Award Number: W81XWH-13-1-0106

TITLE: : Functional Characterization of CENP-A Post-Translational Modifications in Chromosome Segregation

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REPORT DATE: July 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2015	Annual	15Jun2014 - 14Jun2015

4. TITLE AND SUBTITLE

Functional Characterization of CENP-A Post-Translational Modifications in Chromosome Segregation

5a. CONTRACT NUMBER
W81XWH-13-1-0106

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Virginia
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Colorectal cancer is the second leading cause of cancer death in the United States. Approximately 85% of colorectal cancers are CIN+ (Chromosomal instability) and are associated with poor survival. The molecular mechanisms responsible for the CIN phenotype and hence means to target this pattern of genome instability remains poorly defined. I hypothesize that, post-translational modifications (PTM) of the centromeric nucleosome, specifically on the histone variant, CENP-A, will direct centromere activity, and that perturbations of such could lead to aneuploidy and cancer. We proposed to decipher the pathway that leads to CENP-A α-amino methylation and to determine the function it plays in ensuring the fidelity of chromosome segregation. We have shown that CENP-A is methylated by NRMT1 both *in vitro* and *in vivo*. CENP-A is methylated throughout the cell cycle. We established that CENP-A α-amino tri-methylation required for ensuring high fidelity of chromosome segregation, and hence preventing aneuploidy and cancer. Importantly, we found that loss of CENP-A α-amino tri-methylation trigger a proliferation advantage and cells form bigger colonies in colony formation assay. We also found that CENP-A methylation contributes to the cell survival and in the absence of it, cells undergoes senescence. This response is dependent on p53 pathway. But in the absence of p53, cells undergo further proliferation, aneuploidy and tumorigenesis. Methylation of CENP-A is required for the assembly of constitutively centromere associated network proteins and that explain the chromosome segregation defect in CENP-A methylation mutant. The results suggests that α-amino tri-methylation of CENP-A is an important post-translational modification necessary for maintaining accuracy of chromosome segregation.

15. SUBJECT TERMS
colorectal cancer, post-translational modifications, CENP-A, NRMT1, centromere, CENP-A α-N-me3

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	19	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

Colorectal cancer is the second leading cause of cancer death in the United States[1]. Chromosomal instability (CIN) and microsatellite instability (MIN) are two major molecular hallmarks of colorectal cancer[2,3]. 85% of colorectal cancers are CIN+ and are associated poor survival[3]. The molecular mechanisms responsible for the CIN phenotype and hence means to target this pattern of genome instability in colorectal tumors remains poorly defined. I hypothesized that, similar to the nucleosomes of general chromatin, post-translational (PTM) modifications of the centromeric nucleosome, specifically on the histone variant, CENP-A, will direct centromere activity, and that perturbations of such could lead to aneuploidy and cancer. However, the identification of CENP-A PTMs has lagged behind PTMs of other histones because of the lack of a good purification strategy. Using a novel purification strategy we have identified three PTMs on the CENP-A tail, α-amino tri-methylation of initial Glycine, and phosphorylations at S16 and S18[4]. Previously, another phosphorylation was reported at S7[5]. We proposed to decipher the pathway that leads to CENP-A α-amino methylation (CENP-A α-N-me3) and to determine the function it plays in ensuring the fidelity of chromosome segregation. We also proposed to determine how its abrogation may cause carcinogenesis and ask whether targeting this PTM is a viable strategy to target colorectal cancer cells. Overexpression of CENP-A in colorectal cancer leads to its mislocalization to chromosome arms resulting in aneuploidy. Until now the only way to inhibit CENP-A function was through shRNA knockdown. However, understanding CENP-A amino terminal tail modification will provide enzymatic and therefore potentially druggable targets to inhibit this pathway.

In the first year report we have demonstrated that CENP-A is methylated by NRMT1 both *in vitro* and *in vivo*. CENP-A is methylated before it is deposited into the centromere and that the methylation persists throughout the cell cycle. However, we found an increase in CENP-A methylation during prophase of the cell cycle. We established that CENP-A α-amino tri-methylation required for ensuring high fidelity of chromosome segregation, and hence preventing aneuploidy and cancer. Importantly, we found that loss of CENP-A α-amino tri-methylation in colorectal cancer cells trigger a proliferation advantage and they form bigger colonies in colony formation assay. Suggesting α-amino tri-methylation of CENP-A is an important post-translational modification necessary for maintaining accuracy of chromosome segregation.

This year we have made progress in further elucidating the role of CENP-A α-amino tri-methylation. Using a CENP-A knockout cell line we have found the CENP-A α-amino tri-methylation contribute to cell survival. In the absence of α-amino tri-methylation, the cells undergo senescence. CENP-A methylation required for the formation of Constitutive Centromere Associated Network (CCAN) complex. We have found in the absence of CENP-A methylation the localization of CENP-T and CENP-C is reduced. This alteration might be the reason for the chromosome segregation defect we have seen in the methylation mutant cancer cells. We inferred that the impaired formation of kinetochore might leads to an imbalance in the force generated by the motor protein on the chromosomes that leads to multipolar spindle in CENP-A methylation mutant cancer cells. Hence, we partially inhibited one of the motor proteins Eg5 using low

concentration of monastrol and we were able to rescue the multipolar spindle formation. We conclude that CENP-A α-amino tri-methylation required for kinetochore formation and maintenance of bipolar spindle.

Keywords: Centromere, CENP-A, CENP-A α-amino tri-methylation, NRMT, CENP-T, CENP-C, Colorectal cancer, p53, p21

Overall project summary:
In this section we discuss our results that we accomplished during last year. Then we will discuss the importance of our finding in discussion part, and finally we will explain major materials and methods we used.

