A two-step mechanism for epigenetic specification of centromere identity and function

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The basic determinant of chromosome inheritance, the centromere, is specified in many eukaryotes by an epigenetic mark. Using gene targeting in human cells and fission yeast, chromatin containing the centromere-specific histone H3 variant CENP-A is demonstrated to be the epigenetic mark that acts through a two-step mechanism to identify, maintain and propagate centromere function indefinitely. Initially, centromere position is replicated and maintained by chromatin assembled with the centromere-targeting domain (CATD) of CENP-A substituted into H3. Subsequently, nucleation of kinetochore assembly onto CATD-containing chromatin is shown to require either the amino- or carboxy-terminal tail of CENP-A for recruitment of inner kinetochore proteins, including stabilizing CENP-B binding to human centromeres or direct recruitment of CENP-C, respectively.

The centromere is the fundamental unit for ensuring chromosome inheritance. Centromeres have a distinct type of chromatin in which hi- stoneH3is replaced by a conserved homologue initially identified in hu- mans and named CENP-A (refs 1,2). Although specific chromosomal regions containing α-satellite repeats3 are the sites of centromere formation and CENP-A association in humans, α-satellite DNA sequences are neither sufficient nor essential for centromere identity4. Rather, except for budding yeast, it is well accepted that in almost all other organisms, including fission yeast, centromeres depend on one or more as yet unidentified epigenetic marks5. Among the strongest evidence for an epigenetically defined centromere was the discovery in humans of migration of a functional centromere from an initial location to a new site on the same chromosome6–9. These loci, referred to as neocentromeres, are stably loaded with CENP-A (ref. 8) and form at previous euchromaticDNAsites withoutα-satellite repeats6,10,11. Epigenetic inheritance implies that the mark must be self-templating

and be stably maintained across the cell cycle. Centromere-bound CENP-A molecules are indeed quantitatively redistributed to both sister centromeres during centromeric DNA replication, yielding each daughter centromere with half the level of the initially loaded CENP-A (ref. 12). Furthermore, in human12 and Drosophila13,14 cells, loading of new CENP-A into centromeric chromatin occurs once per cell cycle only during or on exit from mitosis.

Although the precise nature of centromeric chromatin is highly controversial15–24, CENP-A is a central component in all models. Reduction or mutation in CENP-A (refs 25–28) has proved it to be essential for continuing centromere function27. Indeed, forced loading of CENP-A by tethering it or components that bind it to specific DNA loci has produced partial ectopic centromere formation29–32 and in one case with full kinetochore function after deletion of the authentic centromere33. These reports reinforce the sufficiency of CENP-A for recruitment of some centromere and kinetochore components, but offer no insight into how centromere identity and function are epigenetically specified and maintained. Approaches25–28 relying on RNA interference (RNAi)-mediated messenger RNA degradation to identify the epigenetic mark have suffered from incomplete silencing, especially for proteins such as CENP-A with long half-lives. To overcome these limitations, we now use gene targeting in human diploid cells and in fission yeast to demonstrate that CENP-A- containing chromatin is the epigenetic mark that can identify, maintain and propagatehumanor yeast centromere function indefinitely.

RESULTS

Conditional CENP-A gene deletion in diploid human cells

Using a single-stranded adeno-associated virus 2 (AAV2) vector to enhance homologous recombination34,35, both human CENP-A alleles were targeted in non-transformed diploid human retinal epithelial (RPE1) cells immortalized with human telomerase. One allele was replaced with a CENP-A null allele in which a neomycin-resistance gene (flanked by FRT sites to facilitate its later removal) replaced exons 3 and 4 of the CENP-A gene (the exons encoding the essential CENP-A centromere targeting domain (CATD) that is comprised of loop 1 and the α2 helix of CENP-A and which when substituted into histone H3 is sufficient to restrict H3 targeting to centromeres36). The other allele was specifically targeted with an AAV2 floxed construct containing 34-base-pair (bp) loxP recombination sites flanking exons 3 and 4 (Fig. 1a). This yielded a final cell line (CENP-A−/F) with one null allele and one floxed CENP-A allele, the latter of which can be converted into a null allele by action of Cre recombinase (Fig. 1a and Supplementary Fig. S1a). A CENP-AF/+ cell line was also produced as a control. CENP-A−/F cells grew at a rate indistinguishable from the control CENP-A+/+ or CENP-AF/+ with only a slight reduction of the total pool of CENP-A protein and no reduction seen at centromeres (Supplementary Fig. S1b–d). Transient expression of Cre recombinase (using a replication- defective adenoviral vector, Ad-Cre, to produce excision of exons 3 and 4 of the floxed allele (Supplementary Fig. S1e)) suppressed cell growth in the CENP-A−/F cells (Fig. 1b). PCR analysis of the few surviving clones revealed that they had escaped inactivation of the second CENP-A allele (Supplementary Fig. S1f). To eliminate confounding effects from these escapers, a LoxPRFP–STOPLoxP–GFP cassette was inserted (by lentiviral integration)

into the CENP-AF/+ and CENP-A−/F cells (Supplementary Fig. S1g). Addition of Cre recombinase converted cells from red to green fluorescence by simultaneous excision of the RFP gene and activation of the GFP gene (Supplementary Video S1). CENP-A-depleted cells were then collected by fluorescence-activated cell sorting (FACS) to recover green fluorescent cells. After depletion of both alleles of CENP-A, cells duplicated at a similar rate as control cells for the first 5 days (~6 divisions), then exhibited slowed cycling from extended mitosis and stopped dividing only 9–11 days (~8–9 divisions) after initial CENP-A gene inactivation (Supplementary Fig. S1h). CENP-A excision led to progressive loss of accumulated CENP-A and its levels decreased by approximately half each cell cycle (Fig. 1c,d), consistent with the expected redistribution of centromere-bound CENP-A to sister centromeres during DNA replication but without addition of new CENP-A (ref. 12). Only 1% of the initial CENP-A level was detectable within 7 days (Fig. 1d; as expected for the 1/27dilution = 0.8%). No centromere-bound CENP-A could be detected 9 days following excision (Fig. 1d). Lowered levels of CENP-A corresponded with increased rates of chromosome segregation defects and increased mitotic duration, accompanied by a significant increase in micronuclei formation from failure in initial kinetochore attachment to spindle microtubules or initially aligned chromosomes lagging in anaphase (Fig. 1e,f and Supplementary Video S2).

Disrupted kinetochore nucleation requires almost complete loss of CENP-A

CENP-A loading has been proposed to be at the foundation of recruitment (direct and indirect) of all of the components of the core centromere26,37–39. Three patterns of centromere/kinetochore protein loss after inactivation of the conditional CENP-A allele in the CENP-A−/FRPE1cells were identified in asynchronous cycling cells. The first group included: CENP-C and CENP-N, two primary components of the constitutive centromere-associated network (CCAN) that have been shown to directly interact with CENP-A chromatin37,38 and CENP-P, a more distal kinetochore protein39. Loss of centromere-bound CENP-C, CENP-N and CENP-P was initially in proportion to loss of CENP-A (Fig. 2a,b,d and Supplementary Fig. S2a), consistent with direct binding to CENP-A chromatin or, in the case of CENP-P, through a CENP-N/CENP-C-dependent complex. Surprisingly, a substantial proportion (~30%) of CENP-C remained even afterCENP-Adepletion to<1%of its initial level (Fig. 2a,d). A second pattern included: CENP-T, a component of the CENP- T/W/S/X complex and whose histone fold domains bind to chromatin adjacent to centromeric CENP-A nucleosomes40,41; CENP-I, which has been proposed to act in complex with CENP-H as another mediator of CENP-A deposition42; and Ndc80, a direct microtubule- binding component of the outer kinetochore43. Surprisingly, all three of these centromere/kinetochore proteins were almost fully maintained at centromeres (Fig. 2c,e and Supplementary Fig. S2b,c) until CENP-A levels dropped below ~1% of the initial level. Only after further loss of CENP-A were these kinetochore components then rapidly lost (Fig. 2e). A final group was represented by the direct DNA-binding protein CENP-B that recognizes a 17 bp sequence (CENP-B box) within α-satellite DNA (ref. 44). As expected, CENP-B binding at centromeres was not initially affected by reduction in CENP-A levels. Quite unexpectedly, however, CENP-B binding dropped by half as centromeric CENP-A was completely depleted (Fig. 2f), thereby identifying a previously unrecognized partial dependency on CENP-A for CENP-B binding at centromeres. Overall,

assessment of loss of kinetochore proteins as centromere-bound CENP-A was depleted identified that kinetochore assembly is not an all-or-nothing event and that a remarkably small amount of CENP-A is sufficient to nucleate assembly of a kinetochore with partial centromere function.

The CENP-A CATD maintains centromere position and templates its replication

Next we investigated whether CENP-A in centromeric chromatin can indefinitely maintain centromere identity by templating loading of new CENP-A onto replicated DNA of natural human centromeres and if so, the mechanism underlying its ability to do this. A series of histone H3 or CENP-A rescue variants were created with EYFP (enhanced yellow fluorescent protein) tags and stably expressed (by retroviral integration) in CENP-A−/F cells (Fig. 3a,b and Supplementary Fig. S3a,b). The remaining CENP-A allele was then inactivated by addition of Ad-Cre. As expected, full-length CENP-A accumulated to the endogenous CENP-A level (Fig. 3c) and was able to support cell viability (Fig. 3d(i–ii),e). H3CATD rescued complete deletion of CENP-A with an efficiency 2/3 of CENP-A itself, whereas H3 alone conferred no survival (Fig. 3d(iii–iv),e). H3CATD continued to be loaded at centromeres at a constant level even 5–6 generations after reduction of CENP-A to below1%of its normal level (see Discussion; Fig. 4a–c).

To determine whether CATD-dependent centromere identity was maintained through replication of centromeric chromatin at mitotic exit12 and in an HJURP-dependent manner45,46 in the absence of endogenous CENP-A, a SNAP-tagged H3CATD construct was stably expressed in CENP-A−/F cells. In cells treated with a control GAPDH short interfering RNA (siRNA), assembly of new SNAP-H3CATD molecules at centromeres was observed only at mitotic exit. This loading was completely dependent on HJURP, as its reduction abolished new SNAP−H3CATD assembly (Fig. 4d–f). Furthermore, in agreement with recent findings47, the continued HJURP-dependent loading of H3CATD missing Ser 68 demonstrated that it is not necessary for CENP-A recognition and loading by HJURP, in contrast with a previous hypothesis48. Whereas cell-cycle-dependent loading of newH3CATD at centromeres continued after CENP-A depletion, other centromeric proteins including CENP-I, CENP-N, CENP-C and CENP-T were inefficiently assembled at the CENP-A-depleted H3CATD-containing centromeres, although all but CENP-C were maintained longer when compared with cells with no rescue construct (Fig. 4c and Supplementary Fig. S3d). Failure to maintain or recruit kinetochore proteins caused a marked increase in chromosome segregation defects (Fig. 4g) that prevented long-term survival (>100 generations; Fig. 5a,b). Thus, substituting the CATD into histone H3 enables it to mediate epigenetic inheritance of centromere position through a mechanism requiring its cell-cycle-dependent recruitment by the chaperone/loader HJURP, but H3CATD-containing centromeric chromatin is not sufficient to nucleate assembly of a functional kinetochore.

N- or C-terminal tail of centromere-bound CENP-A enables long-term centromere rescue

We then investigated how CENP-A confers long-term centromere function. The αN helix of CENP-A has been shown to be important for contacting DNA as it exits a CENP-A-containing nucleosome19,49 and the six-amino-acid C-terminal tail of CENP- A can directly recruit CENP-C and drive aspects of kinetochore assembly in in vitro extracts29. We therefore created stable cell lines expressing H3CATD with the addition of the CENP-A αN helix (αNH3CATD), C-terminal tail (H3CATD+C) or all or part of its N-terminal tail (NH2H3CATD or NH2(1–29)H3CATD; Fig. 3a and Supplementary Fig. S3a). Addition to H3CATD of either CENP- A tail rescued not only growth in the short-term assay—similar to the full-length CENP-A (Fig. 3d(v–vi),e and Supplementary Fig. S3c)—but also supported growth indefinitely (Fig. 5b), with only a mild slow growth phenotype relative to cells still containing the endogenous CENP-A (Supplementary Fig. S4a). Centromere function continued in the complete absence of CENP-A, as deletion of both endogenous CENP-A genes was confirmed by immunoblotting, PCR and immunofluorescence microscopy (Fig. 5c and Supplementary Fig. S4b,c). As expected, loading of the rescue constructs occurred only after mitotic exit and was dependent on HJURP (Supplementary Fig. S4d–f). Redundancy of the N- and C-terminal tails of CENP-A in supporting long-term viability was further confirmed by full rescue with CENP-A constructs deleted in either domain (H3–NH2CENP-A and CENP-AC–H3; Fig. 5b).

The CENP-A C-terminal tail directs CENP-C binding, but is not essential for kinetochore assembly

Our findings suggested that full kinetochore assembly was nucleated by recruitment by either tail domain of one or more components. Indeed, consistent with direct CENP-C recruitment by the CENP-A C-terminal tail29, centromere-associated CENP-C was reduced in cells lacking the CENP-A C terminus (CENP-AC–H3 and NH2H3CATD; Fig. 5d,e). Nevertheless, a fraction ofCENP-Cremained centromereboundin cells rescued long-term in the absence of the C-terminal tail, demonstrating the existence of CENP-A C-terminal-independent loading of CENP-C, possibly through an existing CENP-C that templates new CENP- C loading. This remaining CENP-C was essential for centromere function, as depletion of it with siRNA produced catastrophic chromosome mis-segregation (Supplementary Fig. S4g). Similar reduction was also observed for CENP-I, suggesting its stabilization by CENP-C (Fig. 5e and Supplementary Fig. S5a). In any event, centromeres rescued with CENP-A variants missing its C-terminal tail (CENP-AH3–C and NH2H3CATD) were sufficient for long-term kinetochore function, with normal assembly of associated centromere proteins thereby maintaining centromere function (Fig. 5d,e and Supplementary Fig. S5a).

