Gene replacement strategies validate the use of functional tags on centromeric chromatin and invalidate an essential role for CENP-A[K124ub]

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SUMMARY

Functional tags are ubiquitous in cell biology, and for studies of one chromosomal locus, the centromere, tags have been remarkably useful. The centromere directs chromosome inheritance at cell division. The location of the centromere is defined by a histone H3 variant, CENP-A. The regulation of the chromatin assembly pathway essential for centromere inheritance and function includes posttranslational modification (PTM) of key components, including CENP-A itself. Others have recently called into question the use of functional tags, with the claim that at least two widely used tags obscured the essentiality of one particular PTM, CENP-A[K124] ubiquitination (ub). Here, we employ three independent gene replacement strategies that eliminate large, lysine-containing tags to interrogate these claims. Using these approaches, we find no evidence to support an essential function of CENP-A[K124ub]. Our general methodology will be useful to validate discoveries permitted by powerful functional tagging schemes at the centromere and other cellular locations.

In brief

Using three gene replacement strategies, Salinas-Luypaert et al. demonstrate that CENP-A^{K124ub} is not essential for CENP-A function at centromeres. Thus, functional tags do not mask the role of K124 when it is mutated. These strategies can be employed to interrogate posttranslational modifications at the centromere and other cellular locations.

Graphical Abstract
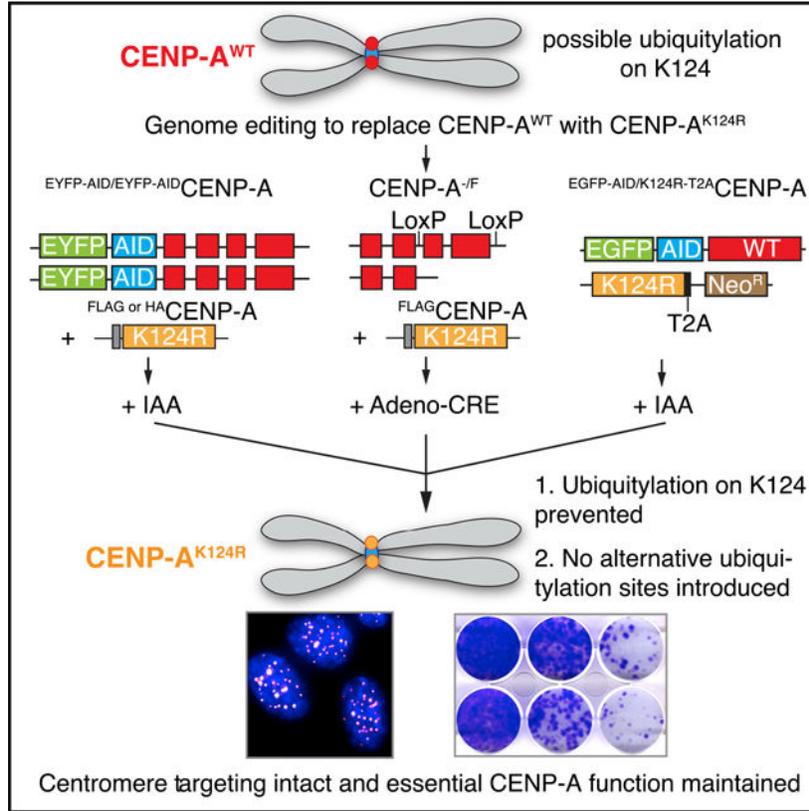
INTRODUCTION

The centromere is the chromosomal locus that recruits the kinetochore at mitosis to connect to spindle microtubules, driving accurate genome segregation (Kixmoeller et al., 2020). The histone H3 variant centromere protein A (CENP-A) (Earnshaw and Rothfield, 1985; Palmer and Margolis, 1985) assembles into nucleosomes that specify the location of the centromere epigenetically (i.e., independent of a particular DNA sequence) (Black and Cleveland, 2011), even on chromosomes that lack the typically repetitive DNA found at the centromeres of many eukaryotes, including mammals (Depinet et al., 1997; Locke et al., 2011; Logsdon et al., 2019; Schueler et al., 2001; Wade et al., 2009). One area of current major investigation is the relationship between the epigenetic (CENP-A nucleosomes) and genetic (centromere DNA repeats) components of the centromere, with molecular evidence recently emerging for centromere strengthening centromeric chromatin via direct (i.e., a special fit with CENP-A nucleosomes; Allu et al., 2019; Iwata-Otsubo et al., 2017) and indirect (i.e., through the

centromere repeat-binding protein CENP-B; Dumont and Fachinetti, 2020; Fachinetti et al., 2015; Hoffmann et al., 2016, 2020; Okada et al., 2007; Otake et al., 2020) interactions. These findings are relevant to a long-standing hypothesis for centromere drive, wherein the epigenetic features rapidly evolve to suppress ever-strengthening genetic features (Henikoff et al., 2001). A second area of current major investigation is on the biochemical steps, and their regulation via posttranslational modifications (PTM), that propagate centromere chromatin (Mitra et al., 2020a), centered on deposition of CENP-A at centromeres by its specific histone chaperone, HJURP (Dunleavy et al., 2009; Foltz et al., 2009).

While not unique to the centromere field, the importance of genetically encoded functional tags to the most seminal findings over the last ~15–25 years cannot be overstated. Notably useful tags on CENP-A-related studies have included fluorescent proteins (i.e., EGFP/EYFP) (Black et al., 2004; Fachinetti et al., 2013), those appropriate for pulse-labeling approaches (i.e., the self-labeling SNAP-tag) (Bodor et al., 2013; Falk et al., 2015; Jansen et al., 2007), those that permit tandem affinity purifications out of cell lysates (Dunleavy et al., 2009; Foltz et al., 2006, Foltz et al., 2009; Nechemia-Arbely et al., 2017, 2019; Okada et al., 2006), those that confer rapid-inducible degradation (i.e., auxin-inducible degron [AID]) (Fachinetti et al., 2015; Hoffmann et al., 2016, 2020; Holland et al., 2012), and those that permit recruitment of centromeric chromatin components to an ectopic locus, such as the LacO-LacI tethering system (Barnhart et al., 2011; Bassett et al., 2012; Chen et al., 2014; Hori et al., 2013; Logsdon et al., 2015, 2019; Mendiburo et al., 2011). The use of fluorescent tags permitted the identification of the CENP-A targeting domain (CATD) within its histone fold domain, as well as the finding that the CATD confers recognition of nascent CENP-A by HJURP (Bassett et al., 2012; Black et al., 2004; Foltz et al., 2009). Also, the use of pulse labeling with the SNAP-tag defined how nascent CENP-A nucleosome assembly is restricted to mitotic exit (Bodor et al., 2013; Jansen et al., 2007). This finding has had a wide-ranging impact on the field, arguably with the most direct influence in thinking of the biochemical regulation of the process involving PTM of centromeric chromatin (Mitra et al., 2020a). Phosphorylation (French and Straight, 2019; McKinley and Cheeseman, 2014; Silva et al., 2012; Stankovic et al., 2017), ubiquitylation (Bade et al., 2014; Niikura et al., 2015), sumoylation (Liebelt et al., 2019; Mitra et al., 2020b), methylation (Hori et al., 2014), and acetylation (Shang et al., 2016) have all been proposed to occur as part of the processes that promote centromeric chromatin assembly or regulate its homeostasis in another manner (Srivastava et al., 2018).