Results:

CENP-A α-amino tri-methylation required for cell survival

In order to see whether CENP-A α-amino tri-methylation is required for cell survival, we used a CENP-A knockout cell line developed by the Cleaveland lab[6]. In this cell line, one allele of the CENP-A gene is knocked out and the second allele is a Flox allele, which upon infection with adenovirus Cre-recombinase, will be removed. We have stably integrated CENP-A wild type or methylation mutant in this cell line. Since it has been shown that CENP-A n-terminal tail and c-terminal tail has some redundant function in survival of the cells[6], we replaced the c-terminal tail of CENP-A with H3 c-terminal tail in one set of mutants while keeping the n-terminus either wild type or methylation mutant (Fig.1A). The cells were infected with Ad-Cre recombinase expressing virus and after 48 hours 500 cells were seeded into 10cm plates in triplicate (Fig.1B). After 14 days, the cells were fixed in methanol and stained with crystal violet stain. Similar to CENP-A knockout cells where only 10% of cells survived, the methylation mutant where c-terminus of the CENP-A is also replaced with H3 c-terminal tail only 10% of the cells formed colonies (Fig.1C&D). Suggesting that CENP-A α-amino tri-methylation contributes to the survival or proliferation of the cells.

Loss of CENP-A α-amino tri-methylation causes senescence

To see why the CENP-A methylation mutant did not form colonies, we checked whether the cells were undergoing senescence. We infected the wild type and mutant CENP-A cells with Ad-Cre recombinase and checked for β-galactosidase activity after 4,7 and 10 days of infection using a fluorescent substrate 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C 12FDG) (Fig.2A&B)[7]. The CENP-A knockout cell line without any CENP-A replacement upon infection with Ad-Cre virus underwent senescence (Fig.2C). However, cells replaced with CENP-A wild type or full-length methylation mutant did not show senescence phenotype. In this case we found only less that 1% cells undergoing senescence compared to 20% in CENP-A knockout cell line. However, the methylation mutant where the c-terminal tail changed to H3 c-terminus underwent senescence at a rate similar to the CENP-A knockout cell lines (Fig.2D). This suggests that in the absence of CENP-A α-amino tri-methylation, the n-terminal tail is no longer fully functional and could not compensate for the loss of c-terminal tail. Suggesting the CENP-A α-amino tri-methylation contributes to the survival of the cells and its loss leads to senescence in normal cells.

We checked the senescence induction is through p53[8]. We found that upon endogenous CENP-A removal, the p53 level is elevated and is reduced in CENP-A wild type replaced cells (Fig.2E). But in the methylation mutant where the c-terminal tail changed to H3 c-terminus we see an overall increase in p53 even without removing the endogenous CENP-A and is further induced upon endogenous CENP-A removal (Fig.2E). This increased p53 level even in the presence of endogenous CENP-A renders the cell susceptible to senescence.