The CENP-A N-terminal tail controls CENP-B levels at centromeres

CENP-C, CENP-T, CENP-N, Ndc80 and Dsn1 were fully maintained at centromeres in the absence of CENP-Afollowing long-term rescue with each variant missing the N-terminal CENP-A tail (H3–NH2CENP-A and H3CATD+C; Fig. 5d,e). Surprisingly, however, the CENP-A N-terminal tail was required for proper loading of the direct DNA-binding protein CENP-B. Without that N-terminal tail, only 50% of the normal centromeric amount of CENP-B was loaded at centromeres (Fig. 6a and Supplementary Fig. S5b). This CENP-A-dependent CENP-B binding was essential for centromere function. Indeed, stable reduction of total CENP-B by

half in CENP-A-deleted cells rescued by NH2H3CATD markedly markedly increased chromosome mis-segregation and nearly eliminated colony survival (Fig. 6b,c and Supplementary Fig. S5c). Similar reduction in CENP-B did not affect rescue with full-length CENP-A, presumably because kinetochore functionality was still maintained through recruitment of CENP-C mediated by the C-terminal tail (Fig. 6b,c). Indeed, further reduction of CENP-B did not affect kinetochore function and survival of the H3CATD+C cells (Fig. 6c). This finding implicates CENP-B stabilization as one important component of kinetochore assembly nucleated in a manner dependent on the CENP-A N-terminal tail.

Accurate chromosome segregation with centromere-targeted histone H3 carrying either CENP-A tail domain

The degree of complementation of normal chromosome segregation in cells deleted in CENP-A and rescued with CENP-A or H3CATD including either CENP-A tail domain was measured using fluorescence in situ hybridization (FISH) to visualize chromosomes 2 and 7. No increase in aneuploidy was observed in CENP-A- or H3CATD+C-rescued cells and only a minor increase was found in NH2H3CATD-rescued cells (Fig. 6d). Direct visualization of chromosome segregation using time-lapse microscopy confirmed almost normal segregation in all rescued lines (Supplementary Fig. S5d,e). Overall, long-term, faithful centromere replication and kinetochore function was mediated by CATD-containing centromeric chromatin with either tail ofCENP-A.

The principles of epigenetic centromere inheritance and function are conserved in fission yeast

We next tested the generality of our findings by examining whether the CATD and tail domains of CENP-A similarly specify centromere identity and kinetochore assembly in the fission yeast Schizosaccharomyces pombe, an organism whose epigenetically defined centromeres contain repeat motifs that are reminiscent of the repetitive arrays found at most higher eukaryotic centromeres50. Various histone H3–CENP-ACnp1 rescue genes were constructed to test the contribution of the fission yeast CATD (which lies within the CENP-ACnp1 histone fold domain (HFD)), the short CENP-ACnp1 N-terminal tail (19 amino acids) and the remaining regions of the CENP-ACnp1 HFD that were not included in the CATD (for simplicity we refer to these as N-HFD and C-HFD; Fig. 7a). GFP- tagged chimaeric CENP-ACnp1 variants were expressed in a yeast strain bearing a tetO array inserted at centromere 2, as well as expressing a tetR–Tomato fusion protein that allows visualization of centromere 2 (Fig. 7c and Supplementary Table S1 and Fig. S6). H3CATD co-localized with the centromere, whereas a Cnp1 whose CATD was exchanged with the corresponding domain of histone H3 (Cnp1CATDswap) was found scattered throughout the nucleus (Fig. 7b,c). Chromatin immunoprecipitation confirmed enrichment of H3CATD, but not Cnp1CATDswap, specifically at centromeres relative to non-centromeric regions (Fig. 7b,d).

The ability of the various Cnp1 rescue constructs to complement

the complete absence of CENP-A was tested by employing a plasmid- shuffling assay (Fig. 7e). H3CATD was unable to confer cell viability, but addition of the Cnp1 N-terminal tail to H3CATD did sustain long-term viability (Fig. 7f). Similar results were observed when the rescue constructs were then introduced in cells harbouring a CENP-ACnp1 temperature-sensitive mutant allele (cnp1-76; ref. 51) as the unique source of CENP-A (Fig. 7g). There was no noticeable difference in growth ratesamongcontrol cells expressing full-lengthCnp1orH3CATD with the Cnp1 N-terminal tail. Only a slight sensitivity was seen for cells rescued with the latter construct and exposed to drugs that drive spindle disassembly, indicating that centromere function is largely restored (Fig. 7h). In contrast with human, CENP-ACnp1 has only one amino-acid C-terminal extension beyond the end of the HFD (ref. 52). The corresponding rescue construct (H3CATD+C−HFD) did not maintain growth in the complete absence of CENP-ACnp1, although it did restore growth of the cnp1-76 mutant at the restrictive temperature (Fig. 7f,g), probably facilitating the recruitment of the thermo-sensitive cnp1-76 mutant to centromeres that in turn may recruit kinetochore components through its N-terminal tail. Thus, in fission yeast the CATD requires the addition of the Cnp1 N-terminal tail to provide long-term viability, as in human cells.

DISCUSSION

A long-standing question in chromosome inheritance is the epigenetic mark of centromere identity. By artificially targeting components to chromatin29–31,33, several groups have demonstrated that large artificial arrays of CENP-A-containing chromatin are sufficient to generate partially functional centromeres, but only after completely abrogating any epigenetic component. One earlier effort had reduced CENP-A levels using siRNA (ref. 27). Such approaches fail to test the epigenetic question as they are plagued by partial suppression that precludes testing epigenetic sufficiency. Indeed, we now demonstrate that as little as 1% of the original CENP-A level is sufficient for retention of at least partial centromere function and assembly of kinetochore proteins. Our use of conditional gene inactivation in human cells and in fission yeast has overcome the previous technical limitations and

has now established that the CATD of CENP-A when substituted into histone H3 can template its cell-cycle-dependent, HJURP/Scm3- dependent centromeric loading at a constant level in the complete absence of CENP-A. Consideration of the number of molecules of CENP-A at the normal centromere offers strong support for this conclusion. The number of CENP-A molecules at the 40–500 kilobase chicken centromeres has been reported to be between 25 and 40 (ref. 53). Increasing that by tenfold to account for the increased centromere size in humans yields a maximal estimate of ~250–400 CENP-A molecules per centromere, with a corresponding prediction of <1 molecule remaining per centromere within 9 divisions after CENP-A gene inactivation (400 molecules per centromere×1/29= 0.8 molecules per centromere). Therefore, our evidence demonstrates that H3CATD continues to be loaded at its initial level at each centromere for 4–5 generations after the CENP-A level has fallen below ~1 molecule per centromere. It is important to note that initial H3CATD assembly occurs in the presence of endogenous CENP-A; therefore, our evidence offers no insight into de novo centromere formation. Despite its necessity and sufficiency for maintaining centromere

identity in the absence of CENP-A, we have shown that H3CATD is not sufficient for long-term centromere function and cell viability because it does not nucleate kinetochore assembly. Rather, we have identified two redundant pathways that function to initiate kinetochore assembly onto an epigenetically defined chromatin core containing the CATD. Our evidence establishes that CENP-A-containing chromatin is the epigenetic mark that can identify, maintain and propagate human centromere function indefinitely through a conserved two-step mechanism in which it templates its own CATD-dependent replication and nucleates subsequent kinetochore assembly (Fig. 8). Furthermore, we demonstrate that the principles of epigenetic centromere inheritance and function are conserved from human to fission yeast. The most plausible model is that theCATDestablishes the epigenetic

mark by physically modifying centromeric chromatin54 mediated by its interaction with histone H4 (ref. 21) and by the exposure of the positively charged Loop1 of the CATD (refs 21,22). Conformationally constrained CENP-A-containing chromatin and the exposed Loop1 can then attract centromeric components such as CENP-N or the

nucleosome-like CENP-T/W/S/X complex. Indeed, recognition of these chromatin features offers an explanation for the slow loss of CENP-Nor CENP-T fromH3CATD-defined centromeres. Further, earlier evidence had shown that the C-terminal CENP-A

tail can recruit CENP-C in vitro and that this is sufficient to initiate assembly of kinetochore components that are capable of microtubule capture29. Adding to that earlier work, our evidence has established that the human CENP-A C-terminal tail not only recruits CENP-C, but also is sufficient to nucleate functional kinetochore assembly required for high-fidelity chromosome segregation indefinitely when linked to a histone H3 variant with the CATD. Nevertheless, our evidence has also demonstrated that the CENP-A rescue construct lacking the CENP-A C-terminal tail still supports kinetochore assembly. Indeed, a fraction of CENP-C remains bound to centromeres even in the absence of the CENP-A C terminus, possibly through its proposed DNA-binding domain or interaction with histone H3 or CENP-B (refs 55–57). This underscores that theCterminus of CENP-A is not essential for centromere function as previously suggested29, although in its absence kinetochore function is slightly impaired, as observed by an increase in chromosome segregation errors and modestly increased aneuploidy. Regarding a previously unrealized influence of CENP-B on kinetochore assembly, binding of CENP-B had previously been reported to affect the formation of heterochromatin58. Added to this, use of chromatin fibres and super-resolution microscopy had revealed CENP-A nucleosomes to be interspersed with chromatin containing H3K4me2 (ref. 59) or H3K9me3 (ref. 53) histone H3 modifications. In addition, evidence for CENP-B binding to the CENP-B box was demonstrated to include an effect on positioning of CENP-A nucleosomes on alphoid DNA (refs 60,61). Our evidence has uncovered that at least a fraction of the centromeric CENP-B that is dependent on the first 29 amino acids of the CENP-A N terminus is required for accurate chromosome segregation (in the absence of the parallel kinetochore formation pathway mediated by the CENP-A C-terminal tail). This provides direct evidence in support of a role for the N-terminal tail of CENP-A in centromere function and for

CENP-B in kinetochore assembly. Finally, we note that an essential impact of CENP-B on reinforcing CENP-A chromatin offers an explanation for the previous finding that the density of CENP-B boxes influences the assembly and maintenance of CENP-A chromatin in human cells62. Our findings, coupled with evidence that direct targeting of CENP-C to a specific DNA site is sufficient to recruit CENP-A to that site in chicken cells33 (but not in human cells32, at least when the endogenous centromere is still present), are consistent with epigenetic centromere identity that is stabilized by CENP-C and CENP-B.

METHODS

Methods and any associated references are available in the online version of the paper. Note: Supplementary Information is available in the online version of the paper

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METHODS

Cell culture. hTERT RPE-1 (ATCC) cells were maintained at 37 _C in a 5% CO2 atmosphere with 21% oxygen. hTERT RPE-1 cells were maintained in DMEM:F12 medium containing 10% fetal bovine serum (Clontech), 0.348% sodium bicarbonate, 100Uml□1 penicillin, 100Uml□1 streptomycin and 2mM l-glutamine. Monastarol was used at 100 _M for 10 h.

CENP-A gene targeting. To generate the CENP-A□=F cell line, two rounds of gene targeting were performed. To generate the first conditional allele the left

homology arm (encompassing a total of 1,373 bp of the genomic CENP-A locus from 43 bp upstream of CENP-A exon 2 to 134 bp downstream of exon 4) was PCR amplified from BAC clone RP11-106G13, carrying the entire genomic CENP-A locus (Invitrogen) and cloned into the BamHI/SpeI sites of the Bluscript derivative pNY (ref. 35). A loxP site was inserted 102 bp upstream of exon 3, such that exons 3 and 4 were flanked between this loxP and a loxP present in pNY. The 1,042 bp right homology arm was amplified from the same BAC clone and runs from 351 bp upstream of exon 5 to 691 bp downstream of the beginning of exon 5 and was subcloned as an XhoI/KpnI fragment into the corresponding sites of pNY. The whole cassette encompassing the left arm, loxP, FRT, Neo cassette, FRT, right arm was cloned into the NotI sites of pAAV-LacZ, replacing the lacZ gene. The CENP-A knockout targeting construct was derived from this floxed construct by transformation into Cre-expressing Escherichia coli, thereby resulting in site-specific removal of exons 3 and 4. A second floxed allele was created by gene synthesis (GenScript) to create 9 polymorphic variants and inserted into the pBluescript derivative pNY using the same approaches as described above. The entire insert was then excised through NotI digestion and ligated to a pAAV vector backbone. Procedures for preparation of infectious AAV particles, transduction of hTERT-RPE1 cells and isolation of properly targeted clones were performed as described previously35.

Generation of stable cell lines, siRNA and SNAP-tag. The different transgenes used in this study were introduced by retroviral delivery as described previously64. Stable integrates were selected in 5 _g ml□1 puromycin or 10 _g ml□1 blasticidin S and single clones isolated using fluorescence activated cell sorting (FACS Vantage; Becton Dickinson). An RFP_STOP_GFP was integrated by lentiviral infection. siRNAs were introduced using Lipofectamine

RNAiMax (Invitrogen). A pool of four siRNAs directed against CENP-C (50-GCGAAUAGAUUAUCAAGGA-30 , 50-GAACAGAAUCCAUCACAAA-30 , 50-CGAAGUUGAUAGAGGAUGA-30 , 50-UCAGGAGGAUUCGTGAUUA-30), CENPB (50-CCAACAAGCUGUCUCCCUA-30 , 50-GGACAUCAAAGCUGAGUCA-30 , 50-GGAGGGUGAUGUUGAUAGU-30 , 50-GGCGGGAGUUCGAGGUCUU-30) or HJURP (targeting nucleotides 1,135_1,153, 1,225_1,243, 1,815_1,833 and 2,017_2,033 of the HJURP open reading frame) and a single siRNA directed against GAPDH (50-UGGUUUACAUGAUCCAAUA-30) were purchased from Dharmacon. Short hairpin RNA (shRNA) against CENP-B and 2 negative controls (an empty and a non-effective 29-oligonucleotide scrambled sequence) were obtained from OriGene. SNAP labelling was conducted as described previously12.

Clonogenic colony assay and Adeno-Cre treatment. Cells were plated in a 12-well plate at 4 _ 104. The next day, cells were washed three times in DMEM:F12 medium containing 2% fetal bovine serum. Ad-Cre virus was added at a multiplicity of infection of 250 in 400 _l of DMEM:F12 medium containing 2% fetal bovine serum. After 3.5 h, cells were washed three times with DMEM:F12 medium containing 10% fetal bovine serum. After 2 days, 500 cells were plated in triplicate on a 10 cm2 dish. After a further 14 days, colonies were fixed for 10 min in methanol and stained for 10 min using a crystal violet staining solution (1% crystal violet and 20% ethanol). The percentage of clonogenic survival was determined by dividing the number of colonies formed in the Ad-Cre-treated condition versus the untreated cells. For live-cell microscopy, immunoblot analysis or immunofluorescence staining, cells were plated at 4_105 on a 10 cm2 dish.

