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CENP-A Ubiquitylation Is Required for CENP-A deposition at the Centromere

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CENP-A is a centromere-specific histone variant that determines centromere identity, but how it localizes to centromeres is not completely understood. Previously, we demonstrated that CENP-A deposition at the centromere requires ubiquitylation on lysine 124 (K124) mediated by the CUL4A-RBX1-COPS8 E3 ligase (Niikura et al., 2015). A K124R mutation reduced interaction with HJURP (a CENP-A-specific histone chaperone) and abrogated centromeric localization, but addition of a mono-ubiquitin at the C-terminus of CENP-A K124R restored interaction with HJURP and centromeric localization (Niikura et al., 2015), indicating that "signaling" ubiquitylation is required for CENP-A deposition at centromeres. We also showed that the CUL4A-RBX1 complex is required for loading newly synthesized CENP-A and maintaining preassembled CENP-A at centromeres by using the SNAP-tagged CENP-A system (Niikura et al., 2015). Recently, we found that pre-existing ubiquitylated CENP-A is necessary for recruitment of newly synthesized CENP-A to the centromere and that CENP-A ubiquitylation is inherited through dimerization during cell division (Niikura et al., 2016). Futhermore, overexpression of mono-ubiquitylated CENP-A creates ectopic functional centromeres (neocentromeres) and causes HJURP accumulation at non-centromeric regions (Niikura et al., 2016). Thus, we proposed that CENP-A ubiquitylation determines centromere location through dimerization (Niikura et al., 2016).

Fachinetti et al. (2017) now report "negative" results, disputing the phenotypes that Yu et al. and we reported independently (Niikura et al., 2015; Yu et al., 2015). They report that the CENP-A S68Q and K124R mutants do not show any defects. However, we believe that the lack of proper controls may explain the discrepancy between their results and ours. We focus the remainder of our response on the K124R mutants.

Fachinetti et al. (2017) criticize our use of an "overexpression system" to characterize K124 ubiquitylation. However, we did detect CENP-A ubiquitylation at the endogenous level (Figure S7, Niikura et al., 2015). Furthermore, we showed that the Cul4A-Rbx1-COPS8 E3 ligase complex ubiquitylates CENP-A in vivo and in vitro (Niikura et al., 2015), and that endogenous CENP-A does not localize to the centromere properly in Cul4A-depleted or Rbx1-depleted cells. Although it is possible that depletion of Cul4A or Rbx1 proteins affects other cellular activities, the addition of mono-ubiquitin to CENP-A-K124R suppressed the defect in centromere localization in Cul4A-depleted or Rbx1-depleted cells, consistent with

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the idea that the loss of CENP-A ubiquitylation caused the centromere localization defects (Niikura et al., 2015).

Furthermore, Fachinetti et al. also use overexpression in their experiments (Figures 1 and 3). In their RPE-1 CENP-A -/F KO system (Figure 1), the 5' LTR promoter (the retroviral promoter) was used to complement the KO cells. This "moderate" overexpression of mutant proteins could still suppresses the mutant phenotype, especially if the mutation causes a defect in protein-protein interactions. As described previously, the binding of CENP-A K124R to HJURP is weaker than that of wild-type CENP-A, but the mutant is still able to bind to HJURP (Niikura et al., 2015). Thus, it is not surprising that an increased amount of K124R can suppress the mutant defects under some conditions (Figure 1H in Fachinetti et al. (2017) indicates that EYFP-K124R was expressed at levels a few times greater than that of wild-type EYFP).

Mutant proteins were also overexpressed from a CMV promoter in the LacO array–LacI-CENP-A (LacO/I) system (Figure 3). Moreover, the levels of mutant proteins were not evaluated by immunoblotting analyses (Fachinetti et al., 2017, Logsdon et al., 2015). As expression levels greatly affect the ectopic incorporation and function of CENP-A (Bodor et al., 2014), a meaningful comparison requires 'normalization' of CENP-A levels not done in these studies.

Another caveat of the RPE-1 CENP-A -/F KO system (Figure 1, Fachinetti et al., 2017), is that the EYFP-fusion protein is much larger than endogenous CENP-A (~45 kDa vs. ~15 kDa). Although this CENP-A fusion does not affect the viability of "cancerous (artificially immortalized)" cells expressing the mutant proteins, it may not faithfully recapitulate the precise endogenous regulation of CENP-A by post-translational modifications. Based on structural predictions, K124 ubiquitin does not directly bind to HJURP. Rather, the addition of mono-ubiquitin likely affects protein conformation. The presence of EYFP, a large fusion protein, may similarly affect protein conformation, perhaps mimicking the structural changes caused by ubiquitylation. Similarly, in the SNAP-tagged CENP-A system (Figure 2), the SNAP-tagged fusion protein was much larger than wild-type CENP-A (~24 kDa vs. ~16kDa), which could again induce a conformational change in CENP-A.

