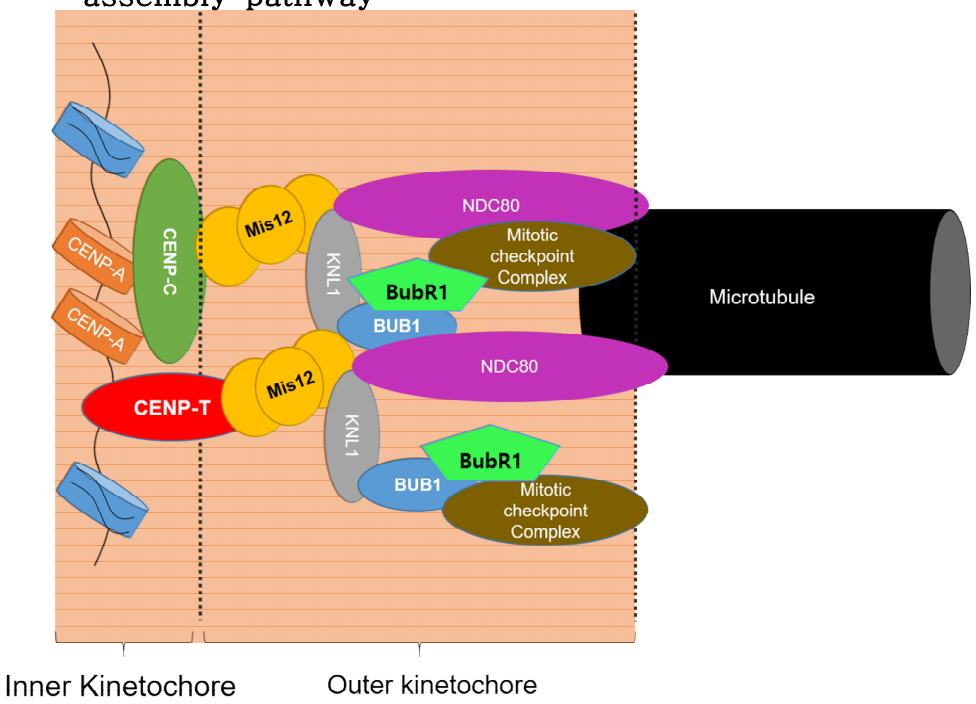
Centromere specification

Through previous studies, CENP-A incorporation and

maintenance at centromeric regions have been shown to mediate the establishment and heritance of centromeric identity. Successfully incorporated CENP-A at centromeres further directly interacts and recruits centromeric proteins C and N (CENP-C & CENP-N) which then further recruits the constitutive centromere associated network (CCAN) consisting of CENP-B, CENP-I, CENP-N, and CENP-W. (Carroll et al., 2010; Guse et al., 2011) CCAN further recruits the KMN network composed of Knl1, Mis12 and NDC80 to the centromere forming the outer kinetochore. Additionally, CENP-T has been shown to be able to recruit the KMN network in a CENP-A and CENP-C independent pathway. (Nishino et al., 2013) (Figure 1)

A fully assembled kinetochore then allows the proper onset of mitosis through events such as allowing kinetochore-microtubule attachment through NDC80s direct association with spindle microtubules, as well as allowing successful onset of the spindle assembly checkpoint through Knl1 association with MCC components such as Bub1, Bub3 and BubR1. (Kixmoeller et al., 2020; Regnier et al., 2005) With CENP-As crucial role established, studies have further explored the upstream mechanism of their centromeric incorporation and maintenance.

Figure 1. Centromere specification and kinetochore assembly pathway



Centromere specification and kinetochore assembly occurs in two main independent pathways: CENP-A/CENP-C dependent and CENP-T dependent pathways.

Mechanism of CENP-A incorporation

The events known to be required to allow successful CENP-A incorporation is divided throughout the cell cycle. During S-phase, pre-existing CENP-A is equally distributed to the template and the newly synthesized DNA. During late M phase and early G1 phase, CENP-A is incorporated into the centromeres by the CENP-A incorporation machinery. The CENP-A incorporation machinery is composed of the Mis18 complex, and the Holiday junction recognition protein (HJURP). The current model suggests that CENP-A is stabilized from a soluble state by forming a complex with the HJURP chaperone protein. This CENP-A:HJURP complex is then recruited to the centromeres by the Mis18 complex present at centromeres. Upon successful centromeric localization, active incorporation of CENP-A occurs in a PLK1 and cyclin dependent kinase dependent manner. (Pan et al,. 2019) Currently, how the Mis18 complex is localized to centromeres is poorly understood. However, previous studies show that Mis18 complex interacts with pre-existing old centromeric CENP-A which was distributed during S phase. This interaction is suggested to mediate the localization of the Mis18 complex to centromeres and hence CENP-A:HJURP complex localization and ultimately CENP-A incorporation. (Pan et al., 2019) Furthermore, current findings also highlight the importance of the epigenetic status of

histone 3 present at the centromeric region for successful CENP-A incorporation.

The epigenetic modification site of histone 3 lysine 9 (H3K9) has been previously shown to be carefully modulated in order for successful incorporation of CENP-A. (Molina et al., 2016) H3K9 is known to be both available for tri-methylation as well as acetylation. Previous studies have observed that the tri-methylation of H3K9 (H3K9me3) and its diffusion across the chromosome, led to abrogated centromeric CENP-A incorporation. (Zhu et al., 2018) On the other hand the acetylation of H3K9 (H3K9ac) and its removal of positive charge have been shown to loosen histone to histone interaction leading to increased histone turn over rate and allow successful CENP-A incorporation. (Zhu et al., 2018) Studies suggest that this tri-methyl:acetyl switch is crucial in modulating CENP-A incorporation and hence the successful downstream event of kinetochore assembly. (Molina et al., 2016; Shang et al., 2016)

Interestingly, CENP-A have also been shown to be recruited to DNA-damage regions during DNA replication in S phase suggesting a role in DNA-repair where its distribution to newly synthesized DNA is simultaneously occurring. (G. Zeitlin et al., 2009) Additionally, the CENP-A incorporation mediating H3K9me3 has been previously been shown to be promoted upon

DNA damage and is also crucial in regulating DNA double-strand repair. (Ayrapetov et al., 2014) However, the potential effect of prolonged replicative stress and or DNA damage on centromere specification by CENP-A incorporation has not yet been explored.