Immunoblotting. For immunoblot analysis protein samples were separated by SDS_PAGE, transferred onto nitrocellulose membranes (BioRad) and then probed with DM1A (_-tubulin, 1:5,000), CENP-A (Cell Signaling, 1:1,000), GFP (Roche,

1:500), HJURP (Covance, 1:1,000; ref. 46), CENP-B (Abcam and Upstate, 1:1,000) or GAPDH (Abcam, 1:10,000). Proteins for fission yeast were extracted with 0.7N NaOH solution and probed with GFP (Roche, 1:500) and _-actin (ACTB, Proteintech, 1:4,000).

Immunofluorescence and live-cell microscopy. Cells were fixed in 4% formaldehyde at room temperature or in methanol at 20 _C for 10 min. Incubations with primary antibodies were conducted in blocking buffer for 1 h at room temperature using the following antibodies: CENP-A (Abcam, 1:1,500), CENP-T (1:5,000), CENP-C (Covance, 1:1,000), CENP-B (Abcam, 1:1,000), ACA (Antibodies Inc, 1:500), Ndc80 (Abcam, 1:1,000), CENP-I (a gift from S-T. Liu, University of Toledo, USA), Dsn1 (1:1,000) and _-tubulin (1:2,000). Immunofluorescence images were collected using a Deltavision Core system (Applied Precision). For quantification of centromere signal intensity, un-deconvolved two-dimensional maximum intensity projections were saved as un-scaled 16-bit TIFF images and signal intensities determined using MetaMorph (Molecular Devices). A 15_15 pixel circle was drawn around a centromere (marked by ACA staining) and an identical circle drawn adjacent to the structure (background). The integrated signal intensity of each individual centromere was calculated by subtracting the fluorescence intensity of the background from the intensity of the adjacent centromere. About 20 centromeres were averaged to provide the average fluorescence intensity for each individual cell. Centromere signal intensity was also quantified using an automated system65. Aliquots treated with Ad-Cre were compared with untreated conditions (Day 0). For CENP-N and CENP-P_SNAP, untreated controls were processed at the same time of the Ad-Cre treatment (day 1 to day 9). For high-resolution spinning-disc fluorescence microscopy, cells were imaged using a _60 1.4NA PlanApochromat oil lens on a spinning-disc confocal mounted on a Nikon TE2000-E inverted

microscope equipped with a solid-state laser combiner (ALC)_491nm and 561nm lines_a Yokogawa CSU10 head and a CCD (charge-coupled device) Clara camera (Andor Technology). Acquisition parameters, shutters and focus were controlled by iQ 1.10.0 software (Andor Technology). Z sections of 5_ 2mM were acquired at 5 min time intervals for RFP and/or EYFP and maximum intensity projection created using MetaMorph. Movies were assembled and analysed using QuickTime (Apple).

Fluorescence in situ hybridization. Fluorescence in situ hybridization was performed as previously described28 with the following alterations: centromerespecific probes for chromosomes 2 and 7 were labelled with tetramethyl-rhodamine-5-dUTP and Fluorescein-12-dUTP (Roche), respectively and coverslips were washed in 2_ SSC containing 60% formamide. More than 100 cells were analysed for each data point.

Yeast strain and plasmid construction. Standard procedures were used for bacterial and fission yeast growth, genetics and manipulations66. CENP-Acnp1/H3 chimaeric constructs were obtained by a combination ofDNAsynthesis (GenScript), PCR and subsequent subcloning into standard LEU2-marked pREP81 plasmids. The codon usage of all constructs was optimized to the initial CENP-Acnp1 gene codon usage (Leto software). S. pombe strains used in this study are described in Supplementary Table S1. Cells were grown at 30 _C in all the experiments unless indicated otherwise. To create a cnp11 strain, a standard auxotrophic strain P013 was transformed with an ura4+-marked plasmid pREP82-cnp1+, thereby producing P162. P162 was subsequently transformed with a NAT cassette replacing the entire cnp1 ORF and thus obtaining a ura+ NATr strain, P164, which was checked by DNA blot and transformed with the aforementioned pREP81-based plasmids harbouring chimaeric constructs. Finally, transformants were employed in plasmid-shuffling

assays by plating serial dilution in PMG−LEUCFOA (containing 50 _g ml−1 uracil) and PMG −LEU−URA as control plates. For the cnp1-76 (ts) complementation assay, P482 strain was transformed with the mentioned pREP81-based plasmids. leu+ transformants were isolated and analysed by serial dilution assays on PMG −LEU at 25 _C and 36 _C. To isolate cnp11-harbouring pREP81-based plasmids as a unique source providing CENP-ACnp1, FOA-resistant colonies were picked directly from the plasmid-shuffling assay plate and subjected to two rounds of streaking onto PMG−LEUCFOA. Loss of the ura+ marker (that is, cnp1+ plasmid) was confirmed. Subsequently, newly isolated strains were assessed by dilution serial assays on PMG containing thiabendazole (TBZ) at 15 _g ml−1 for 4 days at 30 _C.

S. pombe live-imaging. Cells were grown overnight until logarithmic phase in minimal mediumPMG−LEU at 30 _C and then mounted inPMG2% agarose. Cells were imaged on a spinning-disc microscope with a _100 1.4NA PlanApochromat oil lens on a spinning-disc confocal mounted on a Nikon TE2000-E inverted microscope equipped with a solid-state laser combiner (ALC)_491nm and 561nm lines_a Yokogawa CSU10 head and a CCD Clara camera (Andor Technology). Fourteen 0:5-_m z sections were acquired and further image processing, including maximum intensity projections, was performed using ImageJ (National Institutes of Health).

Chromatin immunoprecipitation. Cells were grown at 30 _C in PMG −LEU.

For each immunoprecipitation, 1 _l of anti-GFP (A11122, Invitrogen) was used. Chromatin immunoprecipitation was performed as described previously67 with the following modifications. After incubating the samples (that is, crude extracts and beads) with 250 _l of TES for 6 h at 65 _C, 220 _l of TE and 30 _l of proteinase K (10 mg ml−1) were added for subsequent overnight incubation at 37 _C. The following day, 3 volumes of PB buffer (Qiagen) were added and samples were

purified with Qiagen PCR purification kit as indicated by the manufacturer.
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Figure 1 CENP-A conditional disruption in human cells. (a) Schematic illustration of the _nal CENP-A cell lines after gene targeting. Positions of the exons, the CATD domain, LoxP sites, FRT sites, the neomycin cassette and the start_stop codons are indicated. Xs indicate polymorphic mutations between the initial two CENP-A alleles. KO, knockout. (b) Clonogenic cell survival assays (visualized with crystal violet) for the indicated cell lines with (Ad-Cre) or without (NT, not treated) treatment. (c) CENP-A protein level determined by immunoblotting extracts from CENP-AF=C and CENP-A□=F cells at the indicated days after Ad-Cre addition. (d) Top, representative _uorescence images of CENP-A localization and intensity at the indicated days after Ad-Cre addition. Bottom, quanti_cation of CENP-A protein levels from _uorescence images.

Bars represent the mean of six independent experiments (>30 cells per experiment). Error bars represent the s.e.m. (standard error of the mean). ACA (anti-centromere antibody) staining was used to identify centromeres. (e) Time-lapse images of CENP-A□=F RPE1 cells stably expressing histone H2B_mRFP 5 days after Ad-Cre addition. Time (in minutes) after nuclear envelope breakdown (NEBD) is shown at the top. Yellow arrows point to a lagging chromosome that ultimately forms a micronucleus. (f) Bars represent the mean of >50 cells per condition. Each individual point represents a single cell. Error bars represent the s.e.m. of three independent experiments. Time in mitosis was de_ned as the period from NEBD to chromosome decondensation. Uncropped images of blots are shown in Supplementary Fig. S7. Scale bar, 5 _m.

Figure 2 Disrupted centromere positioning and kinetochore nucleation requires almost complete loss of CENP-A. (a_c) Left, representative _uorescence images show the localization and intensity of CENP-A and CENP-C (a), CENP-N (b) and CENP-T (c) at day 0 and 7 or 9 after Ad-Cre addition to inactivate the remaining CENP-A allele in CENP-A□=F cells. Right, bar graphs (the mean of four independent experiments; >30 cells per experiment) show CENP-A (red), CENP-C (green), CENP-N (orange) and CENP-T (blue) levels 0 to 9 days after Ad-Cre addition. Error bars represent the s.e.m. ACA staining was used to identify centromeres. CENP-N was tracked by covalent labelling with rhodamine-benzyl guanine after stable expression of a gene encoding SNAP-tagged CENP-N. (d_f) Kinetics of loss from centromeres as CENP-A levels diminish de_nes three groups with similar kinetics of loss: proteins lost in proportion to CENP-A (d), those lost more slowly and whose depletion

requires complete loss of CENP-A (e), and CENP-B (f), whose binding is reduced by half by complete loss of CENP-A. Statistics source data are in Supplementary Table S2. Scale bar, 5 _m.

Figure 3 Short-term rescue of centromere maintenance and function following CENP-A gene depletion. (a) Schematic representation of the rescue constructs. Each CENP-A domain is in red; H3 in green. CENP-A domains and amino-acid positions are also indicated. Each construct is tagged with an N-terminal EYFP (enhanced yellow _uorescent protein). (b) Schematic outlining the construction of cell lines expressing each rescue construct and _nal clonogenic assay. (c) Immunoblots of cell extracts with antibodies against GFP, CENP-A and _-tubulin to determine the level of expression of the indicated rescue constructs. (d) Clonogenic survival experiment for the indicated cell lines with or without addition of Ad-Cre. (e) Quanti_cation of clonogenic assays for each rescue construct shown as the mean of the percentage of Ad-Cre-surviving colonies relative to the untreated condition. Each column represents the average of _ve independent experiments and error bars represent the s.e.m. Uncropped images of blots are shown in Supplementary Fig. S7.

Figure 4 H3CATD is suf_cient for centromere identity and to template its chromatin replication. (a) Representative schematic of experimental design in b. (b) Representative _uorescence images of the localization and intensity of endogenous CENP-A and EYFP_H3CATD at days 0, 9 and 12 after Ad-Cre addition. ACA staining was used to identify

centromeres. (c) Bar graphs indicate protein intensity at centromeres of CENP-C, CENP-T, CENP-I, CENP-N and EYFP_H3CATD (in b) at the indicated days after Ad-Cre treatment. Columns represent the mean of _ve independent experiments (>30 cells per experiment) and error bars represent the s.e.m. (d) HJURP and GAPDH levels in cells with or without siRNA treatment for GAPDH or HJURP, determined by immunoblotting. _-tubulin was used as a loading control. (e) Representative schematic of experimental design in f. (f) Representative _uorescence images from the experiment in e to determine the localization and intensity of CENP-A and SNAP-H3CATD after siRNA of GAPDH or HJURP. _-tubulin staining was used to identify telophase-early G1 cells. (g) Columns are the mean of the percentage of micronuclei formation in CENP-A□=F cells rescued with EYFP_H3CATD at the indicated days after Ad-Cre addition. Error bars represent the s.e.m. of three independent experiments (>100 cells per experiment). Statistics source data are in Supplementary Table S2. Uncropped images of blots are shown in Supplementary Fig. S7. Scale bars, 5 _m.

Figure 5 N- or C-terminal tails of CENP-A nucleate kinetochore assembly. (a) Schematic of the long-term viability assay used to isolate survival clones after endogenous CENP-A depletion. (b) Table of viability of various CENP-A_histone H3 chimaera for conferring short- (12_19 generations) and long-term (>100 generations) survival. (c) Immunoblot to quantify levels of each EYFP-tagged rescue variant or endogenous CENP-A, visualized with GFP or CENP-A antibodies. _-tubulin was used as a loading control. (d) Representative _uorescence images show the

localization and intensity of CENP-C or CENP-T in the indicated cell lines. ACA was used to mark centromeres. (e) Column graphs quantifying centromere intensities from experiments such as those in d to measure CENP-C, CENP-T, CENP-I, Ndc80 and Dsn1 protein intensity in the indicated cell lines. Columns represent the mean of three independent experiments (>30 cells per experiment). Error bars represent the s.e.m. Uncropped images of blots are shown in Supplementary Fig. S7. Scale bar, 5 _m.

Figure 6 The CENP-A N-terminal tail controls CENP-B levels at centromeres. (a) Column graphs represent the means of the percentage of CENP-B intensity in the indicated cell lines. Error bars represent the s.e.m. of three independent experiments (>30 cells per experiment. __P < 0:006). (b) Left, clonogenic survival assays after stable insertion to express a control shRNA (scramble) or against CENP-B. Right, immunoblot of cell extracts to measure total CENP-B levels. _-tubulin was used as a loading control. (c) Percentage of cells that undergo chromosome mis-segregation after GAPDH or CENP-B depletion by siRNA in the indicated cell line. Column graphs represent the means of three independent experiments and error bars represent the s.e.m. (d) Faithfulness of chromosome segregation after growth for 30 generations measured with FISH for centromere regions of chromosomes 2 and 7 (CEN2 and CEN7, respectively) in the presence (CENP-A□=F) or absence (CENP-A□=□) of endogenous CENP-A. Bars represent the mean of duplicate experiments in the indicated cell line (>100 cells per experiment). Bottom, diploid or aneuploid cells identi_ed by localization of CEN2 (red) and CEN7 (green) after growth of the

CENP-A□=□CNH2 H3CATD cells. Statistics source data are in Supplementary Table S2. Uncropped images of blots are shown in Supplementary Fig. S7a. Scale bar, 5 _m.