Recent findings from Kitagawa and colleagues have called into question experiments with centromeric chromatin that utilize tags, such as EYFP, SNAP, and the Lac repressor (LacI) (Niikura et al., 2019). They initially reported that a residue outside the CATD, CENP-A^{K124}, is ubiquitylated (K124ub) and that this is essential for CENP-A deposition at the centromere (Niikura et al., 2015). Specifically, CENP-A^{K124ub} was claimed to be crucial for the interaction with HJURP and necessary for the epigenetic propagation mechanism that ensures the faithful inheritance of centromere location on the chromosome (Niikura et al., 2015, 2016). These findings were based on transient overexpression studies using a CENP-A^{K124R} mutant that cannot be ubiquitylated at that particular residue and a version of CENP-A^{K124R} with ubiquitin fused to its C terminus. Using multiple gene replacement strategies and nascent CENP-A assembly assays that included functional tags (EYFP, SNAP,

and LacI-hemagglutinin [LacI-HA]), we failed to find any support for their conclusions; CENP-A^{K124R} mutant interacts with HJURP, accumulates at centromeres, and supports kinetochore function and cell viability (Fachinetti et al., 2017). In a more recent study (Niikura et al., 2019), they counter by proposing that "EYFP tagging induces ubiquitylation at a lysine other than K124, and this ubiquitylation allows EYFP-CENP-A^{K124R} to bind to HJURP" and that "because the SNAP tag (20 kDa) is also a larger tag than CENP-A… and has 10 lysins [sic], SNAP-CENP-A K124R, presumably, is ubiquitylated at a site different than K124" (Niikura et al., 2019). By extension, their conclusion suggests that the LacI-HA-tag may induce yet another ubiquitylation that could functionally substitute for K124ub. Nevertheless, the authors did not provide any experimental evidence or a clear explanation as to how the size or presence of lysines on the tag would cause this substitute ubiquitylation to occur. These new statements also modify their initial claims of the importance of K124 itself (Niikura et al., 2015), as they now propose that CENP-A ubiquitylation does not need to be site specific to bind to HJURP. This model is structurally inconsistent with the well-established mechanism of HJURP binding to CENP-A (Bassett et al., 2012; Hu et al., 2011), and it is unclear how a site-nonspecific ubiquitin moiety would impact CENP-A-HJURP complex formation. One possibility is that EYFP, SNAP, or LacI tagging all mask the importance of CENP-A^{K124}.

We now report findings from rapid protein replacement strategies, including genome editing at the endogenous CENP-A locus and more traditional Cre-mediated gene excision, to test versions of CENP-A^{K124R} that lack any additional lysine residues that could themselves be ubiquitylated, or lead to CENP-A ubiquitylation at sites other than K124.

RESULTS AND DISCUSSION

Stable HA- or FLAG-CENP-A^{K124R} expression rescues endogenous CENP-A loss in RPE-1 cells

We first tested if stably expressed FLAG- or HA-tagged CENP-A^{K124R} (the same short epitope tags used in Niikura et al., 2019) is able to rescue cells in which the endogenous CENP-A was rapidly degraded by an AID tag in genetically stable, diploid hTERT RPE-1 cells (Hoffmann et al., 2016). The FLAG-tag (DY<u>K</u>DDDD<u>K</u>) contains two lysine residues that, in principle, could themselves be ubiquitylated, while the HA-tag (YPYDVPDYA) does not. Importantly, FLAG-tagged CENP-A^{K124R} was reported not to be ubiquitylated at any site, even when co-overexpressed with ubiquitin (Niikura et al., 2015, 2019). $^{EYFP-AID}$CENP-A RPE-1 cells (hereafter named ^{EA}CENP-A) were transduced with retroviral vectors expressing the tagged CENP-A rescue constructs (wild type [WT] or K124R, with FLAG- or HA-tags), and single colonies surviving antibiotic selection were isolated, individually characterized, and pooled (Figure 1A). Upon addition of the auxin hormone indole-3-acetic acid (IAA) ^{EA}CENP-A expressed from the endogenous CENP-A gene locus is fully and rapidly degraded (Figures 1B and 1C), as previously shown (Hoffmann et al., 2016, 2020), leaving the cells to express only the stably integrated rescue proteins (Figures 1B and S1A). We note that in our cell lines, the rescue proteins are uniformly expressed at higher levels than endogenous CENP-A (Figure S1A). HA- or FLAG-tagged CENP-A rescue proteins, both WT and K124R, target to centromeres and

colocalize with CENP-C even when endogenous CENP-A is fully degraded (Figures 1B and S1B).

Long-term cell viability (14 days) was assessed by means of colony-formation assays (Figure 2A). ^{EA}CENP-A cells with one of the four different rescue constructs (WT or K124R mutant, each tagged with either HA or FLAG), were cultured in presence or absence of IAA. After 2 weeks, the cells were fixed and stained with crystal violet (Figure 2B). The percentage of colonies formed in the presence of IAA, relative to the number of colonies formed in its absence for the same rescue, was calculated (Figure 2C). The CENP-A^{K124R} mutant rescue constructs confer long-term cell viability after removal of ^{EA}CENP-A to a similar extent as the CENP-A^{WT} rescue constructs, regardless of the tag used (Figure 2C). Thus, in this context, the FLAG- and HA-tagged constructs provide no evidence to support the claim of impaired function of a CENP-A mutant that cannot be ubiquitylated.

To understand how we arrive at such different results than those reported by Kitagawa and colleagues, we designed an experiment modeled precisely after the strategy they employed (Niikura et al., 2019) using retroviral integration of blasticidin- or puromycin-resistant ^{FLAG}CENP-A WT or K124R rescues in RPE-1 cells that carry one knocked-out (−) and one floxed (F) CENP-A allele (Figure S2). The transduction of CENP-A$^{−/F}$ cells with an adenoviral vector expressing the Cre recombinase (Ad-Cre) leads to CENP-A gene depletion and gradual removal of CENP-A protein (Fachinetti et al., 2013). Populations of blasticidin-resistant cells (either CENP-A^{WT} or CENP-A^{K124R}) with no characterization, including no assessment of CENP-A expression levels (as in Niikura et al., 2019), were tested by colony formation assay after Ad-Cre treatment to score for cell viability, generating similar results as the ones reported by Niikura et al. (2019) (Figure S2B), where CENP-A^{WT} appears to rescue much better than CENP-A^{K124R}. However, upon characterization of single clones from these same blasticidin-resistant populations, we found that the majority do not express the rescue constructs (Figures S2C and S2D), providing a direct explanation to the increased lethality observed in the CENP-A^{K124R} population. We note that, in contrast, most of our puromycin-resistant clones do express the rescue constructs (data not shown). Consistent with our initial conclusions in characterized clones (Figures 1 and 2), in individual blasticidin- or puromycin-resistant clones that do express the ^{FLAG}CENP-A^{WT} or ^{FLAG}CENP-A^{K124R} rescues, we observed that the CENP-A^{K124R} mutant confers cell viability following Ad-Cre treatment to a similar extent as the CENP-A^{WT} rescue construct (Figures S2E and S2F). In summary, the results obtained with the CENP-A gene depletion method match the ones generated using the CENP-A rapid protein degradation (Figure 2).