Previous studies have highlighted the importance of CENP-A incorporation in assuring accurate chromosome segregation and have unveiled key components in CENP-A incorporation. (Regnier et al., 2005; Pan et al., 2019; Kixmoeller et al., 2020) However, despite the fact that pre-exisiting CENP-A distribution, a key event required for CENP-A incorporation is a replication coupled event, the potential effect of disrupted replication by factors such as prolonged DNA damage and or replicative stress on CENP-A centromere incorporation remains uninvestigated. Moreover whilst abrogated CENP-A phenotypes have been observed in many cancers, the underlying cause of such phenotype is poorly understood. Through the investigation of the potential effect of prolonged replicative stress and or DNA damage on CENP-A incorporation, this research provides evidence that prolonged replicative stress and or DNA damage abrogates proper CENP-A incorporation, further unveiling the link between prolonged replicative stress and or DNA damage and chromosomal instability.

II. MATERIALS AND METHODS

II-1. Cell culture

HeLa cells were cultured at 37° C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum with the end concentration of 10% as well as 100units/ml of penicillin and 100ug/mL of streptomycin. Maximum confluency was 80% and the minimum confluency of 40% was maintained.

II-2. Drug treatment

In order to induce replicative stress and DNA damage, 1uM of aphidicolin and 10uM of pyridostatin (PDS) was diluted in the cell culture medium respectively. Each drug was treated for 24 hours.

II-3. Immunofluorescence assay

HeLa cells cultured on cover slides prepared through HCL and ethanol sanitation in 12 well culture plates. Cells were then fixated using 4% paraformaldehyde for 10 minutes, followed by 4 washes using fresh phosphate buffered saline (PBS). Cells were

then permeablized using 0.5% Triton X in PBS solution for 15 minutes. Samples were than blocked using 2% Bovine Serum Albumin in 0.1% Tween-20 in PBS for 1 hour. Cells were then incubated with adequate antibodies over night at 4° C followed by 2 hour wash with gentle agitation with fresh 0.1% tween-20 in PBS which was replaced every 20 minutes. Cells were then incubated with the adequate secondary antibody for 2 hours at room temperature. After final washing of 2 hours using fresh 0.1% tween-20 in PBS with gentle agitation, cells were mounted onto microscope cover slides using VECTASHIELD mounting medium with DAPI.

Images from prepared samples were acquired by a microscope (DeltaVision; Applied Precision, GE healthcare) utilizing its 100X objective lens. The Z-axis was dissected in 0.2um intervals. Numerical aperture 1.514 oil was used. Mitotic cells were exclusively selected for imaging.

II-4. Image analysis and quantification

Images were then analyzed using ImageJ 1.54q. Two ImageJ macros were designed to measure co-localization and immunofluorescence signal intensity. Co-localization was

measured through the composition of a "mask" in order to specify foci regions of one candidate, then the presence of immunofluorescence signal of the other candidate of interest was measured to analyze co-localization of two candidate proteins.
In order to measure immunofluorescence foci intensity, foci positions were determined using the "Find Foci function". Background signals were eliminated through adjustment of threshold values, and to measure its fluorescence intensity to quantify the immunofluorescence signal, the "Analyze particles" and "measure" functions were utilized. Quantitative data analysis, presentation, and statistical analysis was done utilizing GraphPad Prism. The unpaired T-test was used for statistical analysis.

II-4. Western Blot Analysis

Cell lysates were either obtained by mitotic shake off after aphidicolin or PDS treatment for 24 hours or cell lysate was collected after 18 hours of aphidicolin or PDS treatment followed by 6 hours of nocodazole (200ng/ml). Collected cells were lysed at 1*10^5 cells per 50ul in Laemmli buffer with gentle sonication. After gel-running in 12% poly-acrylamide gel in Tris-Glycine buffer, protiens were transfered onto a nitro-cellulose membrane. Then, an appropriate primary antibody

and HRP conjugated secondary antibody was used to observe the protein of interest *(Courtesy of Jiho Mo)*