Figure 7 The _ssion yeast CATD is necessary and suf_cient for centromere identity, but requires addition of the CENP-ACnp1 N terminus to provide long-term centromere function. (a,b) Schematic of the CENP-Acnp1-histone H3 gene constructs (a) and the experimental approach used to test maintenance of centromere position and function in the _ssion yeast S. pombe (b). CENP-ACnp1 domains are red; H3 in green. ChIP, chromatin immunoprecipitation. (c) Representative live-cell imaging of GFP-tagged constructs as outlined in b. The position of the centromere of chromosome 2 was marked by binding of a tetR-tomato fusion protein to a tetO array inserted at the central core of cen2 (ref. 63). (d) Chromatin immunoprecipitation using GFP antibody of cells from b. Wild-type cells were transformed with GFP-tagged constructs as indicated. Enrichment at centromeric central cores (cc1/3 and cc2 products) was compared with input DNA relative to the control non-centromeric locus fbp1. The depiction of _ssion yeast chromosomes is not drawn to scale. (e) Schematic of plasmid-shuf_ing assay for testing CENP-ACnp1 rescue constructs in cnp11 cells. (f,g) Rescue experiment of cnp11 cells using the plasmid-shuf_ing assay outlined in e or of cnp1-76 cells. (h) Serial dilution of cnp11 cells containing NH2H3CATD or CENP-ACnp1 as the unique source of CENP-ACnp1. Growth was assayed in pombe minimal medium with glutamate (PMG) with or without the addition of a spindle poison drug thiabendazole (TBZ; 15 _g ml□1). Wild-type, clr41 (heterochromatin defective mutant) and cnp1-ts controls are also shown. In f_h, a tenfold dilution series is shown

for each strain.

Figure 8 Model for centromeric identity, maintenance and function through a two-step mechanism. In the _rst step, centromere identity is achieved by the CATD-containing chromatin through its loading mediated by HJURP at mitotic exit. Subsequently, new and old CENP-A can nucleate kinetochore assembly on CATD-containing chromatin through the action of the CENP-A N-terminal tail that stabilizes CENP-B or by the direct recruitment of CENP-C by the C-terminal tail of CENP-A.

Figure S1 *CENP-A conditional disruption in human cells.* (a) CENP-A genotypes validated in the indicated cell lines using PCR to distinguish normal (+), disrupted (-), and floxed (F) alleles, as diagramed to right. (b) To monitor growth rate, CENP-A+/+, CENP-AF/+ and CENP-A-/F RPE1 cells were counted every 2 days. Values represent the mean of three independent experiments, and error bars represent the SEM. (c) Immunoblot to determine CENP-A levels in CENP-A+/+, CENP-AF/+ and CENP-A-/F RPE1 cells. (d) Representative fluorescence images of centromeric CENP-A levels. Bar graphs show CENP-A protein intensity in the indicated cell lines. Bars represent the mean of three independent experiments (> 30 cells per experiment). Error bars represent the SEM. ACA staining was used to identify centromeres in CENP-AF/+ and CENPA-/F RPE1 cells. (e) Efficiency of excision of exons 3 and 4 in the overall cell population determined by Q-PCR after Ad-Cre transduction of CENP-A-/F RPE1 cells. Position of the primers is illustrated in the schematic at top. (f) PCR analysis on the DNA extracted from the survival colonies in the CENP-A-/F RPE1

cells after Ad-CRE treatment. Images show representative crystal violet–stained colonies for the indicated cell lines with or without treatment with Ad-CRE. (g) Schematic of introduction of the LoxPRFP-STOPLoxP-GFP gene by retroviral integration into CENP-A-/F cells, followed by FACS sorting to isolate only green fluorescing cells with both CENP-A alleles inactivated. (h) Graph shows the growth rate of CENP-AF/+ and CENP-A-/F with RFP-STOP-GFP construct of the experiment described in f. A parallel-untreated control is also shown. Cells were counted every 2 days. Values represent the mean of three independent experiments, and error bars represent the SEM. Scale bar = 5 mm.

Figure S2 Centromeric proteins dissociate from centromeres with different kinetics during CENP-A depletion. (a) Representative fluorescence images show the localization and intensity of CENP-P (purple bars). ACA staining was used to identify centromeres. Red bars show CENP-A intensity from Figure 1E. Bars represent the mean of three independent experiments (> 30 cells per experiment). Error bars represent the SEM. CENP-P was tracked by covalent labeling with rhodamine-benzyl guanine after stable expression of a gene encoding SNAP-tagged CENP-P (b) As in A, except that CENP-C and Ndc80 *at day 0 and 7* are shown *after Ad-Cre infection*. Cells were arrested in monostarol for 12 hours. Yellow bars show Ndc80 intensity of the fluorescence images at the indicated days after Ad-Cre treatment. (c) As in a, but for CENP-A and CENP-I levels at *day 0 and 9 after Ad-Cre infection. Interphase cells were quantified.* Statistics source data are in supplementary table S2. Scale bar = 5 mm.

Figure S3 Clonogenic and immunofluorescence localization assays

determine that the CATD is sufficient for centromere identity and for templating its replication. (a) CENP-A amino acid sequence and the domains, including the amino terminal tail (red), the aN helix (green), the CATD (light blue), and the carboxyl terminal tail (orange). (b) Representative fluorescence images of the localization of EYFP-rescue constructs of experiment outlined in Figure 3. (c) Clongenic survival assays (as in Figure 3d) for the CENP-A-/F cells with and without incubation in Ad-Cre and with the indicated rescue constructs. (d) Representative fluorescence images of the localization and intensity of CENP-T, CENP-A and EYFP-H3CATD at the indicated days after Ad-Cre treatment. Scale bar = 5 mm.

Figure S4 H3CATD combined with either the amino or carboxyl tails of human CENP-A confer long term centromere identify and kinetochore function. (a) (Top) Clonogenic survival assays for RPE1 cells with the indicated CENP-A genotypes and rescue constructs. (Bottom) Corresponding growth rates determined by counting cell numbers every 2 days for CENP-A-/F cells and CENP-A-/- cells with CENP-A, NH2H3CATD, or H3CATD-C rescue genes. Values represent the mean of three independent experiments, and error bars represent the SEM. (b) Confirmation of excision of endogenous CENP-A alleles in the indicated survival rescue construct cells. A schematic representation of the position of the primers is also shown. (c) (Top) Representative fluorescence images show the loss of endogenous CENP-A at centromeres only for cells lacking the amino-terminus of CENP-A epitopes recognized by our CENP-A antibody. ACA staining was used to identify centromeres. (Bottom) Quantitation of the fluorescence images in the

CENP-A-/- + H3CATD-C and CENP-A-/- + NH2CENP-A cells. (d) Representative fluorescence images showing localization of the NH2H3CATDSNAP in CENP-A-/- cells during different phases of the cell cycle. ACA staining was used to identify centromeres and a-tubulin to identify telophase/early G1 cells. (e) Indirect immuno-fluorescence images of localization and intensity of EYFP-H3CATD-C at centromeres after GAPDH or HJURP siRNA, respectively. ACA staining was used to identify centromeres. (f) Quantitation of EYFP intensity at centromeres in images from (e) after GAPDH, HJURP siRNA, or no siRNA. Error bars represent the SEM of two independent experiments (> 30 cells per experiment). ACA staining was used to identify centromeres. (g) (Left) CENP-C protein intensity after GAPDH or CENP-C siRNA in the indicated cell lines, respectively. Bars represent the mean of two independent experiments (> 30 cells per experiment). (Right) Percentage of cells that undergo division after 72 hours of CENP-C depletion, determined by live cell microscopy. Error bars represent the SEM. Statistics source data are in supplementary table S2. Scale bar = 5 mm.

Figure S5 Accurate chromosome segregation with centromere-targeted histone H3 carrying either CENP-A tail domain. (a) Representative fluorescence images of localization and intensity of centromere-bound CENP-I or Ndc80 in CENP-A-/- cell lines with the indicated rescue genes. ACA staining (red) is also shown. (b) Representative fluorescence images showing localization and intensity of centromere bound CENP-B in the indicated cell lines. (c) Representative fluorescence images showing localization and intensity of CENP-B in the indicated cell lines after 2

days of shRNA against CENP-B. (Right) Quantitation of CENP-B protein intensity of fluorescence images. (d) Time-lapse images of CENP-A-/-+ EYFPNH2H3CATD (green) RPE1 cells also stably expressing histone H2BmRFP (red). Numbers at the top right refer to the time in minutes after nuclear envelope breakdown (NEBD). Yellow arrows show the formation of micronuclei. (e) (Left) Quantitation of misaligned or lagging chromosomes or subsequent micronuclei formation or (right) mitotic timing, determined from images in (d). Time in mitosis was defined by the period from NEBD to chromosome decondensation. Bars represent the mean of > 50 cells per condition. Each individual point represents a single cell. Error bars represent the SEM. Statistics source data are in supplementary table S2. Scale bar = 5 mm.

Figure S6 Protein level expression of the fission yeast rescue constructs. Immuno-blot to quantify levels of each GFP-tagged rescue variant, visualized with a GFP antibody. b-actin is used as a loading control.

Figure S7 Uncropped image of blots from figure 1c, 3c, 4d, 5c, 6b.

Figure 1

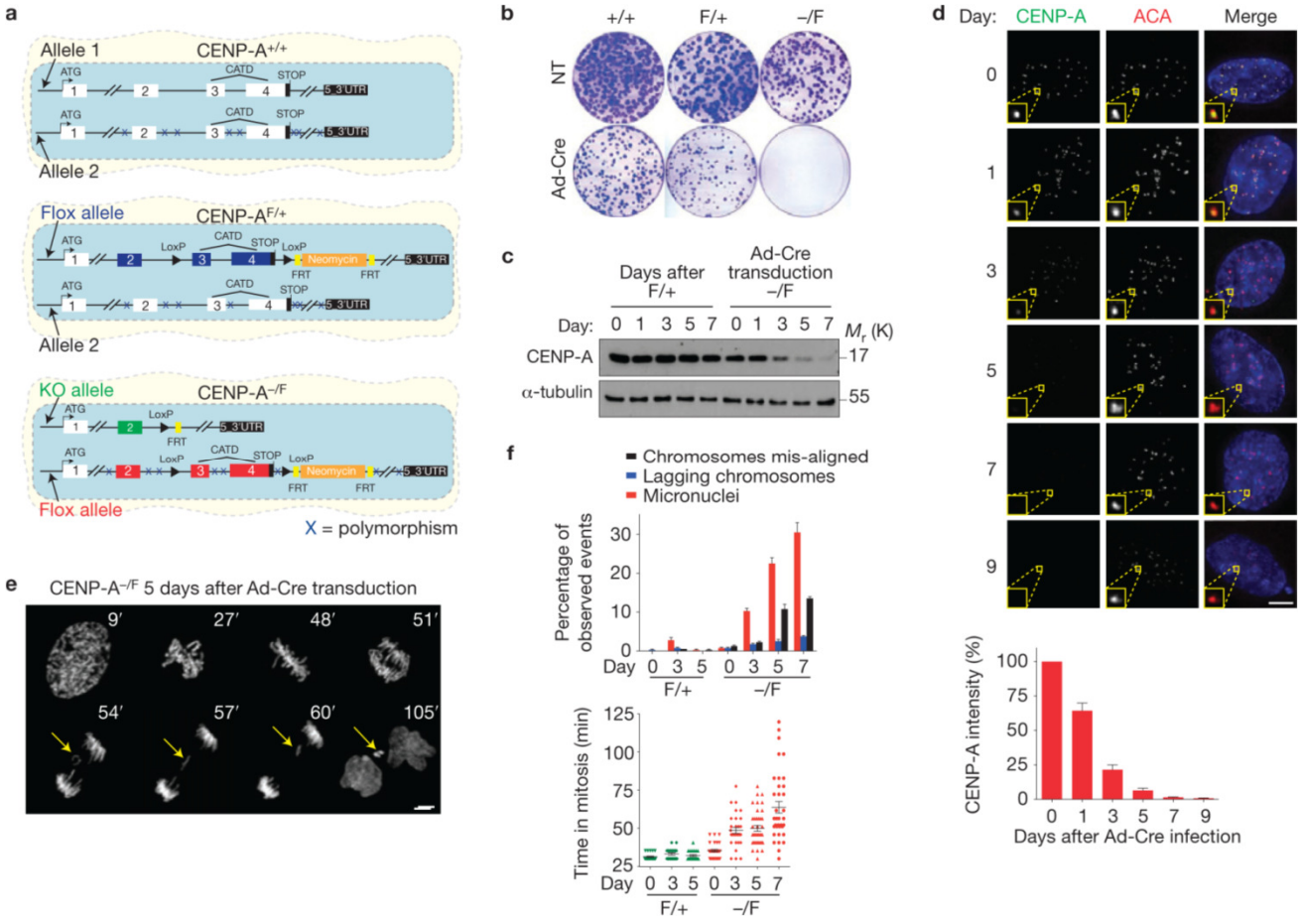

a
Allele 1
CENP-A+/+
ATG
CATD
STOP
5′ UTR
Allele 2
Flox allele
CENP-AF/+
LoxP
Neomycin
FRT
KO allele
CENP-A−/F
X = polymorphism
b
+/+
F/+
−/F
NT
Ad-Cre
c
Days after F/+
Ad-Cre transduction −/F
Day: 0 1 3 5 7 0 1 3 5 7
Mr (K)
CENP-A
17
α-tubulin
55
d
Day:
CENP-A
ACA
Merge
0
1
3
5
7
9
CENP-A intensity (%)
Days after Ad-Cre infection
e
CENP-A−/F 5 days after Ad-Cre transduction
9′
27′
48′
51′
54′
57′
60′
105′
f
Chromosomes mis-aligned
Lagging chromosomes
Micronuclei
Percentage of observed events
Day 0 3 5 0 3 5 7
F/+
−/F
Time in mitosis (min)