CENP-A^{K124R} expression from the endogenous locus supports centromere function and DLD-1 cell viability

To test the CENP-A^{K124R} mutant in a system where it is expressed from the endogenous gene locus, thus avoiding expression at levels exceeding the endogenous ones that could, in principle, somehow bypass an essential function of K124 ubiquitylation, we performed CRISPR-Cas9-based biallelic replacement of the CENP-A gene on DLD-1 cells (Figure 3A). DLD-1 cells are pseudodiploid, colorectal cancer cells with a stable karyo-type and have been previously used in studies to generate CRISPR-Cas9 tagging or replacement to

define many centromere-kinetochore functions (Dumont et al., 2020; McKinley et al., 2015). The system employed is similar to the one we used previously (Fachinetti et al., 2017). One allele is EGFP-AID tagged for rapid protein degradation, while the other is mutated (K124R) or not (WT). In our current effort, we follow the coding sequence of the second allele with a T2A viral cleavage site and an antibiotic selection marker. The T2A site was chosen because it does not leave any lysine residues in the remaining C-terminal sequence that extends only 26 amino acids beyond the natural CENP-A stop site (Figure 3B). This additional sequence cannot be ubiquitylated and, due to its small size (2.65 kDa), is not expected to alter the ubiquitylation status of the endogenous CENP-A. We obtained several monoclonal lines where biallelic replacement is successful. We performed PCR genotyping and Sanger sequencing of the entire CENP-A^{K124R} open reading frame (ORF), confirming the desired edit with no changes to the designed rescue gene product (Figures 3C, 3D, and S3A). The addition of IAA potently degrades ^{EA}CENP-A but leaves the rescue proteins intact (Figures 3E and S3B).

CENP-A^{K124R} remains properly localized to the centromere after a short 24-h IAA treatment (Figures 4A, S3C, and S3D). Further, it clearly confers cell viability, similar to CENP-A^{WT} (Figures 4B and S4A), an opposite outcome relative to the mutant α2.2 that fails to interact with HJURP (Bassett et al., 2012), does not target to centromeres, is unstable at the protein level, and thus effectively serves as a null mutation. After removal of ^{EA}CENP-A with prolonged IAA treatment, CENP-A^{K124R} localized to centromeres in similar amounts as CENP-A^{WT} (10 days) (Figures 4C, 4D, and S4B) and confers long-term viability (14 days) (Figures 4E, 4F, and S4C), to a similar extent as the WT version of the rescue protein.

CENP-A^{K124ub} is dispensable, with or without large tags

The mechanistic basis of centromere identification and maintenance conferred by CENP-A have mostly emerged from studies using tagged proteins. Pulse labeling (SNAP), fluorescent labeling (EGFP/EYFP), LacI-LacO exogenous genomic site targeting, and AID tagging have all offered critical insights to postulate the standing model of epigenetic centromere specification. In our current effort, we found no support for the most recent explanation provided by Kitagawa and colleagues for why we have not been able to find any survival defects on cells with CENP-A that cannot be ubiquitylated at lysine 124 (K124R mutants). Their explanation is that, in the presence of commonly used tags such as the ones we used in our prior studies (Fachinetti et al., 2017), substitute ubiquitylation site(s) can be generated on CENP-A residue(s) other than K124 (Niikura et al., 2019). Our findings in two different cell backgrounds, with three independent methods that avoid any added lysines and eliminate the possibility of alternative ubiquitylation due to tag size, as demonstrated by the authors themselves (Niikura et al., 2015, 2019), refute these claims. Kitagawa and colleagues reached their conclusions with experiments that include the use of uncharacterized polyclonal cells and transient overexpression of relevant components, as in the *in vivo* ubiquitylation assays and mass-spectrometry-based analysis (Niikura et al., 2019), thus yielding unreliable results. Further, the report of a single gene replacement experiment lacks data to sufficiently characterize the edited locus (Niikura et al., 2019). A simple explanation for their loss of cell viability is that unintended mutations might

have been introduced when the authors generated their CENP-A^{K124R} allele and that these mutations led to a reduction/loss of expression and/or function of their rescue protein. Perhaps related to this notion, we found that CENP-A^{K124R} clones can vary somewhat in expression, with the lowest-expressing clone (clone 1, which expresses the rescue protein at less than half of the WT clone; Figure 3E) exhibiting reduced colony formation (Figures 4F and S4C). These results demonstrate that the levels of CENP-A are critical in colony-formation assays, as markedly reduced CENP-A levels within a clone correlate with a decrease in the abundance of centromere/kinetochore components and an increase in the rate of chromosome mis-segregation (Fachinetti et al., 2013). In addition, we have shown that following retroviral integration and selection, many blasticidin-resistant clones (11 out of 16) do not express the desired constructs, and therefore, they act as null allele (Figure S2C). While the basis for the clone-to-clone variability remains unclear, this particular finding illustrates the experimental importance of careful characterization of clonal populations (crucial to determine the phenotypes from mutational studies) and the folly in drawing conclusions from uncharacterized mixtures of cells. We emphasize that we do not dispute that ubiquitylation of CENP-A is possible, but our observations are in strong opposition to the major claims from Kitagawa and colleagues (Niikura et al., 2015, 2016, 2019) that such modifications of K124 (or a substituting lysine in its absence) are essential for CENP-A nucleosome assembly at centromeres and overall centromere function, large tag or no.

Strategies for assessing the functional relevance of particular PTMs

Great interest has been laid on CENP-A PTMs since they could, in principle, impact upon CENP-A nucleosome targeting and affect centromere identity and function. CENP-A PTM sites reported to date lie outside the CATD, and while some of these residues have been shown to be important for centromere identity and function, the specific roles of some of these PTMs remain elusive and, in some cases, controversial (Bailey et al., 2013; Barra et al., 2019; Fachinetti et al., 2017; Hori et al., 2020; Niikura et al., 2015; Sathyan et al., 2017; Yu et al., 2015; Zeitlin et al., 2001). Strategies to test the importance of a specific PTM typically rely on ectopic expression or gene replacement studies that involve the usage of large tags to assess the localization, function, and dynamics of the modified protein of interest (as in the case of CENP-A). It is always possible that protein tagging may alter important characteristics or functions of the protein under investigation. The methods used here with gene replacement strategies using small tags that lack lysines and confer rapid removal of the endogenous WT protein represent a compelling alternative to this problem and can now clearly be applied at the centromere. This type of approach also supports the use of functional tags in the study of CENP-A, its partners, and centromeric chromatin. More broadly, our approach can be readily adaptable as a general methodology in biology to interrogate candidate sites of PTM in diverse cellular contexts.