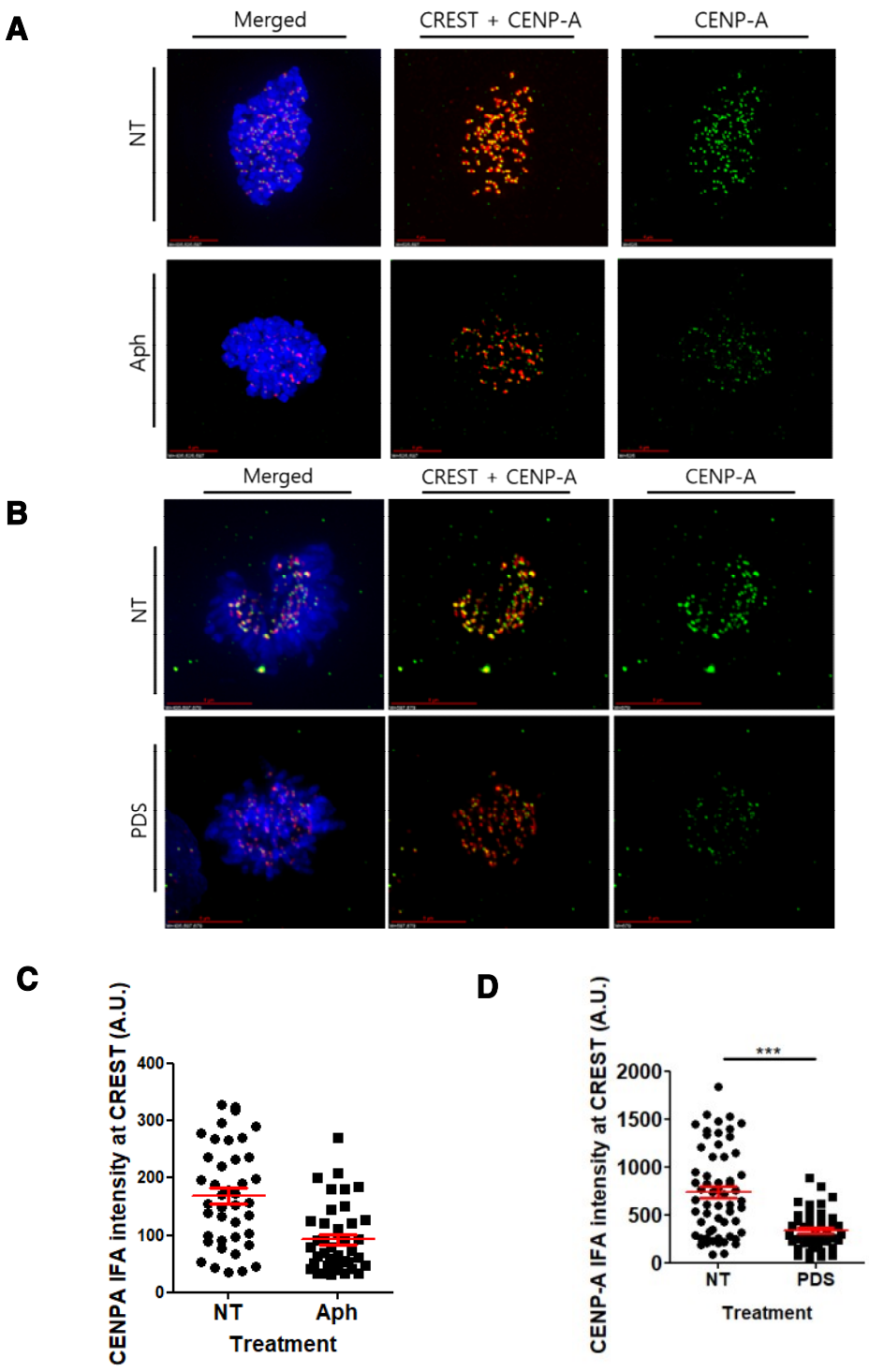
III. Results

III-1. Defective CENP-A assembly upon replicative stress and DNA damage induction.

Successful incorporation of CENP-A is required for successful mitosis. Pre-existing CENP-A distribution during S phase, an important step in assuring CENP-A incorporation, is a replication coupled event. Furthermore, CENP-A have been suggested to be recruited to double stranded breaks. (G. Zeitlin et al., 2009) Based on these facts, it is deducible disrupted replication caused by excessive or prolonged DNA damage and or replicative stress may result in abrogated CENP-A incorporation. In order to investigate this hypothesis, HeLa cells were treated with a DNA polymerase alpha inhibitor, aphidicolin, and a G4 quadruplex stabilizer, PDS, in order to cause prolonged replicative stress and DNA damage respectively. CENP-A was then observed through immuno-fluorescence assay and analyzed utilizing Fiji in order to quantify the fluorescence signal strength at centromeres.

Upon analysis, HeLa cells which progressed into mitosis despite induced replicative stress (aphidicolin) and DNA damage (PDS), showed significantly decreased CENP-A immunofluorescence signals at centromeres. (Figure 2.A,B,C,D)

Furthermore, in order to further understand the abrogated phenotype, cell lysates of mitotic HeLa cells after 24 hours of aphidicolin and PDS treatment were collected through either nocodazole synchronization or mitotic shake off. When compared to non-treated HeLa cell lysates, it can be seen that the overall expression of CENP-A remains unchanged upon replicative stress (aphidicolin) and DNA damage (PDS) induction. (Figure 2.E) Collectively, based on these findings, as CENP-A expression levels remain equal, whilst centromeric CENP-A signal is reduced, it is deducible that CENP-As centromeric incorporation is abrogated upon replicative stress and DNA damage induction through aphidicolin and PDS treatment respectively


A
Merged
CREST + CENP-A
CENP-A
NT
Aph
B
Merged
CREST + CENP-A
CENP-A
NT
PDS
C
CENPA IFA intensity at CREST (A.U.)
400
300
200
100
0
NT
Aph
Treatment
D
CENP-A IFA intensity at CREST (A.U.)
***
2000
1500
1000
500
0
NT
PDS
Treatment

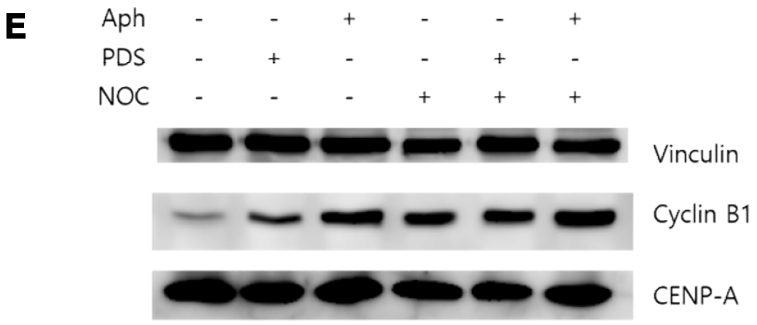

E
Aph - - + - - +
PDS - + - - + -
NOC - - - + + +
Vinculin
Cyclin B1
CENP-A

Figure 2. CENP-A assembly is abrogated upon replicative stress and DNA damage induction.

(A) Immunofluorescence assay of DAPI (BLUE), CREST (RED), CENP-A (GREEN) of non-treated (NT) vs upon aphidicolin treatment (Aph)

(B) Immunofluorescence assay of DAPI (BLUE), CREST (RED), CENP-A (GREEN) of non-treated (NT) vs upon PDS treatment (PDS) *(Courtesy of Jiho MO)*

(C) Graphical representation of immunofluorescence signal of CENP-A at centromeres of non-treated and aphidicolin treated cells. Each value represents the average centromere specific CENP-A flouresence signal of each cell. (NT, n= 41; Aphidicolin treated, n= 41, ***, $p < 0.0001$)