Figure 2

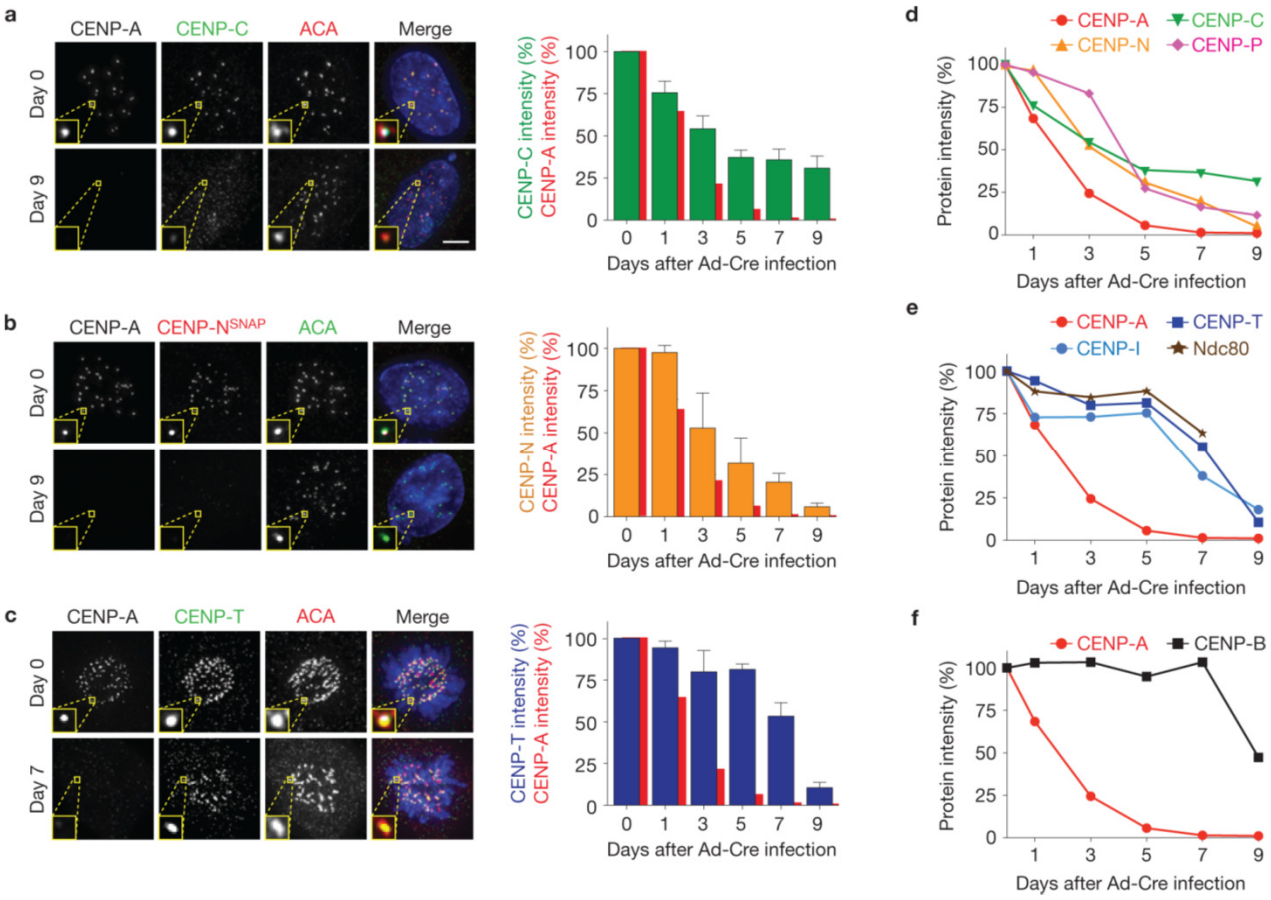

a
CENP-A
CENP-C
ACA
Merge
Day 0
Day 9
CENP-C intensity (%)
CENP-A intensity (%)
Days after Ad-Cre infection
b
CENP-A
CENP-N^SNAP
ACA
Merge
Day 0
Day 9
CENP-N intensity (%)
CENP-A intensity (%)
Days after Ad-Cre infection
c
CENP-A
CENP-T
ACA
Merge
Day 0
Day 7
CENP-T intensity (%)
CENP-A intensity (%)
Days after Ad-Cre infection
d
CENP-A
CENP-C
CENP-N
CENP-P
Protein intensity (%)
Days after Ad-Cre infection
e
CENP-A
CENP-T
CENP-I
Ndc80
Protein intensity (%)
Days after Ad-Cre infection
f
CENP-A
CENP-B
Protein intensity (%)
Days after Ad-Cre infection

Figure 3

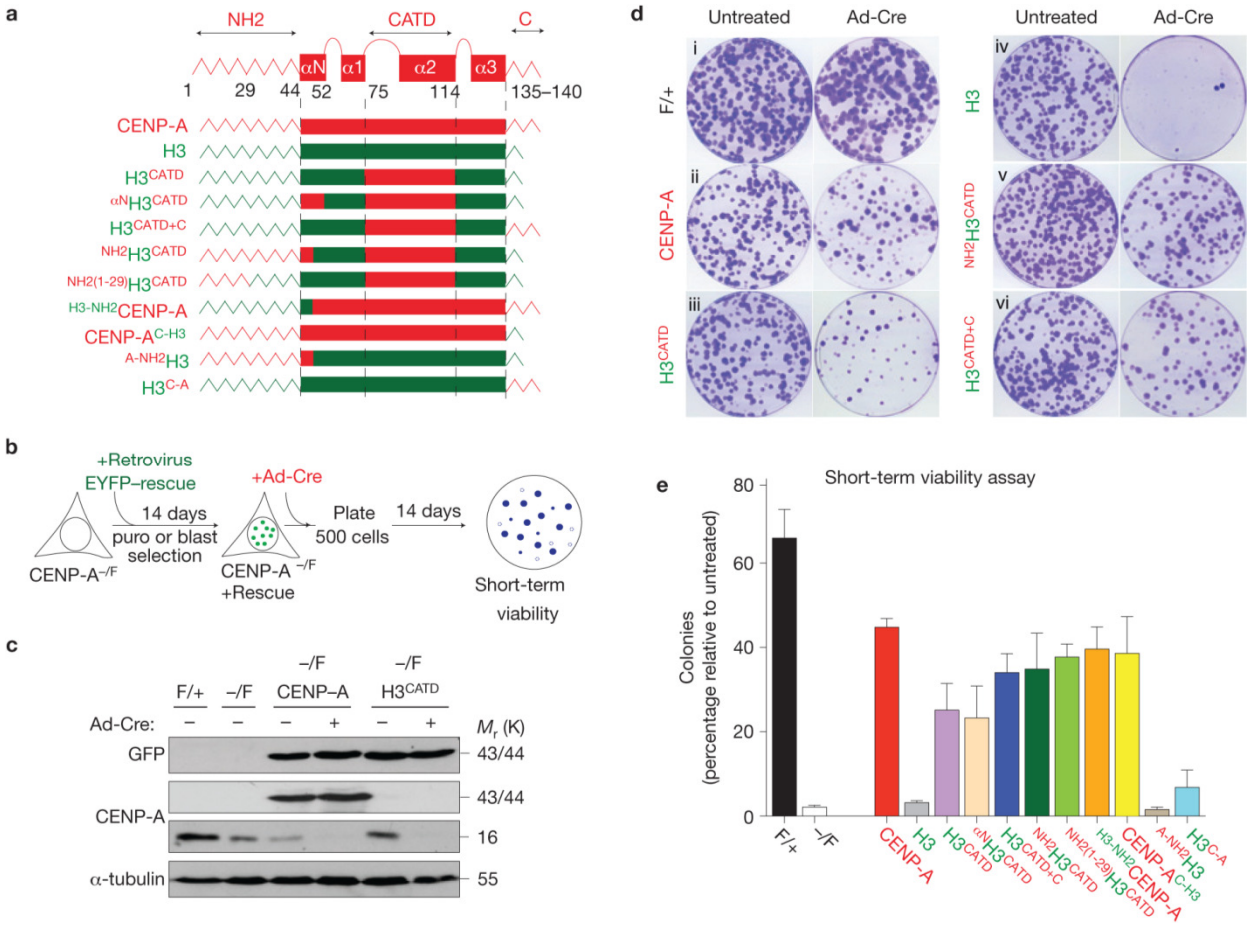

a
NH2
CATD
C
αN
α1
α2
α3
1
29
44
52
75
114
135–140
CENP-A
H3
H3CATD
αNH3CATD
H3CATD+C
NH2H3CATD
NH2(1-29)H3CATD
H3-NH2CENP-A
CENP-AC-H3
A-NH2H3
H3C-A
b
+Retrovirus
EYFP–rescue
14 days
puro or blast
selection
CENP-A–/F
+Ad-Cre
CENP-A –/F
+Rescue
Plate
500 cells
14 days
Short-term
viability
c
–/F
–/F
F/+
–/F
CENP–A
H3CATD
Ad-Cre:
–
–
–
+
–
+
Mr (K)
GFP
43/44
CENP-A
43/44
16
α-tubulin
55
d
Untreated
Ad-Cre
Untreated
Ad-Cre
i
ii
iii
iv
v
vi
F/+
CENP-A
H3CATD
H3
NH2H3CATD
H3CATD+C
e
Short-term viability assay
Colonies
(percentage relative to untreated)
80
60
40
20
0
F/+
–/F
CENP-A
H3
H3CATD
αNH3CATD
H3CATD+C
NH2H3CATD
NH2(1-29)H3CATD
H3-NH2CENP-A
CENP-AC-H3
A-NH2H3
H3C-A

Figure 4

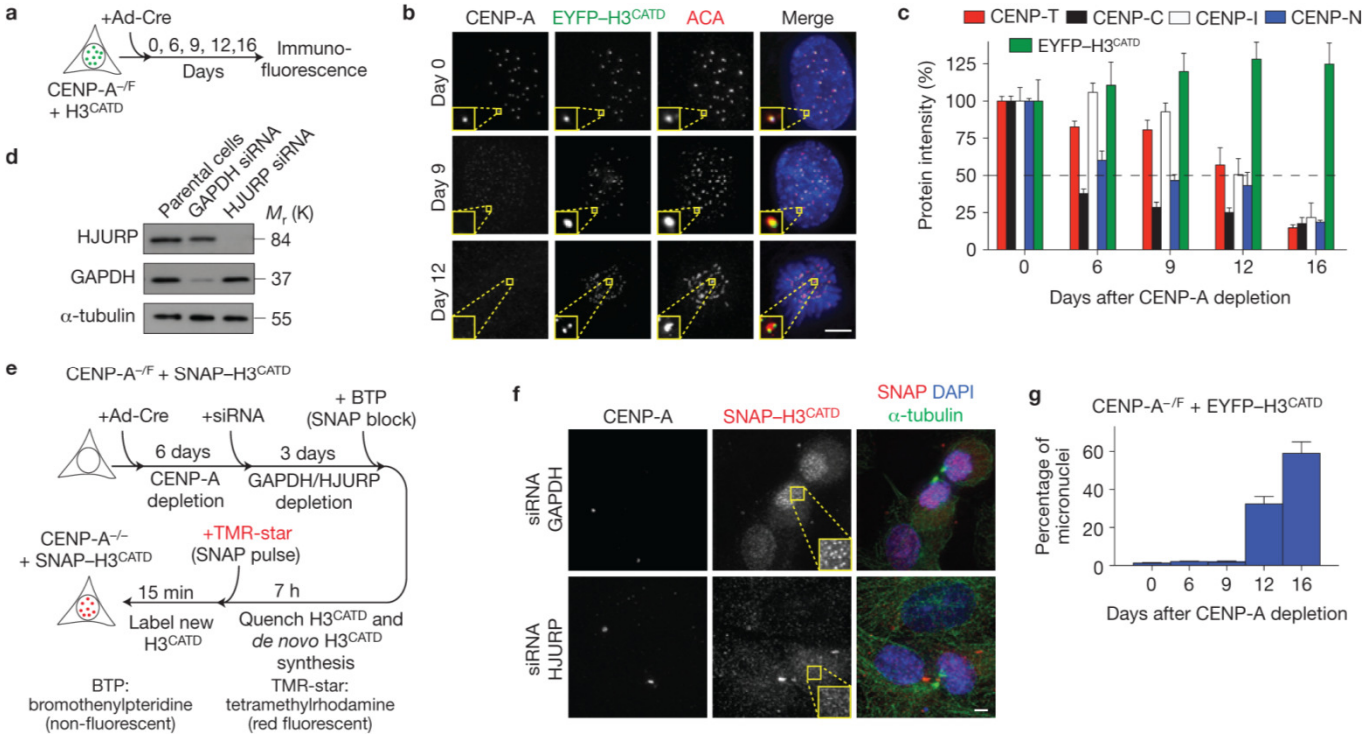

a
+Ad-Cre
0, 6, 9, 12,16
Days
Immuno-
fluorescence
CENP-A−/F
+ H3CATD
b
CENP-A
EYFP–H3CATD
ACA
Merge
Day 0
Day 9
Day 12
c
CENP-T
CENP-C
CENP-I
CENP-N
EYFP–H3CATD
Protein intensity (%)
125
100
75
50
25
0
0
6
9
12
16
Days after CENP-A depletion
d
Parental cells
GAPDH siRNA
HJURP siRNA
Mr (K)
HJURP
84
GAPDH
37
α-tubulin
55
e
CENP-A−/F + SNAP–H3CATD
+Ad-Cre
+siRNA
+ BTP
(SNAP block)
6 days
CENP-A
depletion
3 days
GAPDH/HJURP
depletion
CENP-A−/−
+ SNAP–H3CATD
+TMR-star
(SNAP pulse)
15 min
Label new
H3CATD
7 h
Quench H3CATD and
de novo H3CATD
synthesis
BTP:
bromothenylpteridine
(non-fluorescent)
TMR-star:
tetramethylrhodamine
(red fluorescent)
f
CENP-A
SNAP–H3CATD
SNAP DAPI
α-tubulin
siRNA
GAPDH
siRNA
HJURP
g
CENP-A−/F + EYFP–H3CATD
Percentage of
micronuclei
60
40
20
0
0
6
9
12
16
Days after CENP-A depletion