Figure 1

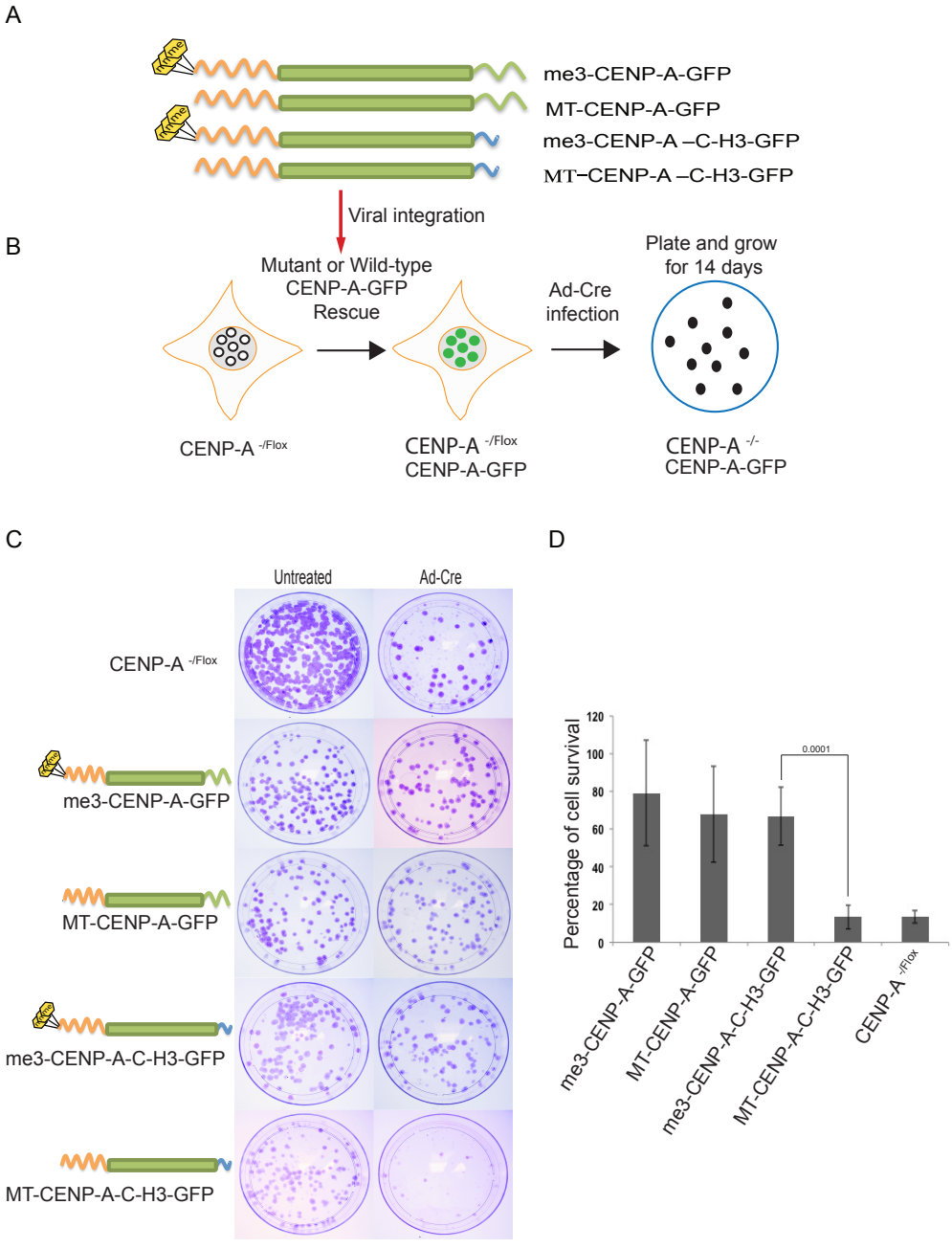

Figure 1. CENP-A α-amino tri-methylation required for cell survival (A). Schematic diagram of the different CENP-A constructs used, (B). Schematics of the experiment. RPE CENP-A knockout cell line RPE CENP-A -/F cells

were virally integrated with CENP-A constructs. The second allele of the endogenous CENP-A was then removed by infecting with Ad-cre virus. 48 hours after infection, the cells were split and 500 cells were seeded onto 10cm plate in triplicate. After 14 days the colonies were visualized by fixing in methanol and crystal violet staining, (C). Representative images of the colony formation assay. The number of colonies formed drastically reduced upon CENP-A removal that is restored with all the wild type and mutant CENP-A except the methylation mutant where c-terminus is also swapped with H3 c-terminus, (E). Graph showing percentage of cell survival calculated based on colony formation assay.

The same is true for p21 level (Fig.2E). Suggesting that loss of CENP-A/CENP-A α-amino tri-methylation induce senescence in a p53 dependent manner.

Figure 2

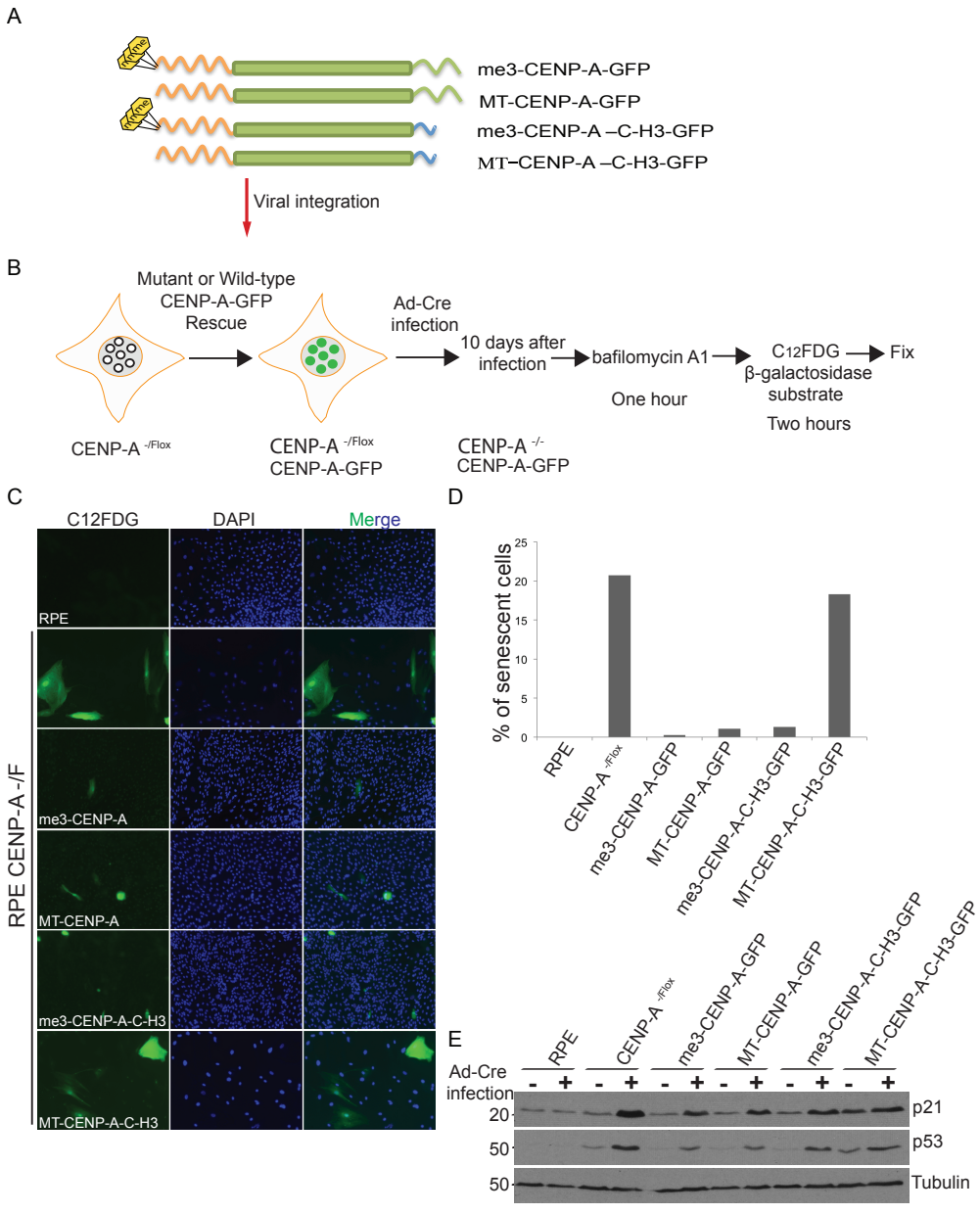

Figure 2. Loss of CENP-A α-amino tri-methylation causes senescence (A). Schematic diagram of the different CENP-A constructs used, (B). Schematics of the experiment. CENP-A knockout cell line RPE CENP-A -/F cells were