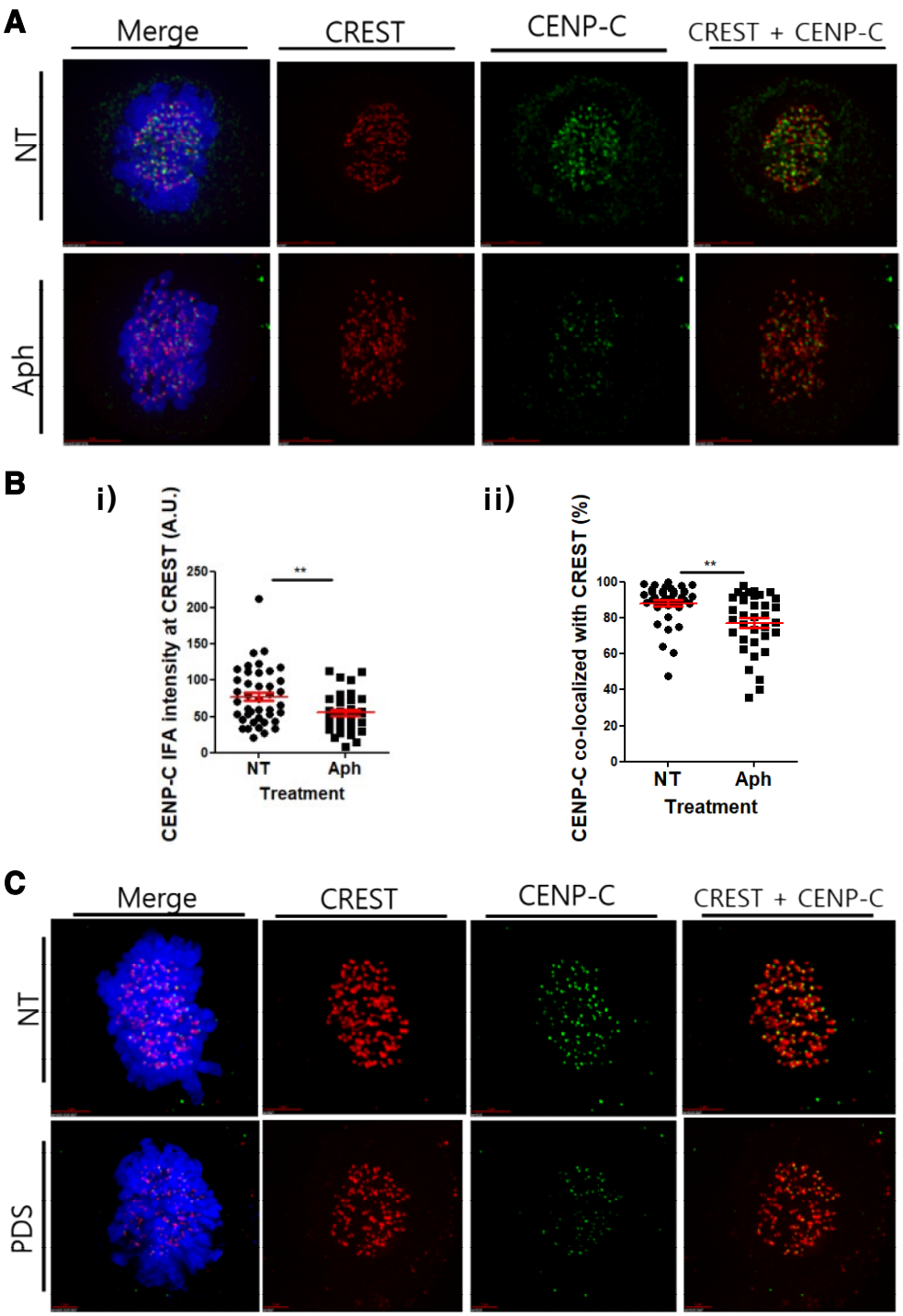
(D) Graphical representation of immunofluorescence signal of CENP-A at centromeres of non-treated and PDS treated cells. Each value represents the average centromere specific CENP-A fluorescence signal of each cell. (NT, n = 59; PDS treated, n= 58, ***, $p < 0.0001$)

(E) Western blot analysis of HeLa cell lysates. Experimental groups of non-treated, aphidicolin treated, and PDS treated was also compared in terms of method of mitotic cell collection. (Mitotic shake-off vs nocodazole synchronization (NOC)) *(Courtesy of Jiho Mo)*

III-2. Defective CENP-C mis-localization upon replicative stress induction.

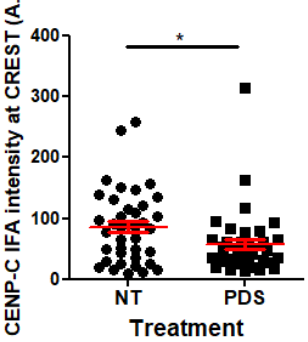
CENP-C, which is part of the inner kinetochore, has been suggested to be recruited by CENP-A during kinetochore assembly. With abrogated CENP-A phenotype observed upon replicative tress and DNA damage induction through aphidicolin treatment and PDS treatment (Figure 2), further down-stream inner kinetochore component CENP-C was observed to investigate the downstream effect of the observed CENP-A phenotype abrogation.

CENP-C phenotype was revealed by immunofluorescence assay after 24 hours of aphidicolin and PDS treatment. (Figure 3. A,C) It was observed that upon replicative stress induction, CENP-C seems to fail to localize to centromeres. This abrogation in centromere localization is represented by the decrease in percentage of CNEP-C colocalized with centromeres (CREST) (Figure 3.B(i),D(i)). This is further shown by the decrease in CENP-C immunofluorescence intensity at centromeres. (Figure 3 B(ii), D(ii)) CENP-Cs failure to localize to centromeres show that the observed replicative stress and or DNA damage induced aberration in CENP-A (Figure 2) leads to further downstream aberrations of CENP-C mis-localization to centromeres.


A
Merge
CREST
CENP-C
CREST + CENP-C
NT
Aph
B
i)
CENP-C IFA intensity at CREST (A.U.)
250
200
150
100
50
0
**
NT
Aph
Treatment
ii)
CENP-C co-localized with CREST (%)
100
80
60
40
20
0
**
NT
Aph
Treatment
C
Merge
CREST
CENP-C
CREST + CENP-C
NT
PDS

D

i)


CENP-C IFA intensity at CREST (A.U.)
400
300
200
100
0
*
NT
PDS
Treatment

ii)

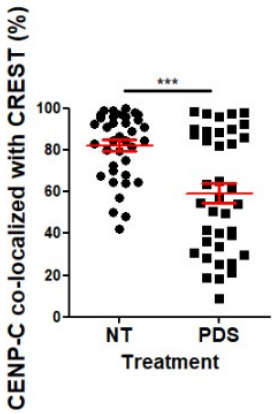

CENP-C co-localized with CREST (%)
100
80
60
40
20
0
***
NT
PDS
Treatment

Figure 3. Defective CENP-C localization to centromeres upon replicative stress induction.