Figure 5

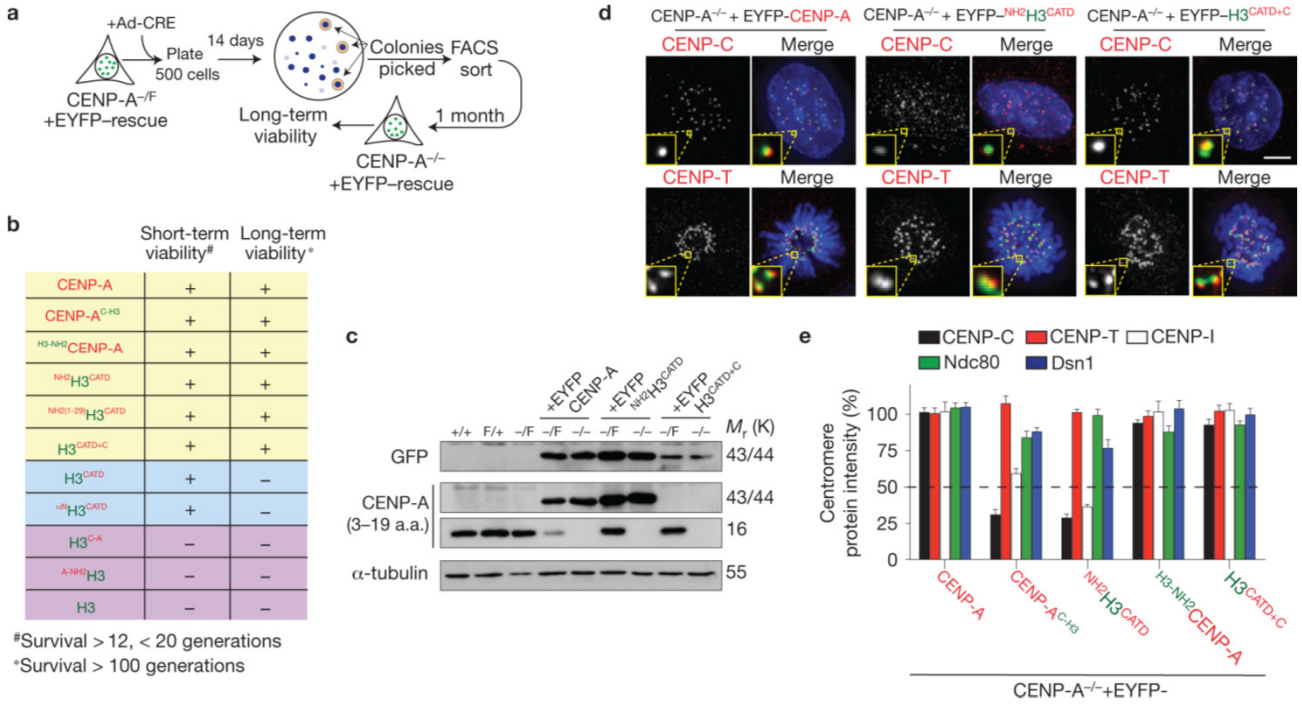

a
+Ad-CRE
Plate
500 cells
14 days
Colonies picked
FACS sort
1 month
Long-term viability
CENP-A−/F +EYFP–rescue
CENP-A−/− +EYFP–rescue
b
Short-term viability#
Long-term viability*
CENP-A
CENP-AC-H3
H3-NH2CENP-A
NH2H3CATD
NH2(1-29)H3CATD
H3CATD+C
H3CATD
αNH2H3CATD
H3C-A
A-NH2H3
H3
#Survival > 12, < 20 generations
*Survival > 100 generations
c
+EYFP CENP-A
+EYFP NH2H3CATD
+EYFP H3CATD+C
+/+ F/+ −/F −/F −/− −/F −/− −/F −/−
Mr (K)
GFP
43/44
CENP-A (3–19 a.a.)
43/44
16
α-tubulin
55
d
CENP-A−/− + EYFP-CENP-A
CENP-A−/− + EYFP–NH2H3CATD
CENP-A−/− + EYFP–H3CATD+C
CENP-C
Merge
CENP-T
e
CENP-C
CENP-T
CENP-I
Ndc80
Dsn1
Centromere protein intensity (%)
100
75
50
25
0
CENP-A
CENP-AC-H3
NH2H3CATD
H3-NH2CENP-A
H3CATD+C
CENP-A−/−+EYFP-

Figure 6

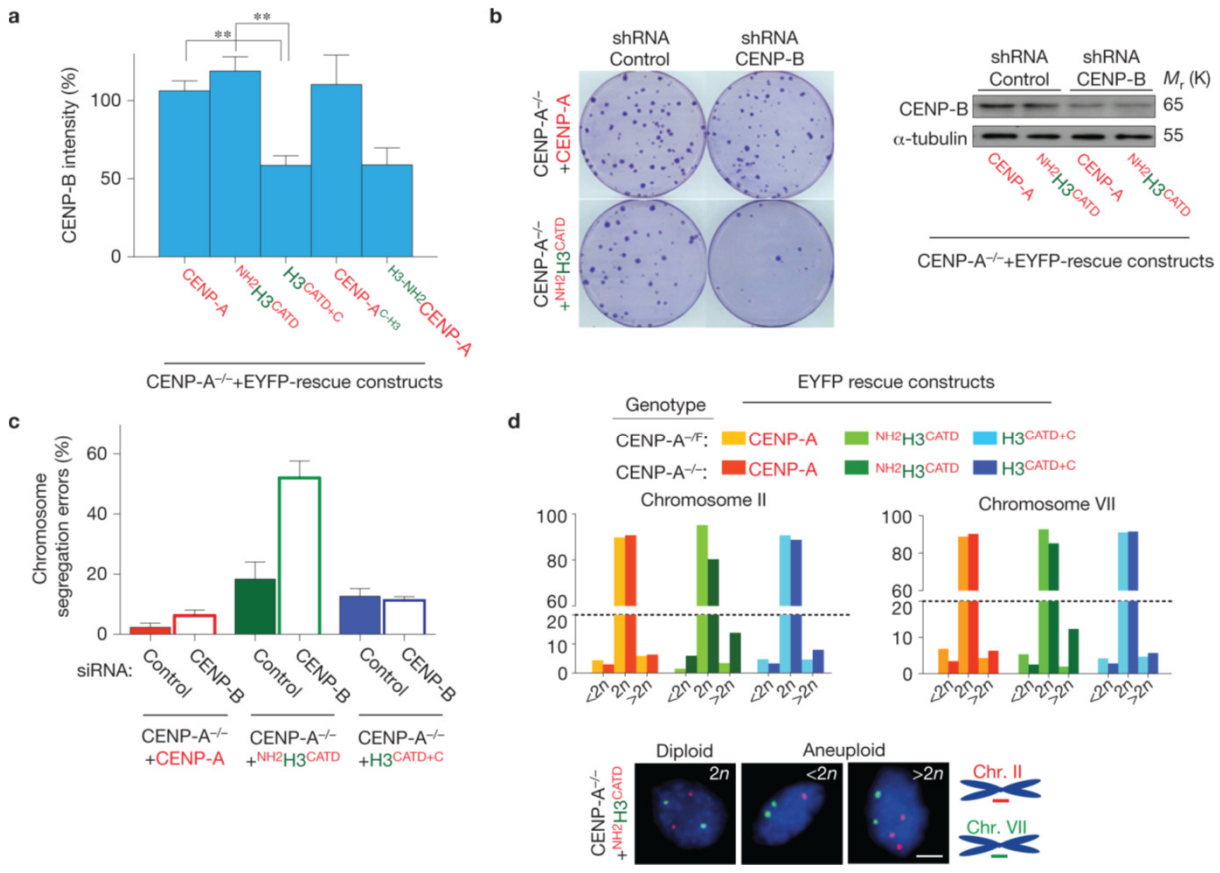

a
CENP-B intensity (%)
100
50
0
**
**
CENP-A
NH2H3CATD
H3CATD+C
CENP-AC-H3
H3-NH2CENP-A
CENP-A−/−+EYFP-rescue constructs
b
shRNA Control
shRNA CENP-B
CENP-A−/− +CENP-A
CENP-A−/− +NH2H3CATD
shRNA Control
shRNA CENP-B
Mr (K)
CENP-B
65
α-tubulin
55
CENP-A
NH2H3CATD
CENP-A
NH2H3CATD
CENP-A−/−+EYFP-rescue constructs
c
Chromosome segregation errors (%)
60
40
20
0
siRNA:
Control
CENP-B
Control
CENP-B
Control
CENP-B
CENP-A−/− +CENP-A
CENP-A−/− +NH2H3CATD
CENP-A−/− +H3CATD+C
d
EYFP rescue constructs
Genotype
CENP-A−/F:
CENP-A
NH2H3CATD
H3CATD+C
CENP-A−/−:
CENP-A
NH2H3CATD
H3CATD+C
Chromosome II
Chromosome VII
100
80
60
20
10
0
<2n
2n
>2n
Diploid
Aneuploid
CENP-A−/− +NH2H3CATD
2n
<2n
>2n
Chr. II
Chr. VII

Figure 7

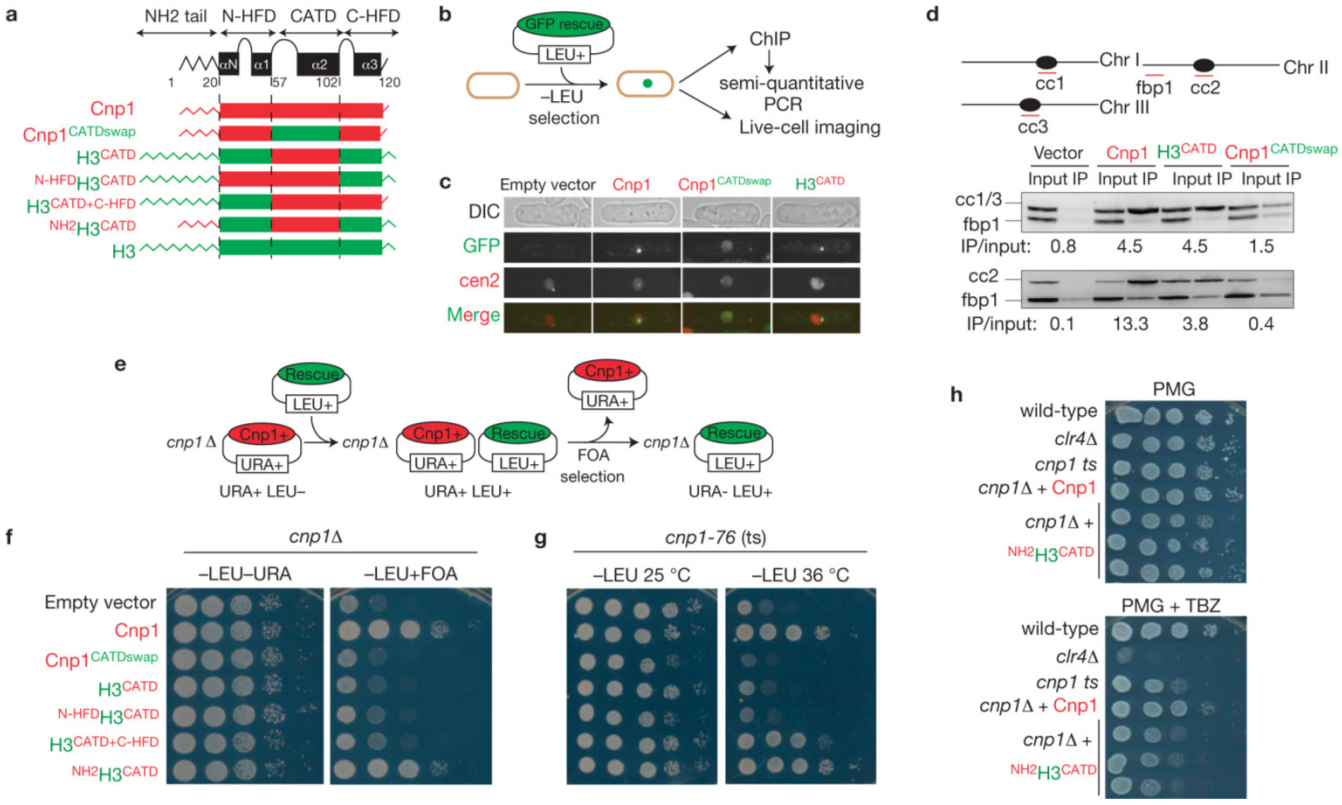

a
NH2 tail
N-HFD
CATD
C-HFD
αN
α1
α2
α3
1
20
57
102
120
Cnp1
Cnp1CATDswap
H3CATD
N-HFDH3CATD
H3CATD+C-HFD
NH2H3CATD
H3
b
GFP rescue
LEU+
–LEU selection
ChIP
semi-quantitative PCR
Live-cell imaging
c
Empty vector
Cnp1
Cnp1CATDswap
H3CATD
DIC
GFP
cen2
Merge
d
Chr I
Chr II
Chr III
cc1
cc2
cc3
fbp1
Vector
Cnp1
H3CATD
Cnp1CATDswap
Input IP
cc1/3
fbp1
IP/input: 0.8 4.5 4.5 1.5
cc2
fbp1
IP/input: 0.1 13.3 3.8 0.4
e
Rescue
LEU+
cnp1Δ
Cnp1+
URA+
URA+ LEU–
URA+ LEU+
FOA selection
URA- LEU+
f
cnp1Δ
–LEU–URA
–LEU+FOA
Empty vector
Cnp1
Cnp1CATDswap
H3CATD
N-HFDH3CATD
H3CATD+C-HFD
NH2H3CATD
g
cnp1-76 (ts)
–LEU 25 °C
–LEU 36 °C
h
PMG
wild-type
clr4Δ
cnp1 ts
cnp1Δ + Cnp1
cnp1Δ + NH2H3CATD
PMG + TBZ