virally integrated with CENP-A constructs. The second allele of the endogenous CENP-A was then removed by infecting with Ad-cre virus. On days 4, 7 and 10, the cells were treated with Bafilomycin A1 for one hour and then added 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), a fluorogenic substrate for β-galactosidase for another hour. The cells were then fixed, permeabilized and DAPI stained. The cells were observed under microscope. A green fluorescent staining indicates senescent cells, (C). Representative images of the β-galactosidase assay. Upon CENP-A removal the cells undergo senescence, which is rescued by all the CENP-A, constructs used except CENP-A methylation mutant where c-terminal of CENP-A is also swapped with H3 c-terminus (lower panel), (D). Graph showing percentage of senescent cells, (E). Western blot showing p53 and p21 induction after CENP-A knockout. P53 and p21 induced upon removal of endogenous CENP-A which is rescued partially by exogenous wild type and mutant CENP-A except CENP-A methylation mutant where c-terminal of CENP-A is also swapped with H3 c-terminus. p53 and p21 remained high in this mutant cells even in the presence of endogenous CENP-A, which is further induced upon endogenous CENP-A removal by infecting with Ad-cre virus.

α-amino tri-methylation of CENP-A required for the accurate formation of CCAN complex

Since CENP-A epigenetically specifies centromere and involved in the assembly of Constitutively Centromere Associated Network (CCAN) complex, we wanted to check whether loss of CENP-A methylation cause defects in CCAN recruitment at the centromere. For this we used the CENP-A knockout RPE cells where we stably integrated either wild type or mutant CENP-A (Fig.3A). The localization of CCAN proteins such as CENP-C, CENP-T, CENP-I and CENP-B where analyzed with and without Ad-Cre virus infection which removes endogenous CENP-A. In our initial analysis, we found a consistent reduction in CENP-T (Fig.3E&F) and CENP-C (Fig.3E&G) in CENP-A methylation mutant compared to wild type replaced cells similar to CENP-A knockout cell line. However, we did not find such a difference in the localization of these proteins where endogenous CENP-A is still present (Fig.3B,C&D). This suggests that CENP-A α-amino tri-methylation is required for the accurate assembly of CCAN components. This result may explain the chromosome segregation defects and multipolar spindle formation that we see in the CENP-A methylation mutant cancer cells (Last year report). Thus, CENP-A methylation is required for the formation of the proper centromere formation and efficient chromosome segregation.

Force imbalance generated by the lagging chromosomes causes multipolar spindle in CENP-A methylation mutant

It has been shown that force balance generated by different motor proteins such as Kif15, Eg5, CENP-E and Dynein is required for the formation of bipolar spindle[9]. Alterations in the centromere and kinetochore structure could lead to improper localization of these factors. To see whether the multipolarity is caused by the force imbalance generated by the unaligned chromosomes, we partially inhibited the motor protein Eg5. CENP-A was knockdown in HeLa cells using shRNA as depicted in the diagram (Fig.4A) and synchronized using a double Thymidine block and release. Twelve hours after release we added either MG132 or MG132 along with Eg5 inhibitor monastrol. We found that percentage of cells with multipolar spindles was reduced to control levels in CENP-A shRNA treated cells after treatment with Monastrol. This suggests that a force imbalance generated by the improper kinetochore formation after knockdown of CENP-A is the reason for the formation of multipolar spindle (Fig.4B&C). To see whether a similar force imbalance causes multipolar spindles in CENP-A methylation mutants, we knockdown and replaced CENP-A wild type or mutant CENP-A. In these cells we treated cells with MG132 along with increasing concentration of

monastrol. With increase in monastrol concentration we observed a decrease in multipolar spindle in CENP-A methylation mutant (Fig.4D). This suggests that in CENP-A methylation mutant force imbalance generated by the defective chromosome segregation causes multipolarity. We were also seen similar effects in HCT116 colorectal cancer cells.