Limitations of the study

The study is limited to two human cultured cell backgrounds, DLD-1 (near diploid; colon cancer) and RPE-1 (diploid; non-transformed retinal pigment epithelial). A role for CENP-A^{K124ub} in other cell types or in the context of an animal cannot be excluded.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ben E. Black (blackbe@pennmedicine.upenn.edu).

Materials availability—Plasmids and cell lines generated in this study will be provided upon request to the Lead Contact.

Data and code availability

- Data reported in this paper will be shared by the Lead Contact upon request.

- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture—Cells were cultured at 37°C in a humidified incubator at 5% CO_2. The DLD-1 TIR1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. hTERT RPE-1 WT, CENP-A$^{F/+}$ and CENP-A$^{-/F}$ cells were maintained in DMEM:F12 (GIBCO) medium containing 10% heat inactivated FBS (biosera), 0.123% sodium bicarbonate and 2 mM L-glutamine.

METHOD DETAILS

Constructs—For DLD-1 TIR1 stable cell lines, repair templates with ^{WT}CENP-A-T2A-Neo^R and ^{K124R}CENP-A-T2A-Neo^R plasmids were generated. To this end, gBlock gene fragment (IDT) T2A-Neo^R was PCR amplified, restriction digested with XhoI and NheI sites and cloned into vectors with ^{WT}CENP-A-SNAP-3xHA-P2A-Neo^R and ^{K124R}CENP-A-SNAP-3xHA-P2A-Neo^R (Fachinetti et al., 2017) under the same sites replacing SNAP-3xHA-P2A-Neo^R with T2A-Neo^R. Retroviral plasmids used in the generation of RPE-1 stable cell lines were generated by cloning the full length of the human CENP-A open reading frame (WT or K124R mutant), tagged with HA or FLAG at the amino terminal, into pBabe-Puro or pBabe-Blast based vectors.

Cell line generation—DLD-1 TIR1 (Holland et al., 2012) stable cell lines were generated by replacing both CENP-A alleles with EGFP-AID-CENP-A (Fachinetti et al., 2017) and CENP-A (WT or K124R)-T2A-Neo^R. To obtain biallelic replacement of CENP-A gene locus with these constructs DLD-1 TIR1 cells were cotransfected with 400 ng of each repair template and 100 ng of each sgRNA-Cas9 (Fachinetti et al., 2017) using lipofectamine 2000 (Invitrogen). After 5 days of transfection antibiotic resistance selection was performed with 750 μg/mL G418-S and after 2–3 weeks of selection GFP-positive monoclonal lines were FACS sorted into 96 well plates. $^{EGFP-AID}$CENP-A expression was assessed by immunofluorescence microscopy and immunoblot. From DLD-1 TIR1 CENP-A

WT or K124R monoclonal lines, genomic DNA was isolated and verified by PCR and Sanger sequencing. hTERT RPE-1 [EYFP-AID]CENP-A OsTIR1 (Hoffmann et al., 2016) and hTERT RPE-1 CENP-A[−/F] (Fachinetti et al., 2013) stable cell lines were generated by retroviral delivery of the HA or FLAG-tagged CENP-A (WT or K124R) transgenes. Briefly, retroviral plasmids (pBabe-Puro or pBabe-Blast based) were cotransfected with the VSV-G pseudotyping plasmid into 293-GP cells. The resulting retroviral supernatant was collected after 48 hours, mixed with 8 μg/ml polybrene and added to the RPE-1 cells in culture. Stable integration was selected with 5 μg/mL puromycin for 7 days or 10 μg/mL blasticidin for 10 days. Single clones were isolated and characterized. For the hTERT RPE-1 [EYFP-AID]CENP-A OsTIR1 experiments, three clones with similar expression levels for each construct were pooled.

Immunoblot analysis—Whole cell lysates were collected from the different cell lines, treated or not with 500 μM IAA (I5148, Sigma) dissolved in ddH$_2$O. For DLD-1 cells, proteins were separated by 15% SDS-PAGE and transferred onto PVDF membranes (GE healthcare). Immunoblot analysis was performed by blocking the membrane with 5% skim milk and probing with anti-ACA (Antibodies Incorporated, 2 μg/mL) and anti-α-tubulin (Clone DM1A, 1:5000) antibodies. Three independent gels were probed to quantify CENP-A-T2A levels using Fiji software (Schindelin et al., 2012). Proteins from RPE-1 whole cell lysates were separated by SDS-PAGE using 12% TGX gels (BioRad) or hand casted 15% gels, and transferred onto nitrocellulose membranes using the Trans-Blot Turbo transfer system (BioRad). Immunoblot analysis was performed by blocking the membrane with 5% skim milk and probing overnight at 4°C with anti-CENP-A (1:1000, Cell Signaling #2186), anti-Vinculin (1:2000, Sigma #V9264) and anti-GAPDH (1:5000, Cell Signaling #2118) antibodies.

Cell viability assay—Cell viability assay was performed in triplicate on DLD-1 TIR1 WT or K124R cell lines by seeding at 1.5×10^5 cells/well with 500 μM IAA. Cells were collected every other day at indicated time points and then Trypan Blue staining (Corning) was performed. The percentage of living cells out of total cells was calculated on a hemocytometer based on Trypan Blue uptake. After 8-day IAA treatment, surviving colonies were grown on poly-D-lysine-coated coverslips in a 6-well plate with 500 μM IAA for 2 days and subjected to immunofluorescence microscopy.

Colony formation assay—For the colony formation assays, 2500 cells were plated on 6-well plates and two 1:5 serial dilutions were performed. After 12 or 14 days, colonies were fixed for 30 minutes in methanol, washed with PBS and then stained for 15 minutes using a 1% crystal violet in 20% ethanol stain before being thoroughly washed with PBS. The plates were air-dried and scanned, and then colonies were manually counted.

Indirect immunofluorescence—For experiments involving DLD-1 TIR1 stable cell lines, cells were fixed with 4% formaldehyde in PBS for 5 minutes and quenched with 100 mM Tris pH 7.5 for 5 minutes. Cell were washed three times with 0.1% Tween in PBS each for 5 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Permeabilized cells were washed with 0.1% Tween in PBS for 5 minutes prior