Figure 8

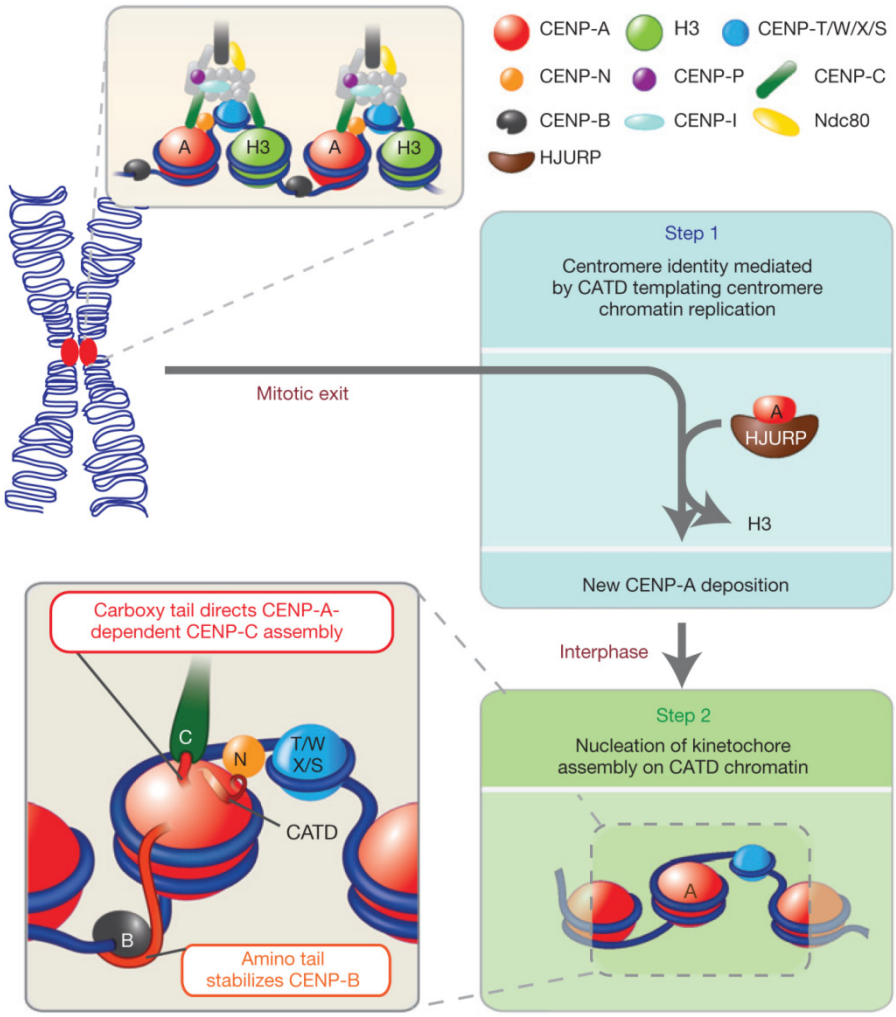

CENP-A
H3
CENP-T/W/X/S
CENP-N
CENP-P
CENP-C
CENP-B
CENP-I
Ndc80
HJURP
A
H3
A
H3
Step 1
Centromere identity mediated by CATD templating centromere chromatin replication
Mitotic exit
A
HJURP
H3
New CENP-A deposition
Interphase
Step 2
Nucleation of kinetochore assembly on CATD chromatin
A
Carboxy tail directs CENP-A-dependent CENP-C assembly
C
N
T/W
X/S
CATD
B
Amino tail stabilizes CENP-B

Figure S1

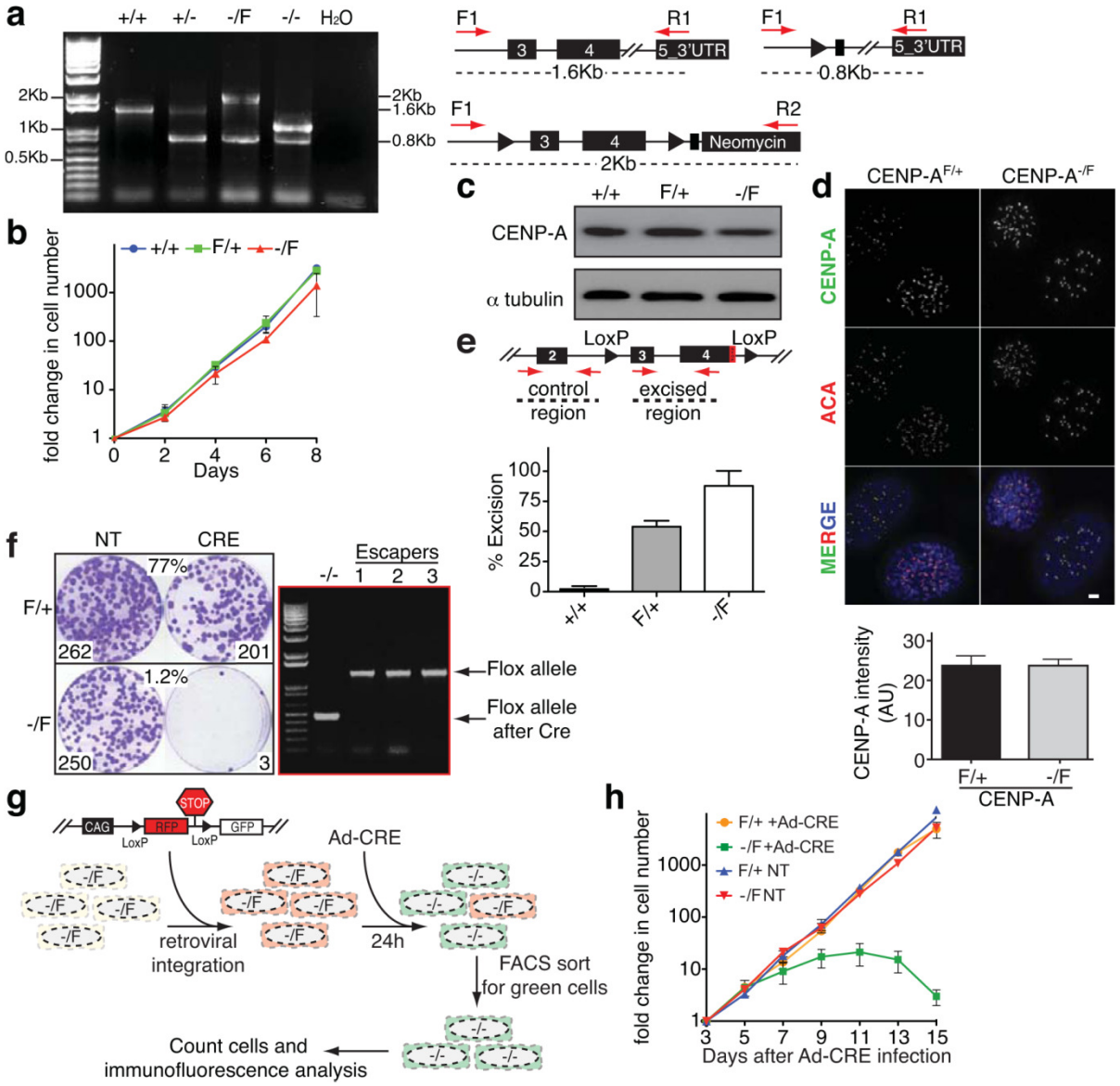

a
+/+ +/- -/F -/- H2O
2Kb
1Kb
0.5Kb
2Kb
1.6Kb
0.8Kb
F1
R1
3
4
5_3'UTR
1.6Kb
F1
R1
5_3'UTR
0.8Kb
F1
R2
3
4
Neomycin
2Kb
b
+/+ F/+ -/F
fold change in cell number
1000
100
10
1
0 2 4 6 8
Days
c
+/+ F/+ -/F
CENP-A
α tubulin
d
CENP-A F/+
CENP-A -/F
CENP-A
ACA
MERGE
e
LoxP
LoxP
2
3
4
control region
excised region
% Excision
100
75
50
25
0
+/+ F/+ -/F
f
NT
CRE
77%
F/+
262
201
1.2%
-/F
250
3
Escapers
-/- 1 2 3
Flox allele
Flox allele after Cre
CENP-A intensity (AU)
30
20
10
0
F/+ -/F
CENP-A
g
STOP
CAG
RFP
GFP
LoxP
LoxP
Ad-CRE
retroviral integration
24h
FACS sort for green cells
Count cells and immunofluorescence analysis
h
F/+ +Ad-CRE
-/F +Ad-CRE
F/+ NT
-/F NT
fold change in cell number
1000
100
10
1
3 5 7 9 11 13 15
Days after Ad-CRE infection

Figure S2

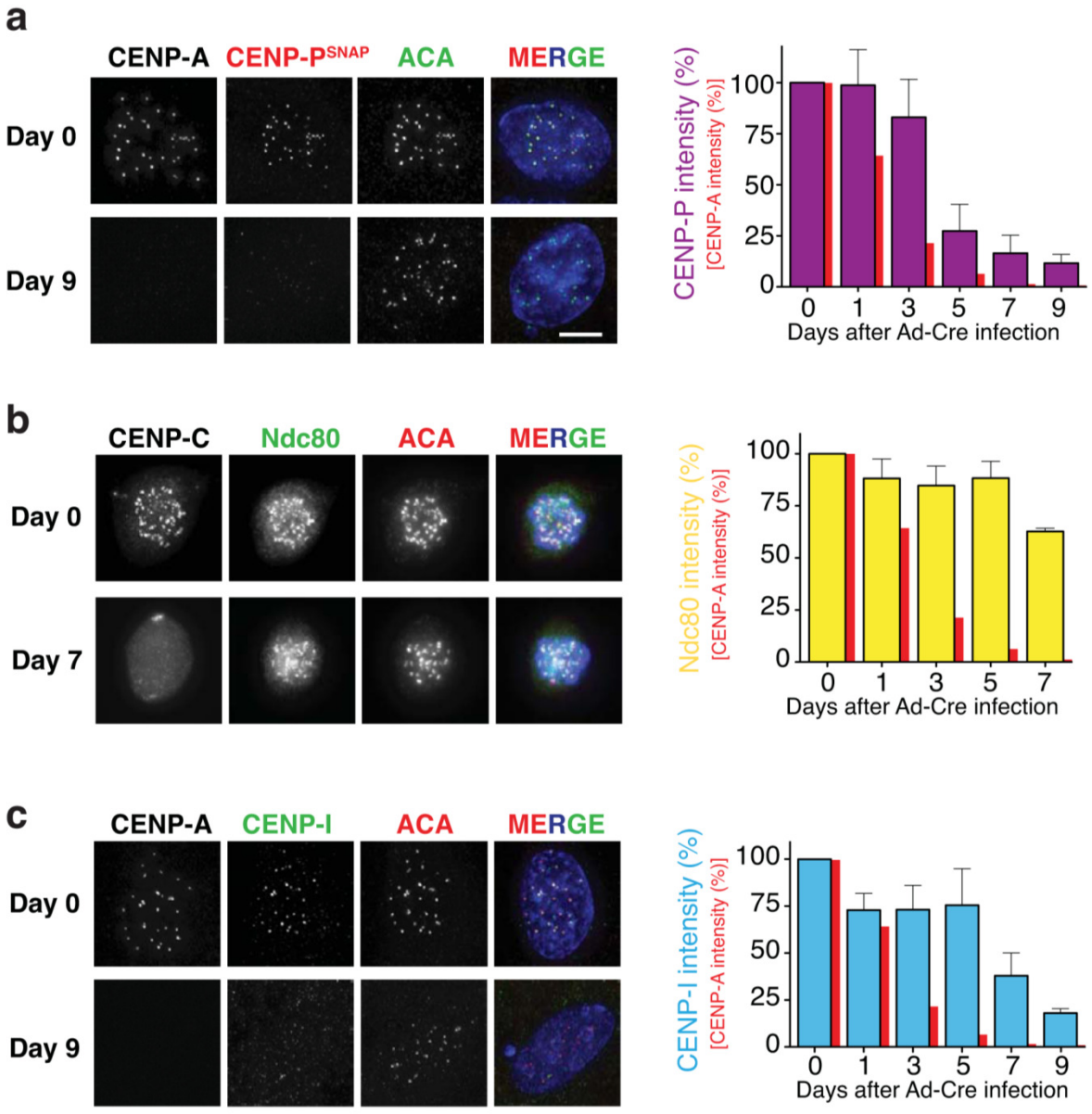
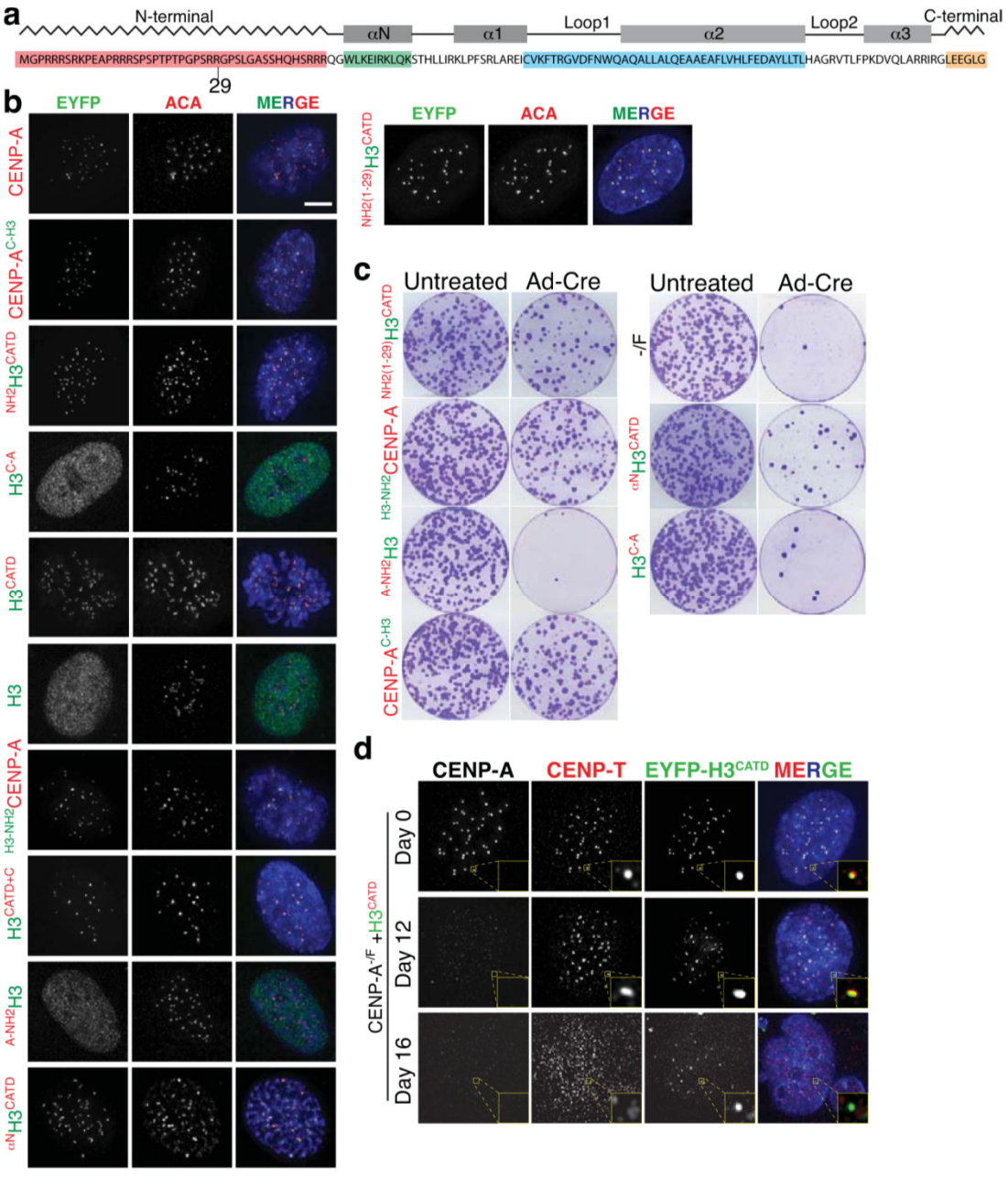

a
CENP-A
CENP-P^SNAP
ACA
MERGE
Day 0
Day 9
CENP-P intensity (%)
[CENP-A intensity (%)]
100
75
50
25
0
0 1 3 5 7 9
Days after Ad-Cre infection
b
CENP-C
Ndc80
ACA
MERGE
Day 0
Day 7
Ndc80 intensity (%)
[CENP-A intensity (%)]
100
75
50
25
0
0 1 3 5 7
Days after Ad-Cre infection
c
CENP-A
CENP-I
ACA
MERGE
Day 0
Day 9
CENP-I intensity (%)
[CENP-A intensity (%)]
100
75
50
25
0
0 1 3 5 7 9
Days after Ad-Cre infection