(A) Immunofluorescence assay of DAPI (BLUE), CREST (RED), CENP-C (GREEN) of non-treated (NT) vs upon aphidicolin treatment (Aph)

(B) i) Graphical representation of immunofluorescence signal of CENP-C at centromeres of non-treated and aphidicolin treated cells. Each value represents the average centromere specific CENP-C fluorescence signal of each cell. (NT, n= 41 ; Aphidicolin treated, n= 37, **, $p < 0.0046$) ii) Graphical representation of the percentage of CENP-C co-localized with centromeres (CREST). Each value is an average percentage of CENP-C colocalized with CREST in each cell. (NT, n= 39; Aphidicolin treated, n= 35, **, p=0.001)

(C) Immunofluorescence assay of DAPI (BLUE), CREST (RED), CENP-C (GREEN) of non-treated (NT) vs upon PDS treatment (PDS)

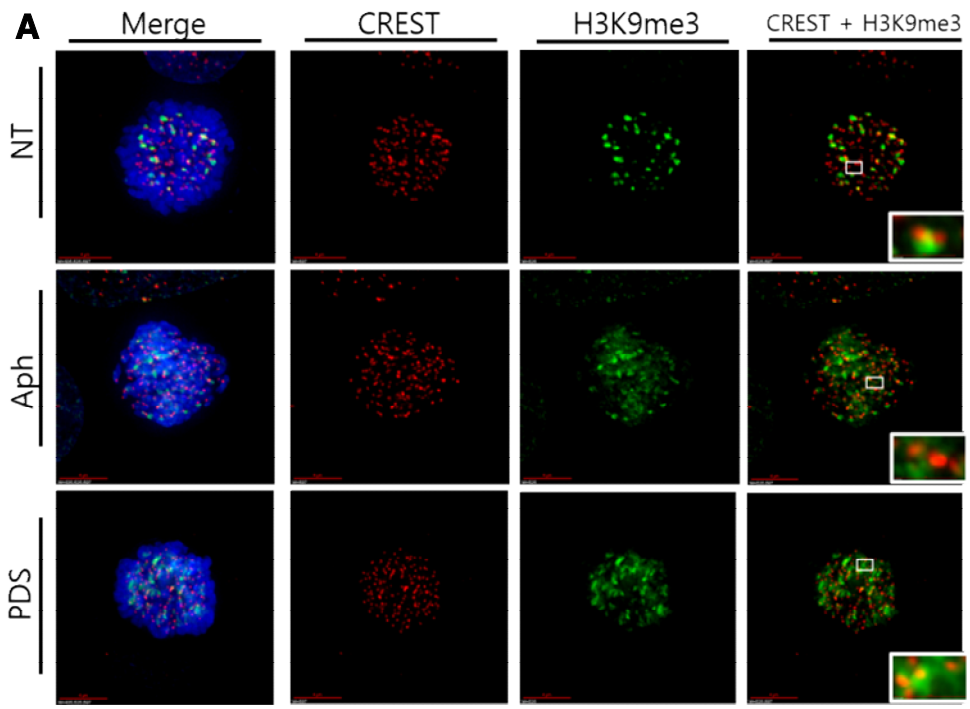
(D) i) Graphical representation of immunofluorescence signal of CENP-C at centromeres of non-treated and PDS treated cells. Each value represents the average centromere specific CENP-C flouresence signal of each cell. (NT, n= 39 ; Aphidicolin treated, n= 39, *, $p = 0.0240$) ii) Graphical representation of the percentage of CENP-C

co-localized with centromeres (CREST). Each value is an average percentage of CENP-C colocalized with CREST in each cell. (NT, n= 38; PDS treated, n= 38, **, $p<0.0001$)

III-3. Changes in epigenetic status of histone 3 upon replicative stress and DNA damage induction.

Previous studies have shown that the tri-methylation: acetylation switch at lysine 9 of histone 3 (H3K9) mediates the incorporation of CENP-A. In order to observe the effect of prolonged replicative stress and or DNA damage on H3K9 epigenetic modification status, HeLa cells were treated with aphidicolin and PDS for 24 hours and were fixated with 4% paraformaldehyde and observed through immunofluorescence assay utilizing H3K9me3 and H3K9ac specific antibodies. (Figure 4.A)

In both cases of replicative stress and DNA damage induction through aphidicolin and PDS treatment respectfully, H3K9me3 shows a distinct phenotype. Whilst the tri-methylation immunofluorescence signal is relatively localized at the centromere in non-treated samples, in both aphidicolin and PDS treated cells, the signal is relatively diffused from the centromere, covering more of the chromosomes shown by DAPI staining. (Figure 4. A). On the other hand, H3K9ac IFA results reveal that upon aphidicolin and PDS treatment, the global level of H3K9ac intensity is significantly decreased. (Figure 4. D,E,F(i),F(ii))