to blocking in blocking buffer (2% FBS, 2% BSA, and 0.1% Tween in PBS). Primary antibody incubation with anti-CENP-A (1 μg/mL of mouse monoclonal antibody ADI-KAM-CC006-E, Enzo) and anti-CENP-B (200 μg/ml rabbit polyclonal sc-22788, Santa Cruz Biotechnology) was performed in the blocking buffer for 1 hour at room temperature. Cells were washed three times with 0.1% Tween in PBS for 5 minutes, followed by Cy3-conjugated donkey anti-mouse and Cy5-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) incubation at 1:200 dilution. Cells were washed with 0.1% Tween in PBS for 5 minutes followed by DAPI staining and mounting with Vectashield mounting medium (Vector Laboratories). For experiments involving hTERT RPE-1 stable cell lines, cells were fixed with 4% formaldehyde in PBS for 10 minutes, then washed and permeabilized with 0.1% Triton X-100 in PBS, and blocked for 30 minutes at room temperature in blocking buffer (0.2 M glycine, 2.5% FBS and 0.1% Triton X-100). Primary antibody incubation was performed for 2 hours at room temperature in blocking buffer, with rabbit anti-GFP (PABG1, ChromoTek) 1:200, guinea pig anti-CENP-C (PD030, MBL) 1:500, and mouse anti-HA (A-M-M#07, Recombinant Antibody Platform (TAb-IP), Institut Curie) 1:200 or mouse anti-FLAG (F3165, Sigma) 1:1000 antibodies. Cells were washed three times with 0.1% Triton X-100 in PBS before incubation for 1 hour at room temperature in blocking buffer with donkey anti-mouse Alexa Fluor® 488, donkey anti-rabbit Cy3 and donkey anti-guinea pig Alexa Fluor® 647 secondary antibodies (Jackson ImmunoResearch Laboratories, 1:500). Cells were washed three times with 0.1% Triton X-100 in PBS before DAPI staining (1 μg/mL in PBS) for 10 minutes and mounting with ProLong Gold Antifade Mountant (#P36934, Invitrogen).

Image acquisition and quantification—For experiments involving DLD-1 TIR1 cells, images were captured as 0.2 μm z sections at room temperature on an inverted fluorescence microscope (Leica DMI6000B) at 100× 1.4 NA oil immersion objective lens, equipped with a charge-coupled device camera (Hamamatsu Photonics ORCA AG). Images were deconvolved using LAS-X software (Leica) and max-projected for fluorescence intensity measurements. The mean fluorescence intensity at centromeres was measured using an ImageJ macro, CRaQ v1.12, under default parameters. Plots were generated from three independent immunofluorescence experiments. For experiments involving hTERT RPE-1 cell lines imaging was performed on a DeltaVision Core system (Applied Precision) consisting of an Olympus IX71 inverted microscope equipped with a CoolSNAP$_{HQ}^2$ camera (Photometrics). Images were captured as 0.2 μm z sections at room temperature with a 100× 1.4 NA oil immersion objective lens (Olympus), deconvolved and 3D maximum intensity projected using DeltaVision's softworx software. The integrated fluorescence intensity at centromeres was measured using Fiji (Giunta et al., 2021).

Ad-Cre transduction—4×10^4 hTERT RPE-1 CENP-A$^{F/+}$, CENP-A$^{-/F}$ +/− rescue constructs cells were seeded in 12 well plates 24 hours prior of transduction. The cells were washed three times in DMEM:F12 medium containing 2% FBS and transduction with variable amount of CsCl purified Ad-Cre (1x, 2x and 4x) was carried out in 400 μL final volume of 2% FBS containing media for 4 hours. The cells were then washed extensively in medium containing 10% FBS, and left to recover for 48 hours before seeding colony formation assays.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details, which include the value of n (where n represents the number of centromeres measured) can be found in the figure legends. All statistical tests were performed using GraphPad Prism 9.0 for Mac (GraphPad Software, San Diego, California USA, https://www.graphpad.com:443/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Highlights

- Preventing CENP-A ubiquitylation on K124 does not interfere with cell viability

- Lysine-free tagged CENP-A^{K124R} supports long-term centromere function

- Common functional tags do not mask an essential function for K124 modification

- General strategies presented for interrogating the function of specific PTM sites
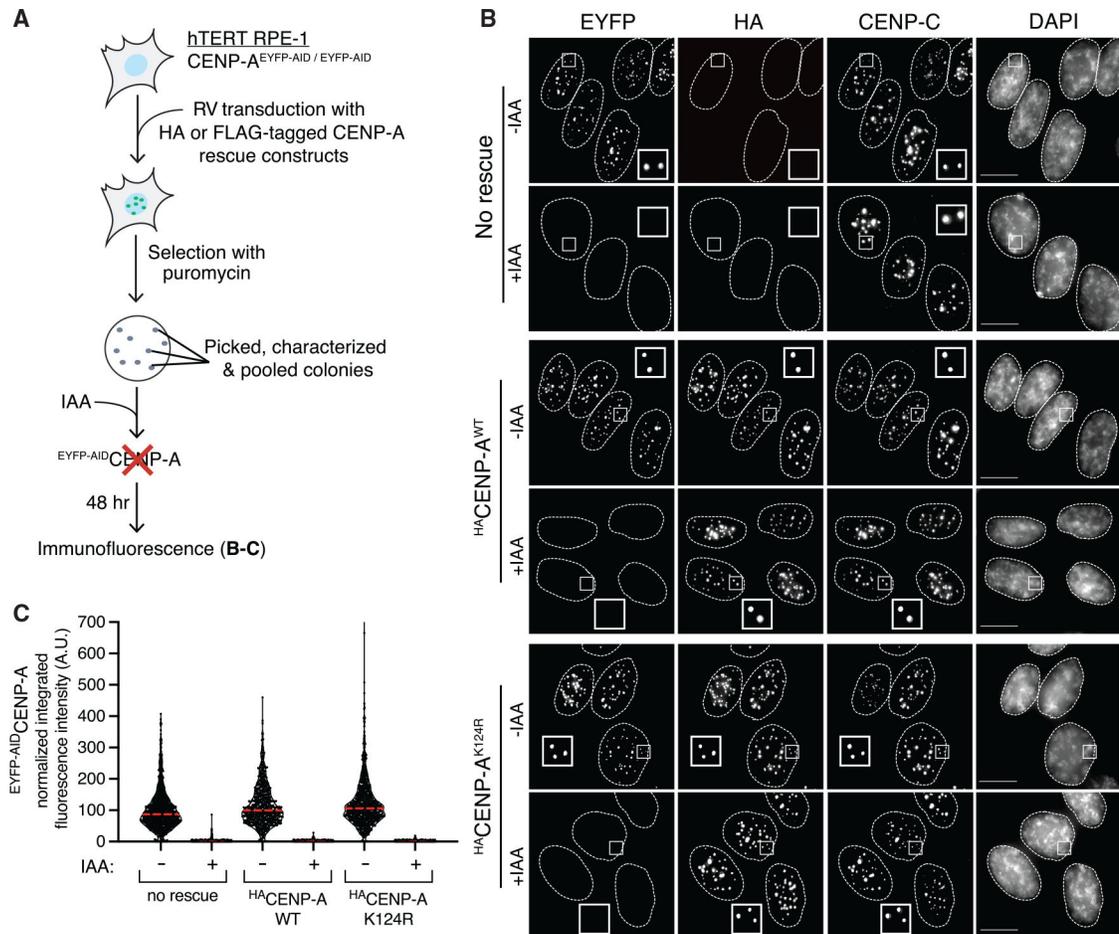
Figure 1. Lysine-free tagged CENP-A^K124R localizes efficiently at centromeres

(A) Schematic of the experimental strategy for generating CENP-A rescues in hTERT RPE-1 cells with ^{EA}CENP-A. EA, EYFP-AID; RV, retrovirus; IAA, indole-3-acetic acid.