Figure 3

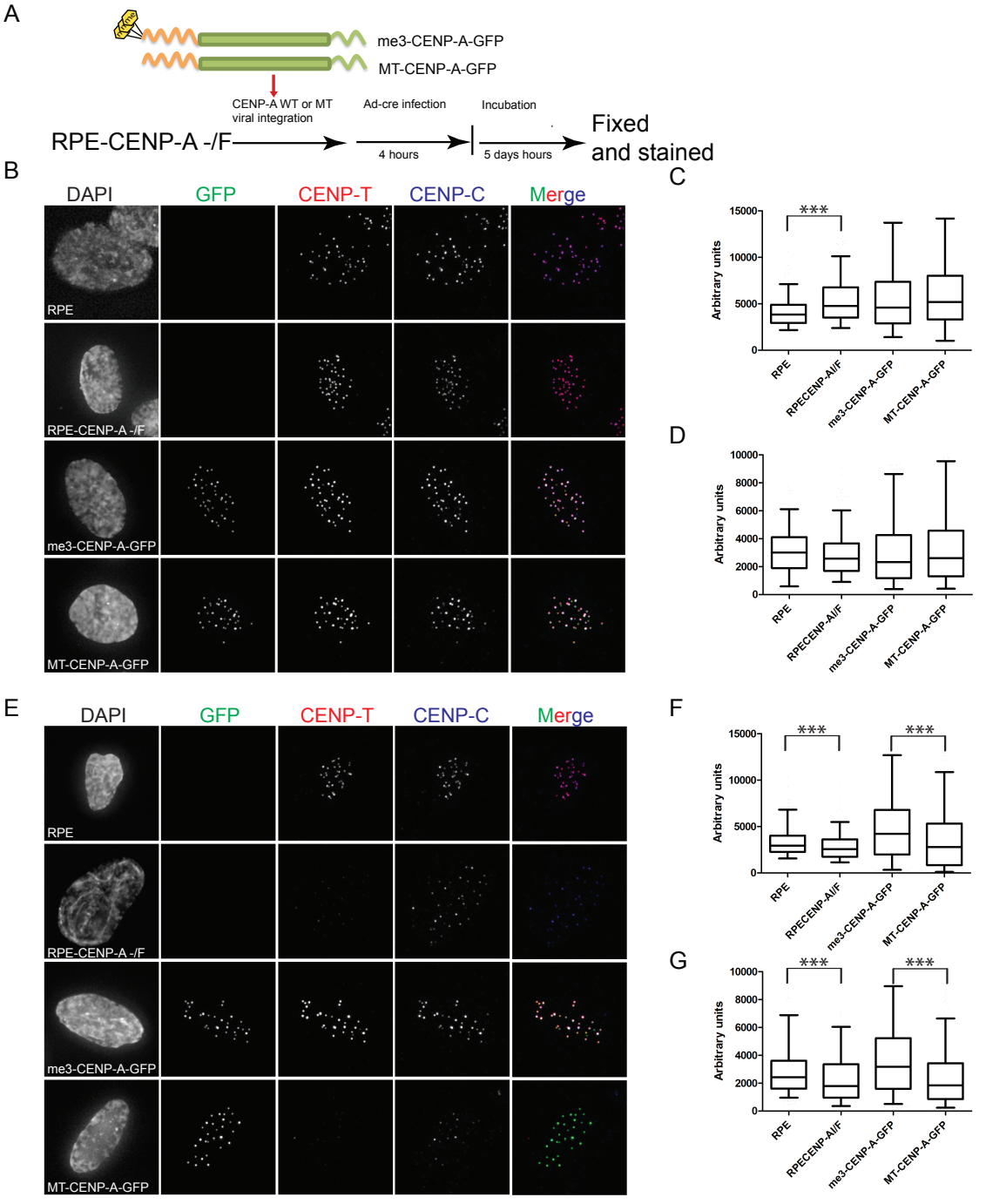

Figure 3. α-amino tri-methylation of CENP-A required for the accurate formation of CCAN complex (A). Schematic diagram of the experiment. CENP-A knockout cell line RPE CENP-A -/F cells were virally integrated with

full-length wild type or mutant CENP-A constructs. The endogenous CENP-A was then removed by Ad-cre virus infection. On 5th day the cells were pre-extracted, fixed and stained with CENP-T and CENP-C antibodies, (B). CENP-T and CENP-C staining before Ad-cre infection. The rescue construct shown as green (GFP), (C&D). Quantitation of CENP-T and CENP-C at the centromere before infecting with Ad-cre virus respectively, (E) CENP-T and CENP-C centromere localization after the removal of endogenous CENP-A by Ad-cre virus, (F&G). Quantitation of CENP-T and CENP-C at the centromere respectively after the removal of endogenous CENP-A by Ad-cre virus. *** p value <0.0001.