A
Merge
CREST
H3K9me3
CREST + H3K9me3
NT
Aph
PDS

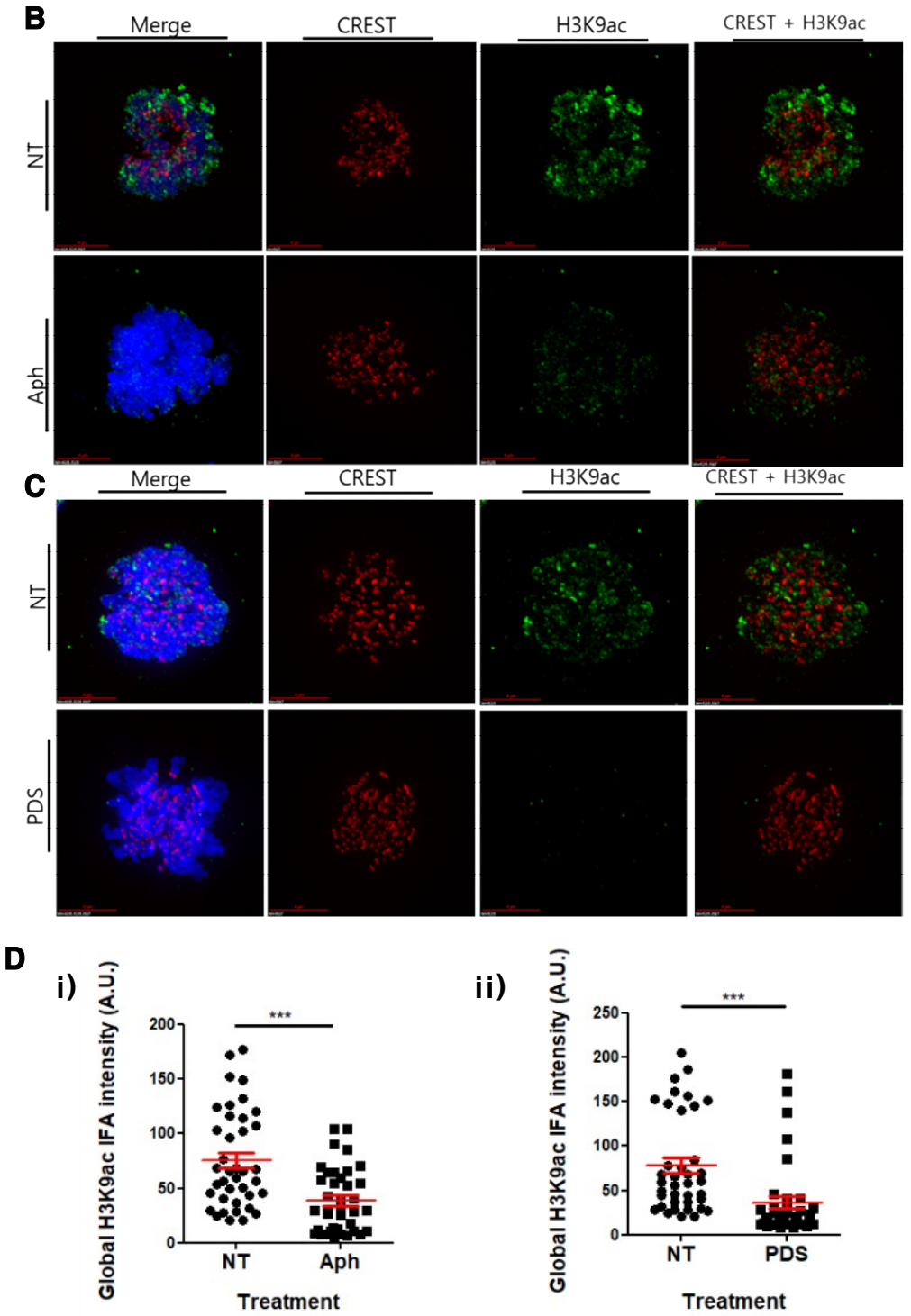

B
Merge
CREST
H3K9ac
CREST + H3K9ac
NT
Aph
C
Merge
CREST
H3K9ac
CREST + H3K9ac
NT
PDS
D
i)
Global H3K9ac IFA intensity (A.U.)
***
200
150
100
50
0
NT
Aph
Treatment
ii)
Global H3K9ac IFA intensity (A.U.)
***
250
200
150
100
50
0
NT
PDS
Treatment

Figure 4. Epigenetic modification status of lysine 9 of histone 3 is effected upon replicative stress and DNA damage induction.

(A) Immunofluorescence assay of DAPI (BLUE), CREST (RED), H3K9me3 (GREEN) of non-treated (NT), aphidicolin treated (Aph) and PDS treated (PDS) cells. (NT; n= 59, Aphidicolin treated; n= 58, PDS; n= 40)

(B) Global immunofluorescence assay of DAPI (BLUE), CREST (RED), H3K9ac (GREEN) of non-treated (NT) vs upon aphidicolin treatment (Aph)

(C) Global immunofluorescence assay of DAPI (BLUE), CREST (RED), H3K9ac (GREEN) of non-treated (NT) vs upon PDS treatment (PDS)

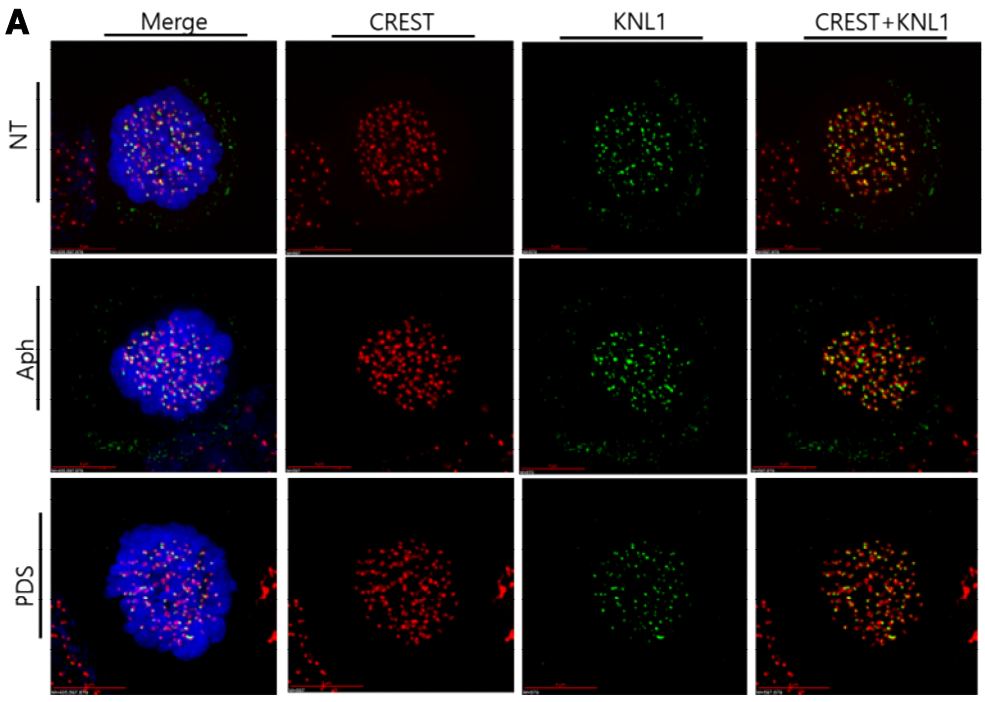
(D) i. Graphical representation of immunofluorescence signal of global H3K9ac non-treated and aphidicolin treated cells. Each value represents the average global H3K9ac fluorescence signal of each cell. (NT, n= 39; Aphidicolin treated, n= 38, ***, $p < 0.0001$)

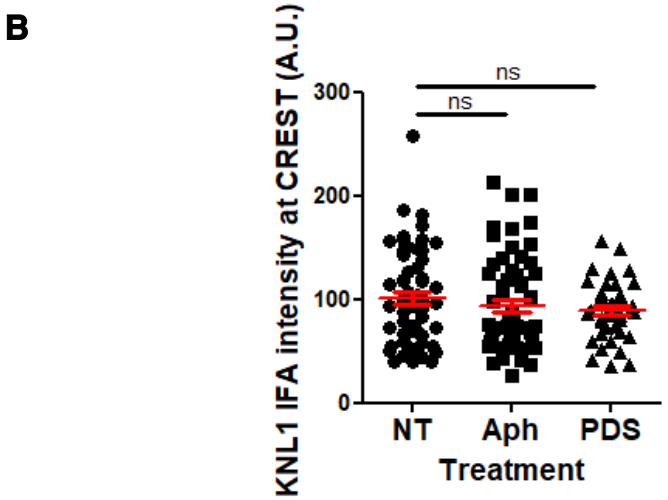
ii. Graphical representation of immunofluorescence signal of global H3K9ac non-treated and PDS treated cells. Each value represents the average global H3K9ac fluorescence signal of each cell. (NT, n= 38; PDS treated, n= 37, ***, $p < 0.0005$)