(B) Representative immunofluorescence images showing the localization of EYFP-AID-tagged CENP-A and HA-tagged WT or K124R CENP-A rescues at centromeres. Cells were treated with 500 μM IAA for 48 h to degrade ^{EA}CENP-A. α-CENP-C was used as a marker to determine centromere position. Nuclei are contoured with white dashed lines. Scale bar, 10 μm.

(C) Quantification of ^{EA}CENP-A integrated fluorescence intensity at centromeres of n > 780 centromeres per condition in presence or absence of 500 μM IAA for 48 h. The red lines represent the median. The data are normalized to "no rescue -IAA."
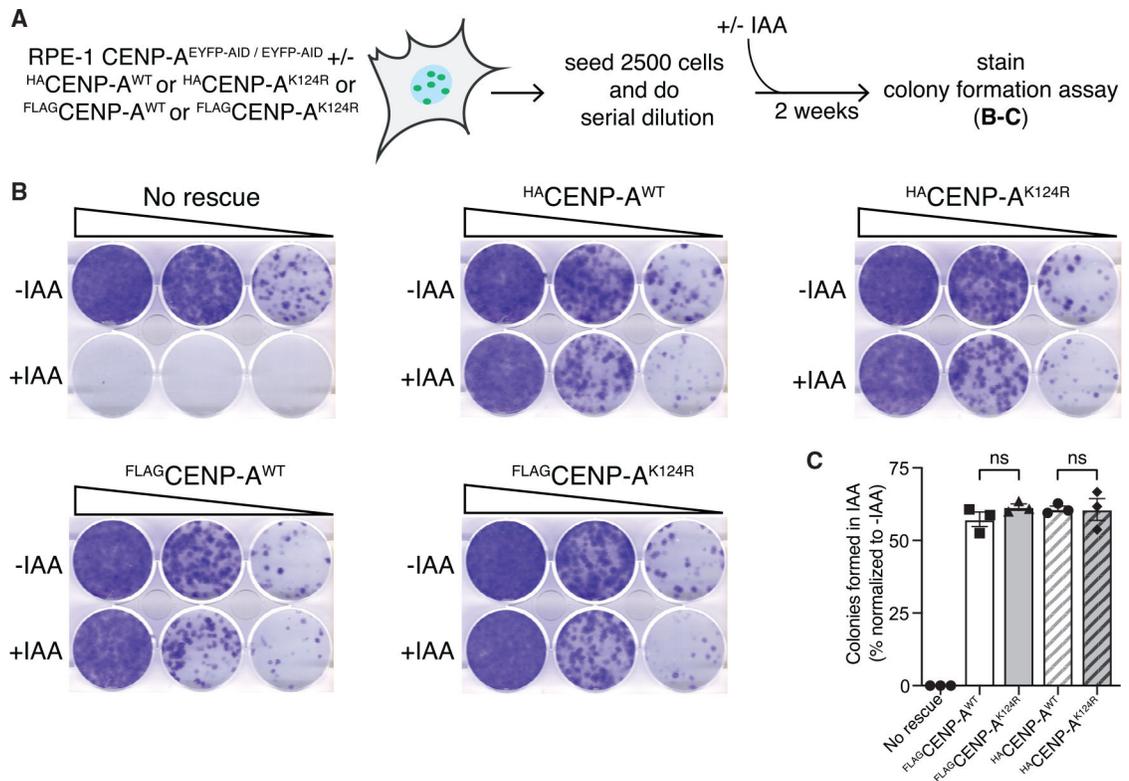
Figure 2. Preventing CENP-A ubiquitylation on K124 does not interfere with long-term centromere function and viability in RPE-1 cells

(A) Schematic of the experimental strategy for colony-formation assays.

(B) Representative images of crystal-violet-stained colonies in the indicated cell lines from the colony-formation assays described in (A). Serial dilution of cells grown for 2 weeks without (top wells) or with IAA (bottom wells).

(C) Quantification of the colony-formation assays. Bars represent the percentage of colonies formed after 2 weeks of culture with 500 μM IAA normalized to the untreated. Each dot represents one experiment, and error bars represent SEM. ns: non-significant differences, unpaired t test.

See also Figure S2.
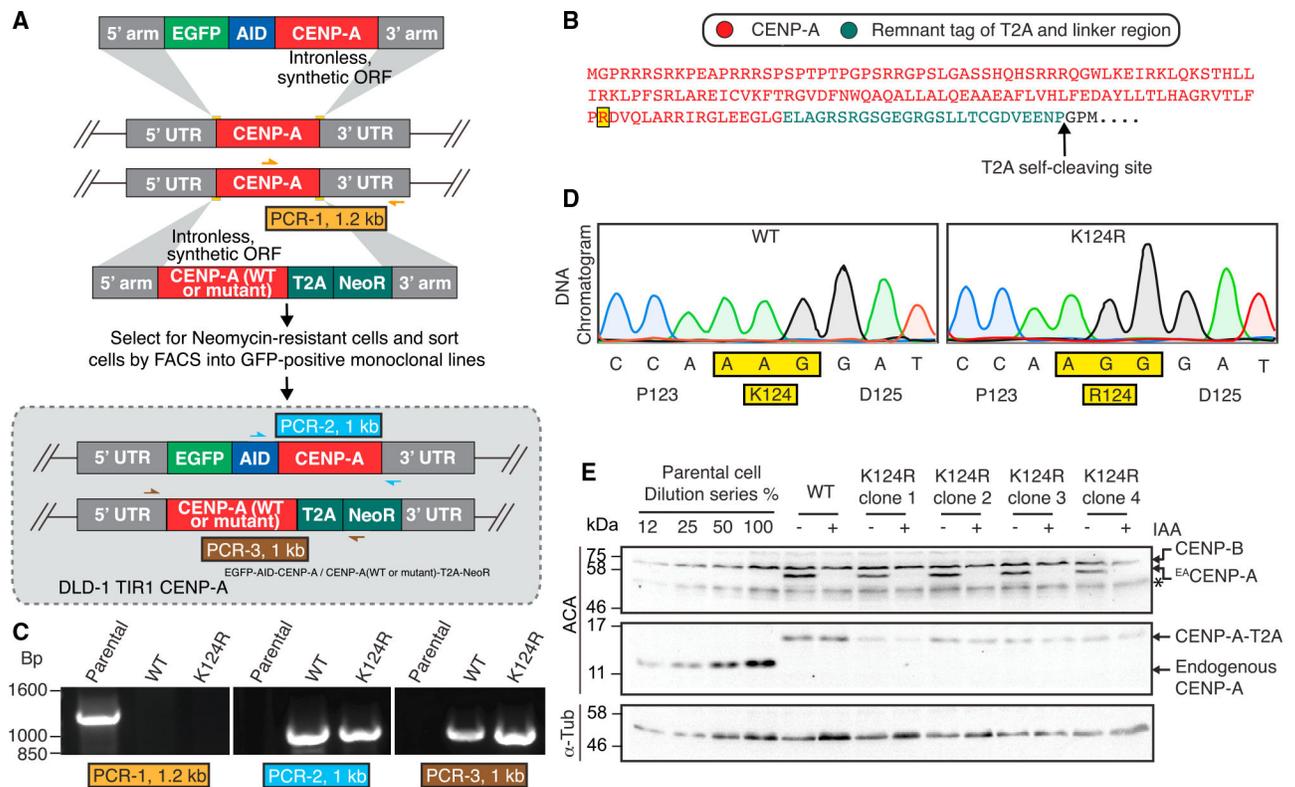
Figure 3. Biallelic gene replacement for the expression of lysine-free tagged CENP-A K124R mutant from the endogenous CENP-A locus in DLD-1 cells