Figure 4

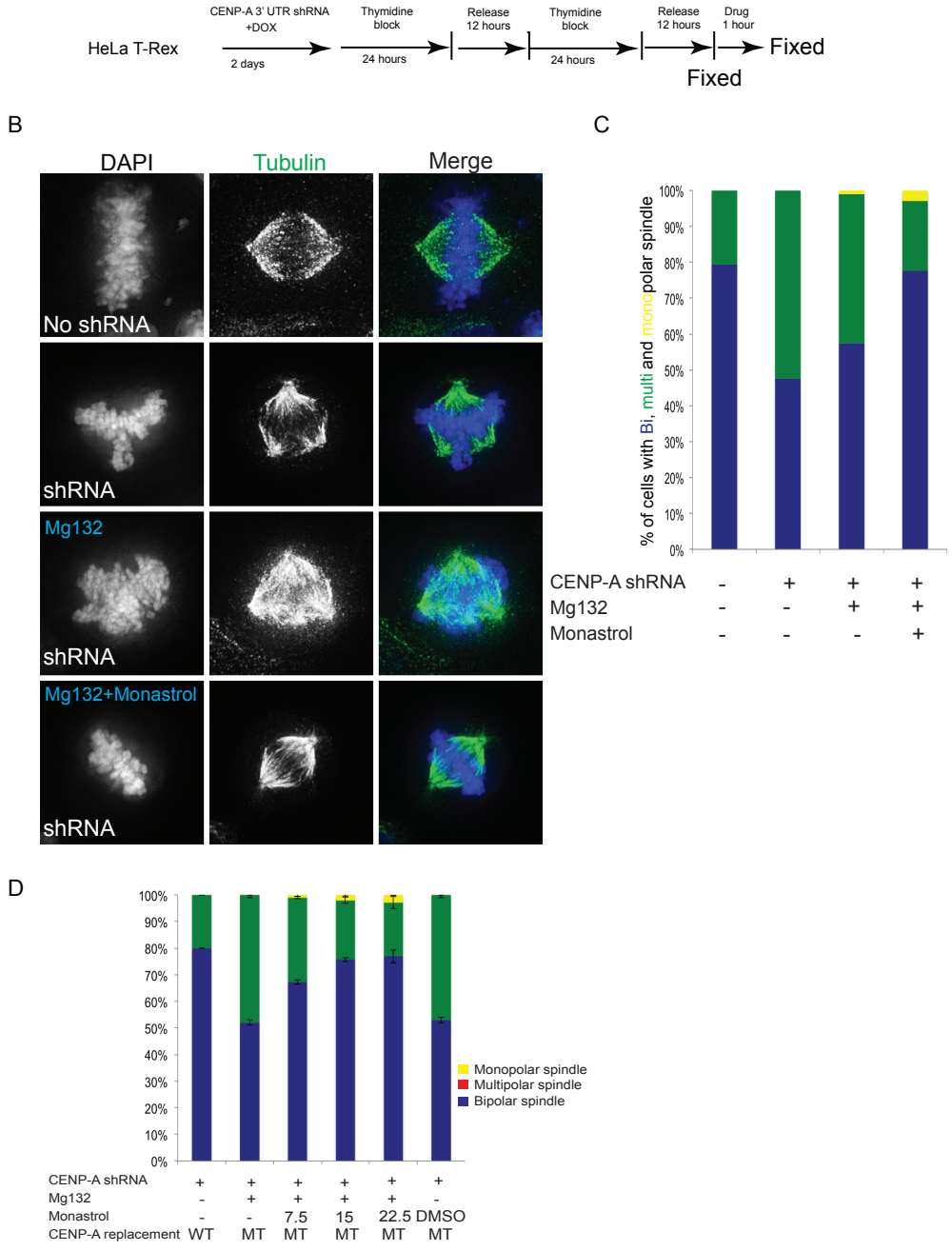

Figure 4. Force imbalance generated by the misaligned chromosomes causes multipolar spindle in CENP-A methylation mutant (A). Schematic diagram of the experiment. Endogenous CENP-A was knocked down either with or without replacement for eight days. The cells then double thymidine blocked and released. 12 hours after second release cells were treated with indicated drugs for one hour and cells were fixed and stained, (B). Mitotic cells

stained for tubulin after with and without CENP-A knock down and treated with Eg5 inhibitor monastrol and proteasome inhibitor MG132. Treatment with monastrol decreases the multipolar spindle formation, (C&&D). Percentages of the monopolar, bipolar and multipolar cells with CENP-A knockdown (C) and replacement cells (D) treated with monastrol.

Discussion:

The post-translational methylation of alpha-amino groups was first discovered over 30 years ago[10,11]. However, its biological function remains obscure except in the case RCC1 (Ran guanine nucleotide-exchange factor) where it is required for its localization into chromosomes[11]. We identified a novel function of alpha-N-methylation. We found that alpha-N-methylation of CENP-A is required for maintaining genomic stability and its loss leads to aneuploidy. We demonstrated that CENP-A is methylated throughout the cell cycle and methylated CENP-A increases during prophase. Reduction in CENP-A level increases the propensity of multipolar spindle pole formation and lagging chromosome in p53 null colorectal cancer cell HCT116. Where as in isogenic p53+/+ cells there is no significant increase in multipolar spindle formation after CENP-A knockdown. In our knockdown replacement experiment, wild type CENP-A stably integrated cells had similar percentage of multipolar cells as that of parental cell lines both in colorectal cancer cell HCT116 p53-/- and HeLa. But the methylation mutant CENP-A stably integrated cells showed significantly increased multipolar spindle in p53-/- HCT116 and p53 inactive HeLa cells suggesting CENP-A methylation mutants are hypomorphic. We also analyzed chromosome missegregation defects in these cells and found that there is an increase in lagging chromosomes compared to wild type irrespective of p53 status. We also found further evidence that CENP-A methylation has a protective effect in tumorigenesis.

Surprisingly, here we demonstrated that CENP-A methylation contributes to the survival and/or proliferation of the immortalized RPE cells. CENP-A's n-terminal and c-terminal tails have some redundant function in cell survival[6]. In the absence of c-terminal tail, the CENP-A methylation mutant could not form colonies, suggesting the n-terminal is non functional without methylation. We also found that loss of CENP-A methylation in CENP-A without c-terminal tail cells trigger a senescence response. CENP-A knockout cell lines also undergo senescence in a p53 dependent manner. In the methylation mutant CENP-A, we found an overall increase in p53. Suggesting that cells sensing a functional CENP-A in the cells and in the absence of functional CENP-A it induce senescence to reduce aneuploidy. But in the absence of p53, cells accumulate chromosome segregation defects and become more tumorigenic.

Our preliminary results showed that there is a reduction in the centromere localization of CCAN components in CENP-A methylation mutant cells. The centromere localization of CENP-C and CENP-T is significantly reduced in methylation mutant cells. This suggests that CENP-A methylation is partially involved in the localization of these proteins to the centromere. Such changes in CCAN might be the reason for the chromosome segregation defects and multipolar spindle in the CENP-A methylation mutant cancer cells due to defective kinetochore formation.

Since we have seen chromosome segregation defects and multipolar spindle formation in CENP-A methylation mutant in cancer cells, we hypothesize that force imbalance generated by different motor proteins may be the reason for multipolar spindle formation. In such cases, we will see a reduction in multipolarity if we partially

inhibit such motor proteins. Upon partial inhibitions of Eg5 with low concentrations of monastrol at which concentration it does not induce monopolar spindle, we found that multipolarity reduced to the control level. Suggesting that indeed force imbalance generated by the improper formation of kinetochore is the mechanism for the formation of multipolar spindle.