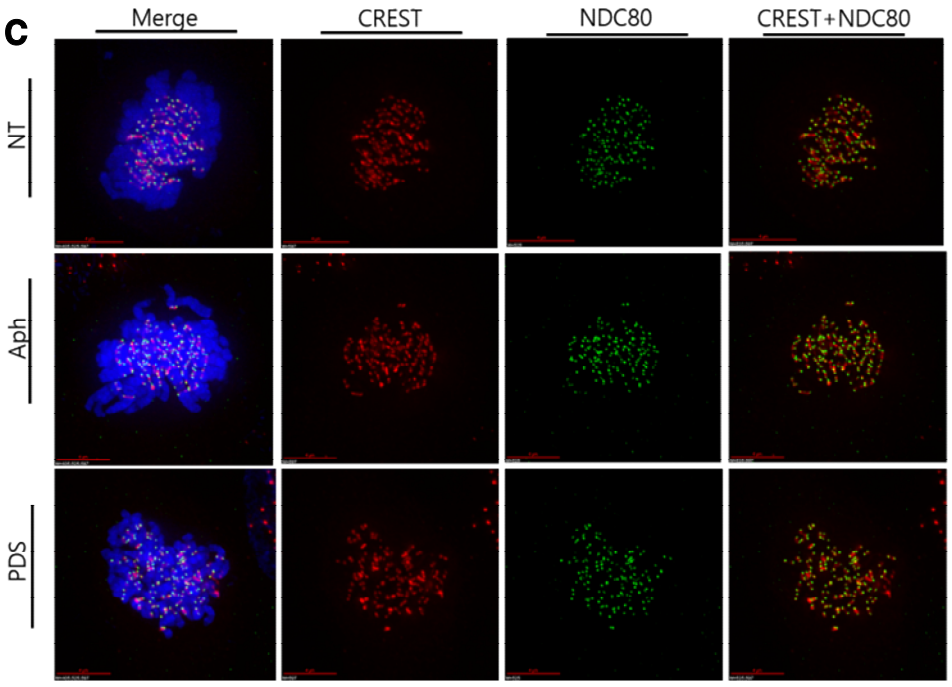
III-4. Outer kinetochore components successfully localizes to centromeres.

As previously mentioned, the KMN network is composed of Mis12, KNL1, and NDC80. The KMN netowrk protects genomic integrity through mediating cellular events such as the spindle assembly checkpoint and allowing kinetochore-spindle microtubule binding. Based on the previous observation of the abrogated inner kinetochore components of CENP-A and CENP-C, we further investigated the potential effect of replicative stress and DNA damage on outer kinetochore components KNL1 and NDC80 through immunoflouresence assay. (Figure 5. A,C)

Upon, replicative stress and DNA damage induction through aphidicolin and PDS treatment respectively, the KMN components of KNL1 and NDC80s fluorescence intensity at centromeres did not change. (Figure 5. B,D) This suggests that KNL1 and NDC80s localization is not disturbed upon replicative stress and DNA damage induction.


A
Merge
CREST
KNL1
CREST+KNL1
NT
Aph
PDS


B
KNL1 IFA intensity at CREST (A.U.)
300
200
100
0
ns
ns
NT
Aph
PDS
Treatment


C
Merge
CREST
NDC80
CREST+NDC80
NT
Aph
PDS

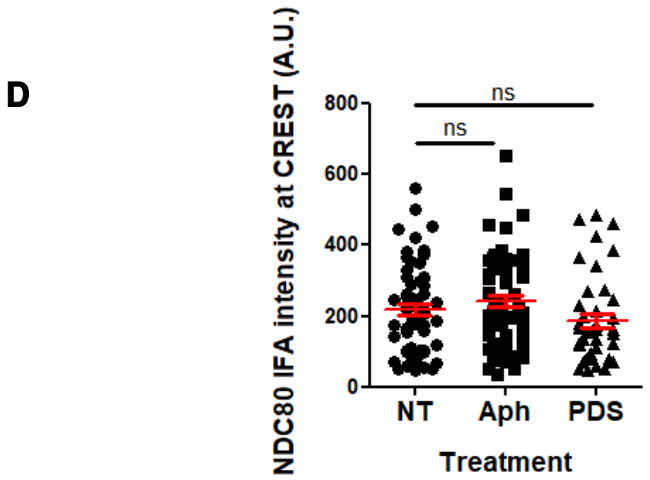

D
NDC80 IFA intensity at CREST (A.U.)
800
600
400
200
0
ns
ns
NT
Aph
PDS
Treatment

Figure 5. Successful assembly of outer kinetochore components KNL1 and NDC80.

(A) Immunofluorescence assay of DAPI (BLUE), CREST (RED), KNL1 (GREEN) of non-treated (NT), aphidicolin treated (Aph) and PDS treated (PDS) cells.

(B) Graphical representation of immunofluorescence signal of KNL1 at centromeres of non-treated, aphidicolin treated, and PDS treated cells. Each value represents average centromere specific KNL1 fluorescence signal of each cell. (NT, n= 60; Aph, n= 59; PDS, n= 39; (ns) NT-Aph, p =0.4205; NT-PDS, p=0.1550)

(C) Immunofluorescence assay of DAPI (BLUE), CREST (RED), NDC80 (GREEN) of non-treated (NT), aphidicolin treated (Aph) and PDS treated (PDS) cells.

(D) Graphical representation of immunofluorescence signal of NDC80 at centromeres of non-treated, aphidicolin treated, and PDS treated cells. Each value represents average centromere specific NDC80 fluorescence signal of each cell. (NT, n= 60; Aph, n= 61; PDS, n= 40; (ns) NT-Aph, p= 0.3057; NT-PDS, p= 0. 2187)

IV. Discussion

CENP-A incorporation is crucial in specifying the centromeric region and serves as the platform for downstream kinetochore assembly. Due to the requirement of successful kinetochore assembly for chromosomal stability protecting events such as the onset of the spindle assembly checkpoint, the incorporation of CENP-A is crucial in maintaining chromosomal stability. (Regnier et al., 2005; Carroll et al., 2010; Guse et al., 2011)

Previous studies have shown that distinct cell cycle specific events are required for successful CENP-A incorporation. This includes the S-phase specific distribution of pre-existing centromeric CENP-A to the template and the newly synthesized DNA. (Pan et al,.2019) Furthermore, recent studies suggest the importance of the epigenetic status of centromeric histone 3 for the successful incorporation of CENP-A such as the tri-methyl;acetyl switch of H3K9. (Ayrapetov et al., 2014) CENP-A have also been suggested to localize to double stranded breaks. (G. Zeitlin et al., 2009) The fact that the crucial S-phase specific equal distribution of pre-existing CENP-A to template and newly synthesized DNA is a replication coupled event, collectively with previous studies suggestion of the role CENP-A in DNA damage response,

provides a new question: the potential effect of prolonged replicative stress and or DNA damage caused during interphase on CENP-A incorporation.