Interestingly, Fachinetti et al. (2017) report a significant difference in viability between cells expressing wild-type CENP-A and those expressing the K124R mutant (Figure 1E). Considering that endogenous CENP-A is stable for as long as seven days after the induction of Ad-Cre (Fachinetti et al., 2013), the 25% reduction in mutant cell viability is significant. In the long-term viability assay (Figure 1F), cells surviving after 100 generations could represent reversions of the original mutation or suppression by secondary mutations. Intriguingly, the number of micronuclei increased by approximately 50% in the K124R cells (Figure 1J), suggesting the cells were deficient in chromosome segregation at some point.

Regarding suppression, we have shown that addition of a mono-ubiquitin to the C-terminal end of CENP-A K124R can suppress the mutant phenotype (Niikura et al., 2015). Thus we know that the ubiquitin group can function at different positions. When CENP-A K124R is maintained in a cell for a certain period of time, one of the other six lysines in CENP-A

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could be ubiquitylated, and this ubiquitylation can suppress the defects of the mutant, which may explain “surviving” cells. We have shown that the Cul4A-Rbx1-COPS8 E3 enzyme ubiquitylates CENP-A K124 (Niikura et al., 2015). In their SNAP-tagged CENP-A system (Figure 2), Fachinetti et al. (2017) used the Cul1-Rbx1-TIR1 E3 enzyme (Holland et al., 2012) to ubiquitylate EGFP-AID-CENP-A. Thus, especially in this system, it is plausible that ubiquitylation activity is increased and CENP-A K124R could be ubiquitylated at another site.

In immunofluorescence experiments (Figure 1C), Fachinetti et al. (2017) counted “positive” centromeres instead of quantifying signals of CENP-A at the centromere; this counting could fail to detect mislocalization of CENP-A. Also, Fachinetti et al. claim that their system should have no fixation artifacts because proteins were labeled fluorescently in living cells. However, the cells were later fixed for CENP-A and tubulin staining. Thus, it remains possible that the authors failed to find an optimal fixation condition to see the phenotypes that Yu et al. and our group observed and reported (Niikura et al., 2015; Yu et al., 2015).

In addition, positive controls were missing in some assays in Fachinetti et al. (2017). For example, in Figure 2C, the CENP-A alpha2.2 mutant behaved as a null and served as a negative control. However, a positive control, e.g. a point mutant that is stable but deficient in centromere function, is lacking.

Such a positive control was also lacking in experiments testing whether mutant CENP-A proteins are efficiently assembled at centromeres during the G1 phase of the cell cycle. Here Fachinetti et al. (2017) measured TMR-Star versus CENP-A signals at the centromere in a SNAP assay (Figure 2H), reporting no difference in centromere propagation between wild-type and mutants. But why were TMR-Star signals normalized to CENP-A signals at the centromere? The TMR-Star signals are detected at a time point when wild-type CENP-A - EGFP-AID-CENP-A, - is degraded (Figure 2C). Thus, most if not all of CENP-A in the cells was SNAP-tagged mutant CENP-A, and the ratio of TMR-Star to CENP-A signalis thus 1. Indeed, their results are 1 in all three cases (Figure 2H). A positive control, e.g., a point mutant that is stable but deficient in centromere function, would likely also yield a normalized value of 1 in this assay. Therefore, these results do not resolve whether K124 ubiquitylation is required for efficient centromere propagation.

Finally, the authors used the LacO/I system to test the function of K124R during early events in centromere establishment (Figure 3), concluding that the K124R mutant interacts normally with HJURP. However, in our hands, with the same system, the K124R mutant shows defects in recruiting HJURP (Niikura et al., 2016). Furthermore, the mono-ubiquitin fusion protein CENP-A K124R-Ub(K48R) recruits more HJURP in this system (Niikura et al., 2016). We found that CENP-A K124R interacts with HJURP inefficiently in vivo (Niikura et al., 2015) and that mono-ubiquitylated CENP-A interacts with HJURP more efficiently in vitro. Results obtained by different methods are consistent with those from the LacO/I system (Niikura et al., 2016).

In conclusion, given the caveats discussed above, we believe that the results of Fachinetti et al. (2017) are insufficient to refute our own recent results (Niikura et al., 2016; Niikura et al., 2015).

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