Overall we have discovered that CENP-A methylation contributes to cell survival and in the absence of methylation cells undergo senescence. However, in the absence of p53, loss of CENP-A methylation causes multipolar spindle and confer more tumorigenic potential to the cancer cells. We still need to further confirm the CCAN localization defects in CENP-A methylation mutant and the role of CENP-A methylation in cancer,[12] for that a continuous support is necessary.

Materials and methods:

Colony formation assay: RPE CENP-A knockout cell line generated by the Cleaveland Lab[6] used for this study. In this cell lines one allele of CENP-A is knocked out and the other allele is a Flox allele, which upon Ad-cre recombinase virus infection will be removed. We made CENP-A wild type and mutant stable lines in these cells. 2500 cells were infected with Ad-cre for four hours and then the virus was washed out. 48 hours after infection, 500 cells were split into 10cm plates. The colonies were crystal violet stained after 14 days and counted.

Senescence assay: RPE CENP-A knockout cell lines integrated with either wild type or mutant CENP-A was infected with Ad-cre virus as mentioned above. After 4,7 and 10 days cells were treated with Bafilomycin-A1 for one hour and then added 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), a fluorogenic substrate for b-galactosidase. Cells were fixed after one hour and permeabilized with 0.1% triton in PBS. The nucleus was stained with DAPI and mounted.

Monastrol treatment: CENP-A shRNA integrated into HeLa T-Rex cells where induced by adding doxycycline for seven days. The cells were then double thymidine block and release. 12 hours after second release cells were either directly fixed or treated one hour with MG132 or MG132 with low concentration of monastrol. Cells were fixed and stained for tubulin. Similarly, in the knockdown replacement experiment, endogenous CENP-A was knockdown in wild type or mutant replaced cells. The cells were double thymidine blocked and released. 12 hours after second release cells were either directly fixed or treated one hour with MG132 or MG132 with increasing concentration of monastrol. Cells were then fixed and stained for tubulin. The multipolar cells were counted.

Immunofluorescence: Cells were either pre-extracted with 0.1% triton in 1X PBS for 3 minutes and then fixed in 4% formaldehyde for 10 minutes. The cells were then blocked in 2% BSA and 2% fetal calf serum with 0.1% triton in 1XPBS. It was incubated with primary antibodies one hour and then secondary antibody was added for one hour. All washes between each step were done in 1XPBS + 0.1% triton. DNA was stained with DAPI and were mounted in prolong. Cells were examined and images were acquired using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) using a X100 oil-immersion Olympus objective lens connected with Photometrics

CoolSNAP HQ^2 camera and Softwrox acquisition software. Images were deconvolved and presented as maximum stacked images. The antibodies used for immunofluorescence were: mouse anti-CENP-A (1:1000), Rabbit anti-me3CENP-A (1:200), Rabbit anti-CENP-T (1:1000), Mouse anti-CENP-C (1:1000), Rabbit anti-CENP-I (1:1000, Mouse anti-CENP-B (1:250), Mouse anti-α-tubulin (1:1000).

Following antibodies were used for western blotting. Mouse anti-p53 (DO7, Santa Cruz) (1:1000), Rabbit anti-p21(Santa Cruz) (1:500), Mouse anti-tubulin (1:1000).

Key accomplishments:

Following are the key accomplishments of the proposed work

Year I

1. Identified NRMT1 methylates CENP-A at its α-amino terminal.
2. CENP-A is methylated throughout the cell cycle.
3. Pre-nucleaosomal CENP-A is methylated.
4. We made several methylation resistant CENP-A mutants.
5. Loss of CENP-A cause aneuploidy by forming multipolar spindle and missegregation of chromosomes.
6. α-amino tri-methylation of CENP-A ensures high fidelity of chromosome segregation. Its loss cause multipolar spindle and lagging chromosomes in p53 null background and lagging chromosomes in the presence of p53. Both these alterations fundamentally cause aneuploidy, a form of genetic alterations prevalent (85%) in colorectal cancer.
7. We also found that methylation mutant CENP-A forms bigger colonies in colony formation assay suggesting role of methylation in controlling proliferation.

Year II

1. CENP-A α-amino tri-methylation required for the survival of the cells.
2. Loss of α-amino tri-methylation of CENP-A leads to senescence in normal cells.
3. Loss of CENP-A induces p53 and that leads to senescence. This explains the increased tumorigenic potential seen in CENP-A methylation mutant, which is less functional, in p53 null cells.
4. α-amino tri-methylation of CENP-A required for the localization of CCAN components to the centromere and centromere homeostasis.
5. Force imbalance generated by the improper kinetochore formation and resultant misaligned chromosomes may be the reason for the multipolar spindle formation in p53 null CENP-A mutant cell lines.

My abstract was selected for a short talk in the prestigious FASEB (Federation of American Societies for Experimental Biologist) meeting - Mitosis: Spindle Assembly and Function. Moreover I gave talk and did poster presentation in several meetings within

the University and outside. A list of the talk and poster presentation that I gave during the last year is given in the publication, abstract and presentation part.