Here we have shown that upon replicative stress induction through aphidicolin treatment and DNA damage induction through PDS treatment, CENP-A incorporation is abrogated. Furthermore, we have shown that the observed abrogated phenotype further affects the downstream recruitment of CENP-C. This collectively suggests that the often observed mitotic errors and or chromosomal instability upon extended replicative stress and or DNA damage induction may potentially be due to the abrogation of CENP-A incorporation.

Furthermore, we have observed the changes in epigenetic status of centromeric histone 3 which have been suggested to affect the incorporation of CENP-A: H3K9me3 and H3K9ac. Previous studies have observed that the diffusion of H3K9me3 throughout the chromosome have resulted in abrogated CENP-A incorporation whilst H3K9ac has been suggested to promote CENP-A incorporation. (Zhu et al., 2018) Here we have shown that H3K9me3 signals diffuse from the centromere upon replicative stress or DNA damage induction. We have also shown that a significant decrease in global immunofluorescence signal

intensity of H3K9ac occurs upon replicative stress and DNA damage induction. Collectively, the observed changes of CENP-A mediating H3K9me3 and H3K9ac, suggest that replicative stress and DNA damage induced diffusion of H3K9me3 and the decrease of global H3K9ac may be the cause of the observed abrogated CENP-A incorporation. We believe that further investigation regarding the direct link between the observed replicative stress and DNA damage induced epigenetic changes and abrogated CENP-A will further our understanding of the link between CENP-A abrogation and disrupted replication. Whilst not shown here, over-expression of histone 3 mutants which are methylation-deficient, acetylation-deficient, and acetylation mimetic have been designed and are under current experimentation in order to acquire such understanding.

Furthermore, we have shown that the outer-kinetochore components KNL1 and NDC80 successfully localize to centromeres upon replicative stress and DNA damage induction. It is interesting to note that, whilst it is expected that kinetochore assembly is not completely abrogated upon replicative stress and DNA damage, there was no significant change in NDC80 and KNL1s centromeric phenotype despite the previously observed CENP-A and CENP-C abrogation. Collectively, this perhaps, hints at the existence of a potential

pathway which is able to compensate for the loss of the CENP-A:CENP-C dependent recruitment of the outer kinetochore which is activated upon replicative stress and or DNA damage. Perhaps, the DNA direct binding CENP-T pathway could be further explored to further understand the observed results.

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국문 초록

복제 스트레스 및 유전자 손상으로 인한 중심절 단백질들의 위치 변화 연구

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세포 분열 시에 유전정보의 안정성을 유지하기 위해서는 중심적 단백질들의 올바른 centromeric recruitment가 중요하다고 알려져 있다. 이는 중심적 단백질들의 성공적인 세포 분열에 필요한 kinetochore assembly의 기반으로 작용하기 때문이다. 완전한 kinetochore는 kinetochore-microtubule 결합 또는 spindle assembly checkpoint의 기능을 허용하여 유전정보의 안정성을 유지하는 데 중요한 역할을 한다. 이러한 역할에 필요한 centromere 식별을 위한 여러 기작 중 CENP-A incoporation 이 있다. CENP-A 는 Histone 3의 변이체로 써 centromeric region에 incorporation 함으로써 세포의 centromere 식별을 가능하게 한다. 현재의 연구는 CENP-A 의 incorporation 이 세포 주기 중에서 각 주기마다 다른 event를 통해 이루어진다고 알려져 있다. 신규 합성 DNA에 대한 기존 CENP-A의 동일한 분포는 S-phase에서 발생하며 CENP-A 단백질의 안정화 및 통함은 holiday-junction-recognizing-protein (HJURP) 및 Mis18 복합체

dependent 한 기작으로 M-phase 후반기 및 G1 초기에 발생한다. 또한, CENP-A incorporation은 histone lysine 9의 (H3K9) tri-methyl:acetyl 스위치를 통해 조절되는 것으로 밝혀진 바 있다. 현재 CENP-A의 이상이 암에서 자주 관찰된다는 사실에도 불구하고, abrogated CENP-A incorporation의 잠재적인 생리학적 원인은 명확하지 않다. 본 연구를 통해 replicative stress 및 DNA 손상이 CENP-A incorporation에 미칠 수 있는 잠재적인 영향을 관찰하고자 한다.

본 연구를 통해 복제 스트레스와 DNA 손상을 겪은 후 mitosis로 진행되는 세포가 CENP-A incorporation 이상뿐만 아니라 CENP-A downstream 구성 요소 CENP-C의 이상을 초래한다는 것을 관찰하였다. 또한, 앞서 언급한 H3K9의 후성유전학적 상태가 복제 스트레스 및 DNA 손상 유도 후 관찰 하였을 때에, H3K9의 tri-methylation은 kinetochore 에선 diffused 한 형태를 보이는 반면, global H3K9의 acetylation은 현저하게 감소하는 것을 관찰하였다. H3K9의 후생유전학적 상태 변화와 CENP-A incorporation의 직접적인 연관성은 현재 추가적 연구가 진행되고 있다. 또한 본 연구는 관찰된 CENP-A incorporationd의 이상을 불구하고 성공적으로 centromere에 outer kinetochore의 일부인 KNL1 과 NDC80의 성공적인 localization을 관찰하였다.

통합적으로, 본 연구는 복제 스트레스 및 DNA 손상은 CENP-A incorporation의 이상을 유발한다는 것을 보여줬으며 downstream inner kinetochore의 구성인 CENP-C의 localization의 이상도 관찰하였다. 또한, 본 연구는 복제 스트레스 및 DNA 손상으로 인한 Histone 3의 후생유전학적 변화가 CENP-A incorporation에 가질 수 있는 잠재적인 영향에 대한 질문을 제기하며 관찰된 CENP-A incorporation의 이상을

불구하고 outer kinetochore를 성공적으로 localize 할 수 있는 잠재적 보상 경로에 대한 존재 가능성을 제기한다.

감사의 글

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