(A) Schematic of CRISPR-Cas9-mediated gene editing in DLD-1 TIR-1 cells. In this biallelic gene replacement approach, one of the endogenous alleles of the CENP-A gene is replaced with ^{EA}CENP-A and the second allele with CENP-A (WT or K124R) in-frame with T2A-neomycin resistance gene (Neo^R). Color-coded arrows indicate primer pairs of each PCR for genotype verification shown in (C). EA, EGFP-AID.

(B) Protein translation of the K124R-edited (highlighted in yellow) CENP-A gene at the endogenous locus with the 26-amino-acid linker and T2A remnant (2.65 kDa). The T2A self-cleavage site is indicated by the arrow. Amino acids in black are lost after cleavage.

(C) Genomic DNA was isolated from the monoclonal lines, and PCR was performed with primer pairs as shown in (A). Positive PCR-1 for parental DLD-1 TIR-1 endogenous CENP-A alleles and no PCR product for either of WT or K124R genome-edited cells. Positive PCR-2 for the endogenous CENP-A allele replaced with ^{EA}CENP-A. Positive PCR-3 for the other allele of the CENP-A gene replaced with either CENP-A^{WT}-T2A-Neo^R or CENP-A^{K124R}-T2A-Neo^R.

(D) PCR-3 product from (C) was purified by agarose gel extraction and Sanger sequenced. Raw DNA sequencing data for CENP-A^{WT}-T2A-Neo^R or CENP-A^{K124R}-T2A-Neo^R (clone 4) alleles showing the mutation.

(E) Immunoblot analysis of whole-cell lysates from the indicated cells with or without 500 μM IAA treatment for 24 h to degrade ^{EA}CENP-A using an anti-centromere (ACA) and anti-α-tubulin antibodies. Asterisks (*) mark unspecific bands.

See also Figure S3.
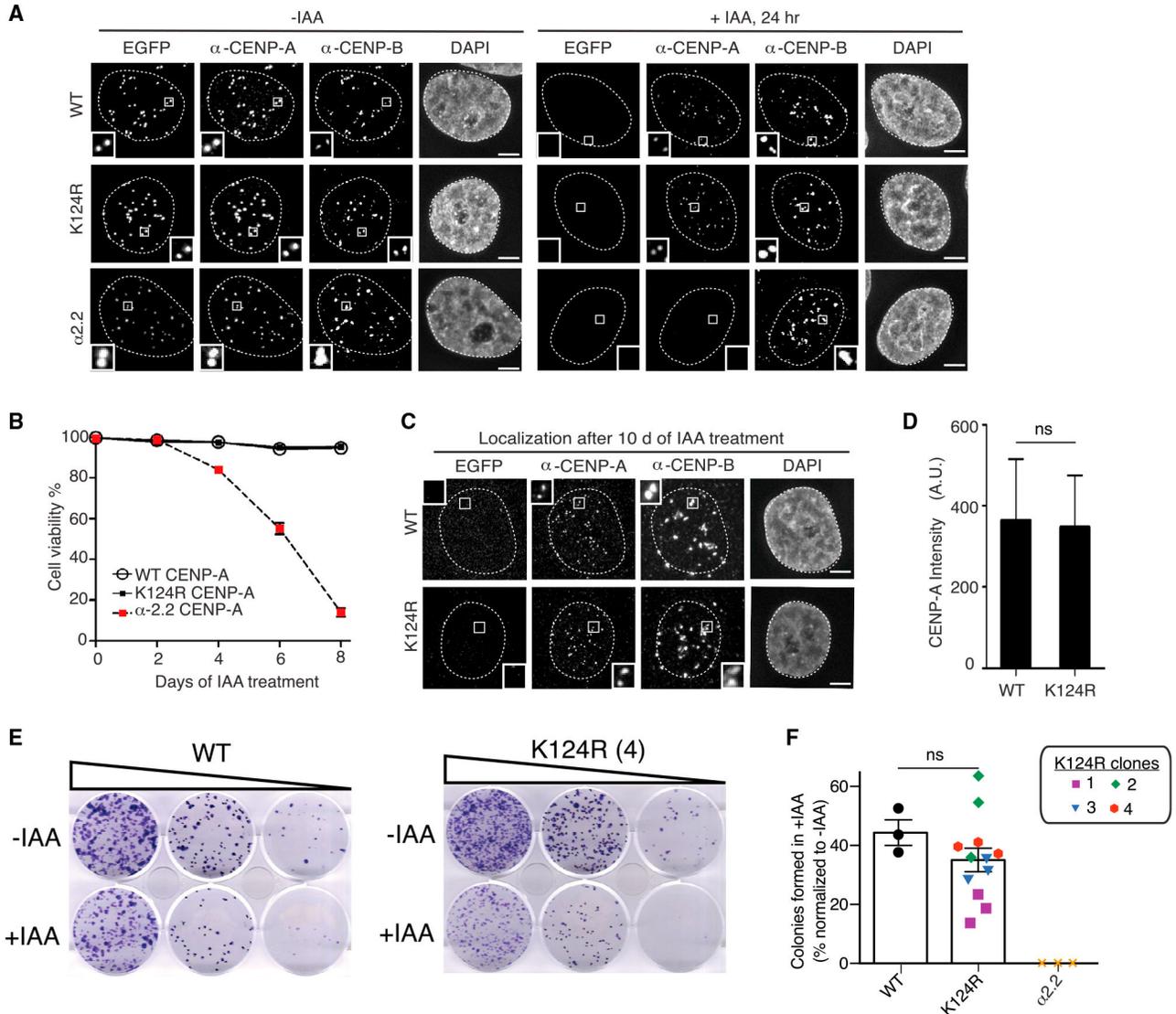
Figure 4. Lysine-free tagged CENP-A^{K124R}, expressed from the endogenous CENP-A locus, efficiently targets centromeres and confers cell survival and centromere functions

(A) Representative immunofluorescence images of CENP-A^{WT}, CENP-A^{K124R} (clone 4), and CENP-A$^{\alpha 2.2}$ cells showing localization of ^{EA}CENP-A and WT or K124R CENP-A-T2A at centromeres. Cells were treated with 500 μM IAA for 24 h to degrade ^{EA}CENP-A. α-CENP-B was used as a marker to determine centromere position. Nuclei are contoured with white dashed lines. Scale bar, 5 μm.