Conclusion:

We have made significant progress in elucidating the function of α-amino tri-methylation of CENP-A. We achieved several of the proposed aims. We found that CENP-A is methylated by NRMT1 and this modification persists throughout the cell cycle. We also found that α-amino tri-methylation of CENP-A is critical in orchestrating chromosome segregation and its abrogation may leads to aneuploidy and cancer. We also found that this modification required for the survival of the cells and in the absence of this modification, normal cells undergo senescence. We were able to show that α-amino tri-methylation of CENP-A is required for the efficient recruitment of CCAN proteins at the centromere. The accurate formation of CCAN and kinetochore is essential for high fidelity chromosome segregation. We have made significant progress in the proposed study but further support is necessary to fully understand the function of α-amino tri-methylation of CENP-A and its role in colorectal cancer.

Publications, Abstracts, and Presentations:

1. FASEB (Federation of American Societies for Experimental Biologist) meeting - Mitosis: Spindle Assembly and Function, Big Sky, Montana USA, June 21-26, 2015. N-terminal a-amino tri-methylation of centromere histone CENP-A required for maintaining bipolar spindle and regulated cell proliferation. KM Sathyan and Daniel R. Foltz (Short talk)

Abstract: FASEB

N-terminal α-amino trimethylation of centromere histone CENP-A required for maintaining bipolar spindle and regulated cell proliferation

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The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for accurate segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14

PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here we demonstrate that the N- terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also found increase in methylation of centromeric CENP-A towards mitosis. The CENP-A methylation is required for cell survival. When CENP-A methylation mutant was replaced in a CENP-A knockout cell lines, they form significantly lower number of colonies compared to wild type replaced cells. Moreover, the CENP-A methylation resistant mutants show multipolar spindles without centriole duplication in p53 inactivated HeLa cells. A significant increase in chromosome segregation defects in HCT116 cells was also evident. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, where as flatter cells in p53+/+ cells may be due to induction of senescence. When injected into mice the methylation mutant cells formed significantly more tumors than the wild type CENP-A cells. We are currently analyzing how the loss of CENP-A α-amino trimethylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability through changes in centrosome number and cell proliferation.

2. Chromatin, ncRNA, Methylation & Disease Symposium – NIH Bethesda, Maryland, April 16-17, 2015. α-N-methylation of CENP-A essential for chromosome segregation and cell survival. KM Sathyan and Daniel R. Foltz (Poster)

Abstract: NIH

N-TERMINAL α-AMINO TRIMETHYLATION OF CENTROMERE HISTONE CENP-A REQUIRED FOR MAINTAINING BIPOLAR SPINDLE AND REGULATED CELL PROLIFERATION

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The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is

unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14 PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here we demonstrate that the N-terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also found that methylation of CENP-A increases through cell cycle with highest being found during mitosis. The CENP-A methylation is required for cell survival. When CENP-A methylation mutant was replaced in a CENP-A knockout cell lines, they form significantly lower number of colonies compared to wild type replaced cells. Moreover, the CENP-A methylation resistant mutants show multipolar spindles without centriole duplication in HeLa cells. We also found similar results in p53-/- HCT116 cells. A significant increase in chromosome segregation defects in p53-/- and p53+/+ HCT116 cells was also evident. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, where as flatter cells in p53+/+ cells may be due to induction of senescence. When injected into mice the methylation mutant cells formed significantly more tumors than the wild type CENP-A cells. We are currently analyzing how the loss of CENP-A α-amino trimethylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability.

3. Mitosis Meeting- NIH, January 22nd 2015. α-N-methylation of CENP-A essential for chromosome segregation and cell survival KM Sathyan and Daniel R. Foltz (talk)

4. Annual departmental retreat. Biochemistry and Molecular Genetics, University of Virginia. December 4, 2014. α-N-methylation of CENP-A essential for chromosome segregation and cell survival. KM Sathyan and Daniel R. Foltz (Poster)

5. Journal club. Biochemistry and Molecular Genetics, University of Virginia. November 11, 2014. α-N-methylation of CENP-A essential for chromosome segregation and cell survival (talk)

Inventions, Patents and Licenses:

Nil

Reportable outcome:

We have got exciting results regarding the function of α-amino tri-methylation, which is under studied. We have found that the α-amino tri-methylation of CENP-A is essential for the survival of the cells and formation of the centromere. In the absence of this PTM, cells undergo senescence probably due to chromosome segregation defects caused by the improper formation of the centromere and the kinetochore. The results suggest that CENP-A α-amino tri-methylation is a crucial post-translational modification in maintaining high fidelity of chromosome segregation and any defect in this modification may results in aneuploidy and cancer. We are writing a manuscript regarding the function of α-amino tri-methylation of CENP-A, which will be submitted in the Nature Cell Biology Journal.

Other Achievements:

Nil

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Appendices:

Abbreviations used

PTM	Post-translational modifications
CIN	Chromosomal instability
MIN	microsatellite instability
NRMT1	N-Terminal RCC1 Methyltransferase 1
NRMT2	N-Terminal RCC1 Methyltransferase 2
shRNA	short hairpin RNA
CENP-A	Centromere protein-A
CCAN	Constitutive Centromere Associated Network
α-N-me3	alpha N-terminal amino acid trimethylation
MT	Mutant

Opportunities for training and professional development: Last year I had several opportunities for my professional development. I got an opportunity to give a short talk in the prestigious FASEB meeting - Mitosis: Spindle Assembly and Function. I also presented a poster in a NIH organized meeting. Moreover Foltz lab had joint meeting with six different labs who are working in the mitosis field. The meeting was held in NIH. I gave a talk about my work in that meeting. I also had several opportunities to present my work in various meetings in the University of Virginia.

I meet my mentor every week and discuss research progress. We have lab meetings every other week. Moreover, we have joint meeting with Stukenberg and Burke labs every week. These meeting and interactions helped me in my professional development.