(B) Quantification of the percentage of viable cells expressing CENP-A^{WT}, CENP-A^{K124R} (clone 4), or α2.2 CENP-A upon treatment with 500 μM IAA for 8 days. Every 2 days, media with 500 μM IAA was changed. Cells were collected every other day and stained with trypan blue. Viability was calculated based on trypan blue uptake. Mean ± SEM from three independent experiments is shown for each time point.

(C) Representative images of viable cells expressing CENP-A^{WT} or CENP-A^{K124R} (clone 4) after 10 days of treatment with 500 μM IAA. CENP-A^{K124R} is still present at endogenous

centromeres (marked by CENP-B). Nuclei are contoured with white dashed lines. Scale bar, 5 μm.

(D) Quantitation of centromeric CENP-A^{WT}-T2A and CENP-A^{K124R}-T2A (clone 4) intensity after 10 days of 500 μM IAA treatment. Mean ± 95% confidence interval from three independent experiments is shown (n > 700 centromeres for each replicate). ns: non-significant differences (unpaired t test).

(E) Representative images of a colony formation assay and its quantitation in the indicated cells.

(F) Bars represent the percentage of colonies formed after 2 weeks of culture with IAA, normalized to the untreated. Each dot represents one experiment. The four CENP-A^{K124R} clones are depicted with different symbols and colors, and error bars represent SEM. ns: non-significant differences (unpaired t test).

See also Figures S3 and S4.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-GFP	ChromoTek	Cat# PABG1-20; RRID: AB_2749857
Guinea pig polyclonal anti-CENP-C	MBL International	Cat# PD030; RRID: AB_10693556
Mouse monoclonal anti-HA (clone 12CA5)	Recombinant Antibody Platform (TAb-IP), Institut Curie	Cat# A-M-M#07
Mouse monoclonal anti-FLAG (clone M2)	Sigma-Aldrich	Cat# F3165; RRID: AB_259529
Rabbit polyclonal anti-CENP-A	Cell Signaling Technology	Cat# 2186; RRID: AB_10828491
Human polyclonal anti-centromere antisera (ACA)	Antibodies Incorporated	Cat# 15-235-0001; RRID: AB_2797146
Mouse monoclonal anti-CENP-A (clone 3-19)	Enzo Life Sciences	ADI-KAM-CC006-E RRID: AB_2038993
Rabbit polyclonal anti-CENP-B	Santa Cruz Biotechnology	Cat# sc-22788, RRID: AB_2078775
Mouse monoclonal anti-αTubulin (clone DM1A)	Sigma-Aldrich	Cat# T9026; RRID: AB_477593
Rabbit monoclonal anti-GAPDH (clone14C10)	Cell Signaling Technology	Cat# 2118; RRID: AB_561053
Mouse monoclonal anti-Vinculin (clone hVIN-1)	Sigma-Aldrich	Cat# V9264; RRID: AB_10603627
Donkey polyclonal anti-mouse - Cy3	Jackson ImmunoResearch Laboratories	Cat# 715-165-151; RRID: AB_2315777
Donkey polyclonal anti-rabbit - Cy5	Jackson ImmunoResearch Laboratories	Cat# 711-175-152; RRID:AB_2340607
Donkey polyclonal anti-mouse - Alexa Fluor® 488	Jackson ImmunoResearch Laboratories	Cat# 715-545-150; RRID: AB_2340846
Donkey polyclonal anti-rabbit - Cy3	Jackson ImmunoResearch Laboratories	Cat# 711-165-152; RRID: AB_2307443
Donkey polyclonal anti-guinea pig - Alexa Fluor® 647	Jackson ImmunoResearch Laboratories	Cat# 706-605-148; RRID: AB_2340476
Donkey polyclonal anti-human - HRP	Jackson ImmunoResearch Laboratories	Cat# 709-035-149; RRID: AB_2340495
Sheep anti-mouse - HRP	GE Healthcare	Cat# NA931l; RRID: AB_772212
Donkey anti-rabbit - HRP	GE Healthcare	Cat# NA934, RRID: AB_772206
Bacterial and virus strains		
RV-FLAG-CENP-A(WT)-Puro	This paper	N/A
RV-FLAG-CENP-A(K124R)-Puro	This paper	N/A
RV-FLAG-CENP-A(WT)-Blast	This paper	N/A
RV-FLAG-CENP-A(K124R)-Blast	This paper	N/A
RV-HA-CENP-A(WT)-Puro	This paper	N/A
RV-HA-CENP-A(K124R)-Puro	This paper	N/A
AdCre	Fachinetti et al., 2013	N/A
Chemicals, peptides, and recombinant proteins		
Indole-3-acetic acid (IAA) sodium salt	Sigma-Aldrich	Cat# I5148; CAS: 6505-45-9
Polybrene®	Santa Cruz Biotechnology	Cat# sc-134220; CAS 28728-55-4
VectaShield	Vector Laboratories	Cat# H-1000; RRID: AB_2336789

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Human: hTERT RPE-1 [EYFP-AID/EYFP-AID]CENP-A OsTIR1	Hoffmann et al., 2016	N/A
Human: hTERT RPE-1 CENP-A$^{-/F}$	Fachinetti et al., 2013	N/A
Human: hTERT RPE-1 CENP-A$^{+/F}$	Fachinetti et al., 2013	N/A
Human: DLD-1 Flp-In T-Rex	Holland et al., 2012	N/A
Human: DLD-1 TIR1 CENP-A[EGFP-AID-CENP-A/CENP-A(a-2.2)-SNAP-3xHA-P2A-NeoR]	Fachinetti et al., 2017	N/A
Human: DLD-1 TIR1 CENP-A[EGFP-AID-CENP-A/CENP-A(WT)-T2A-NeoR]	This paper	N/A
Human: DLD-1 TIR1 CENP-A[EGFP-AID-CENP-A/CENP-A(K124R)-T2A-NeoR]	This paper	N/A
Recombinant DNA		
pBabe-FLAG-CENP-A(WT)-Puro^R	This paper	N/A
pBabe-FLAG-CENP-A(K124R)-Puro^R	This paper	N/A
pBabe-FLAG-CENP-A(WT)-Blast^R	This paper	N/A
pBabe-FLAG-CENP-A(K124R)-Blast^R	This paper	N/A
pBabe-HA-CENP-A(WT)-Puro^R	This paper	N/A
pBabe-HA-CENP-A(K124R)-Puro^R	This paper	N/A
pUC19-EGFP-AID-CENP-A	Fachinetti et al., 2017	N/A
pUC19-CENP-A(WT)T2A-Neo^R	This Paper	N/A
pUC19-CENP-A(K124R)-T2A-Neo^R	This Paper	N/A
Software and algorithms		
Fiji	Schindelin et al., 2012	https://imagej.net/software/fiji
CRaQ v1.12	Bodor et al., 2012	http://facilities.igc.gulbenkian.pt/microscopy/macros/CRaQ_v1.12.ijm
Centromere intensity	Giunta et al., 2021	Available upon request
GraphPad Prism	https://www.graphpad.com:443/	RRID:SCR_002798