Figure S3


a
N-terminal
αN
α1
Loop1
α2
Loop2
α3
C-terminal
MGPRRRSRKPEAPRRRSPSPTPTPGPSRRGPSLGASSHQHSRRRQGWLKEIRKLQKSTHLLIRKLPFSRLAREICVKFTRGVDFNWQAQALLALQEAAEAFLVHLFEDAYLLTLHAGRVTLFPKDVQLARRIRGLEEGLG
29
b
EYFP
ACA
MERGE
CENP-A
CENP-A C-H3
NH2H3CATD
H3C-A
H3CATD
H3
H3-NH2CENP-A
H3CATD+C
A-NH2H3
αNH3CATD
NH2(1-29)H3CATD
c
Untreated
Ad-Cre
NH2(1-29)H3CATD
H3-NH2CENP-A
A-NH2H3
CENP-AC-H3
-/F
αNH3CATD
H3C-A
d
CENP-A
CENP-T
EYFP-H3CATD
MERGE
CENP-A-/F +H3CATD
Day 0
Day 12
Day 16

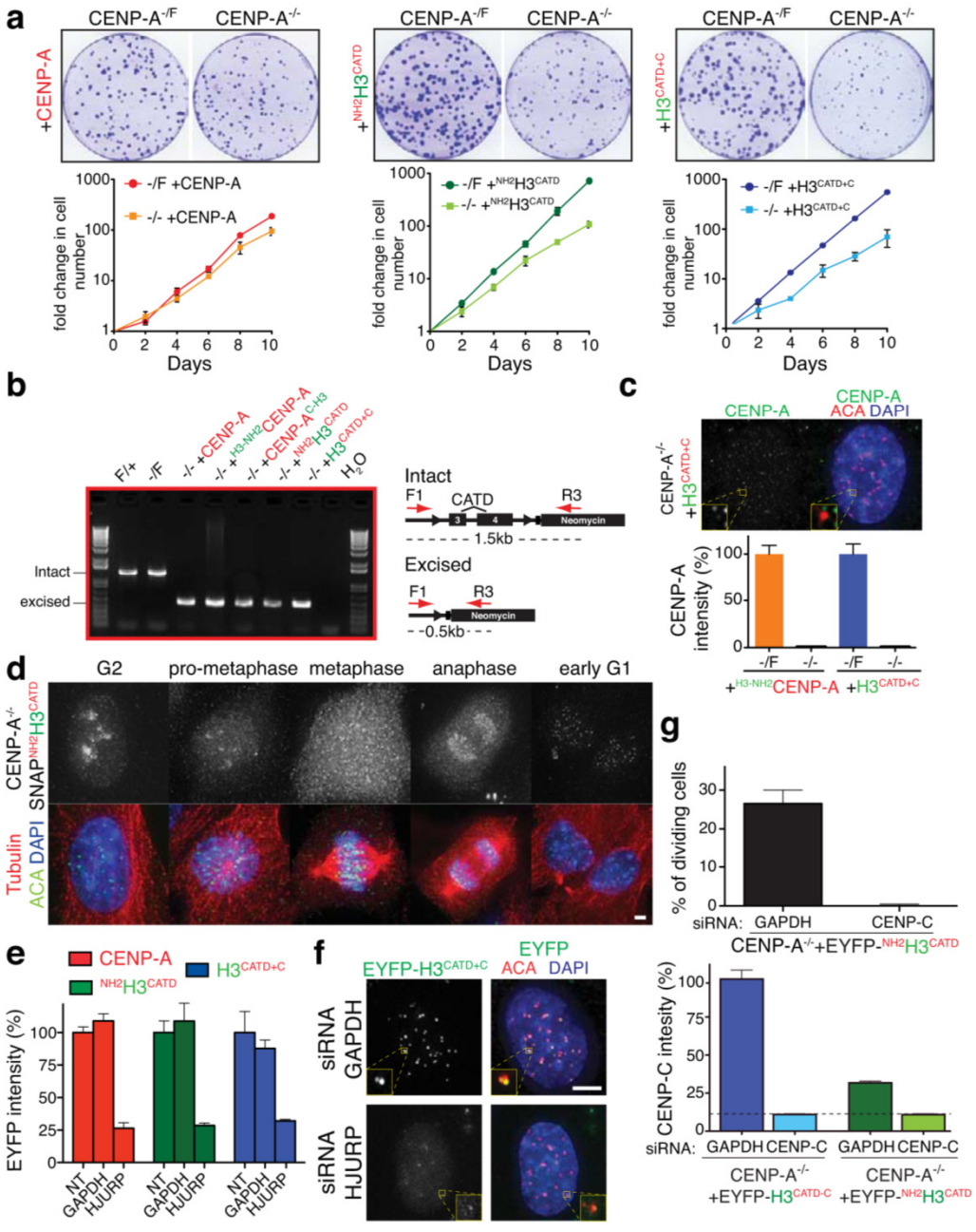

a
CENP-A-/F
CENP-A-/-
+CENP-A
+NH2H3CATD
+H3CATD+C
-/F +CENP-A
-/- +CENP-A
-/F +NH2H3CATD
-/- +NH2H3CATD
-/F +H3CATD+C
-/- +H3CATD+C
fold change in cell number
Days
b
F/+
-/F
-/- +CENP-A
-/- +H3-NH2CENP-A
-/- +CENP-AC-H3
-/- +NH2H3CATD
-/- +H3CATD+C
H2O
Intact
excised
Intact
F1
CATD
R3
Neomycin
1.5kb
Excised
0.5kb
c
CENP-A
CENP-A ACA DAPI
CENP-A-/- +H3CATD+C
CENP-A intensity (%)
+H3-NH2CENP-A
+H3CATD+C
d
G2
pro-metaphase
metaphase
anaphase
early G1
CENP-A-/- SNAPNH2H3CATD
Tubulin ACA DAPI
e
CENP-A
H3CATD+C
NH2H3CATD
EYFP intensity (%)
NT
GAPDH
HJURP
f
EYFP-H3CATD+C
EYFP ACA DAPI
siRNA GAPDH
siRNA HJURP
g
% of dividing cells
siRNA: GAPDH
CENP-C
CENP-A-/-+EYFP-NH2H3CATD
CENP-C intesity (%)
CENP-A-/- +EYFP-H3CATD-C
CENP-A-/- +EYFP-NH2H3CATD

Figure S5

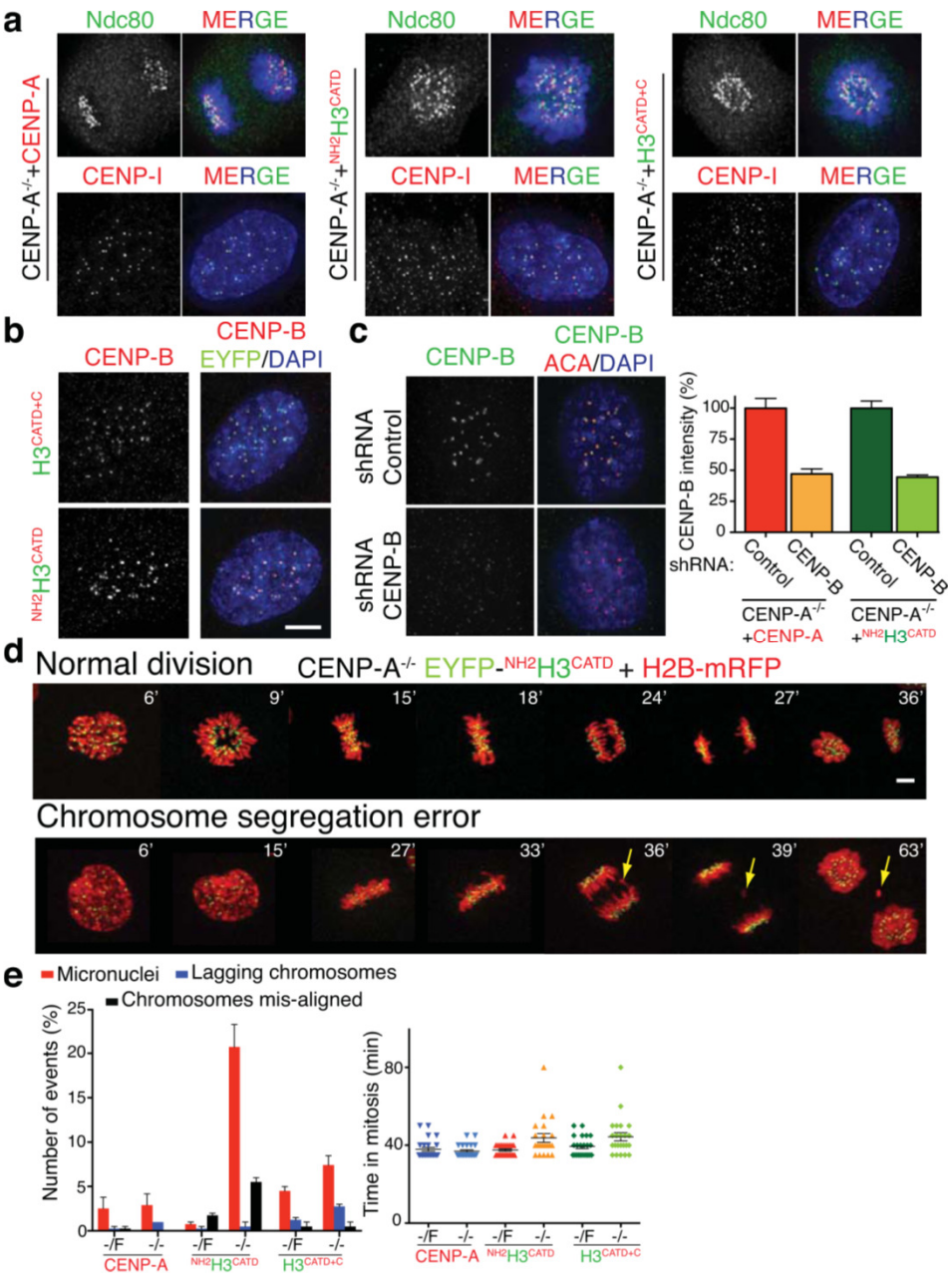

a
Ndc80
MERGE
CENP-I
MERGE
CENP-A-/-+CENP-A
CENP-A-/-+NH2H3CATD
CENP-A-/-+H3CATD+C
b
CENP-B
CENP-B EYFP/DAPI
H3CATD+C
NH2H3CATD
c
CENP-B
CENP-B ACA/DAPI
shRNA Control
shRNA CENP-B
CENP-B intensity (%)
100
75
50
25
0
shRNA:
Control
CENP-B
CENP-A-/- +CENP-A
CENP-A-/- +NH2H3CATD
d
Normal division
CENP-A-/- EYFP-NH2H3CATD + H2B-mRFP
6'
9'
15'
18'
24'
27'
36'
Chromosome segregation error
6'
15'
27'
33'
36'
39'
63'
e
Micronuclei
Lagging chromosomes
Chromosomes mis-aligned
Number of events (%)
25
20
15
10
5
0
-/F
-/-
CENP-A
NH2H3CATD
H3CATD+C
Time in mitosis (min)
80
40
0

Figure S6

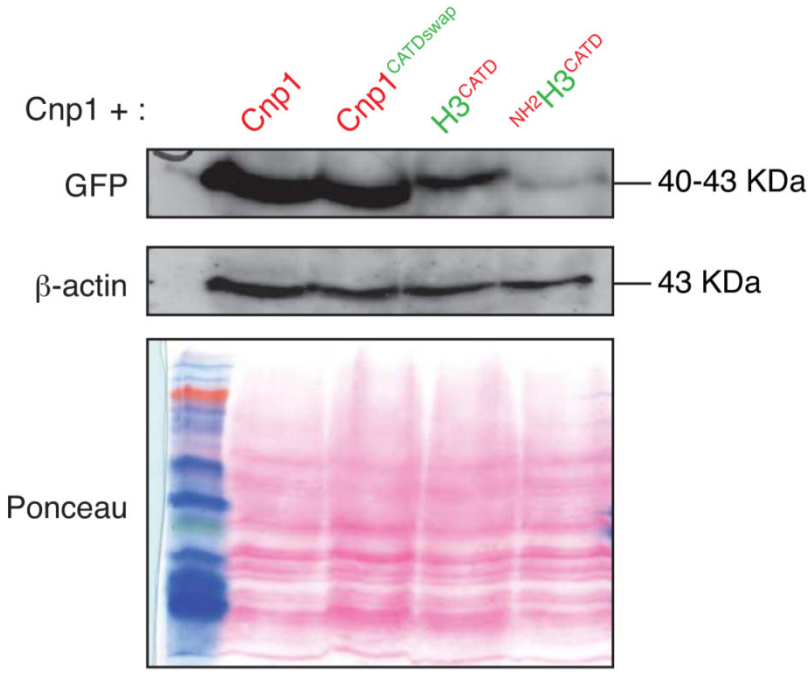

Cnp1 + :
Cnp1
Cnp1CATDswap
H3CATD
NH2H3CATD
GFP
40-43 KDa
β-actin
43 KDa
Ponceau

Figure S7

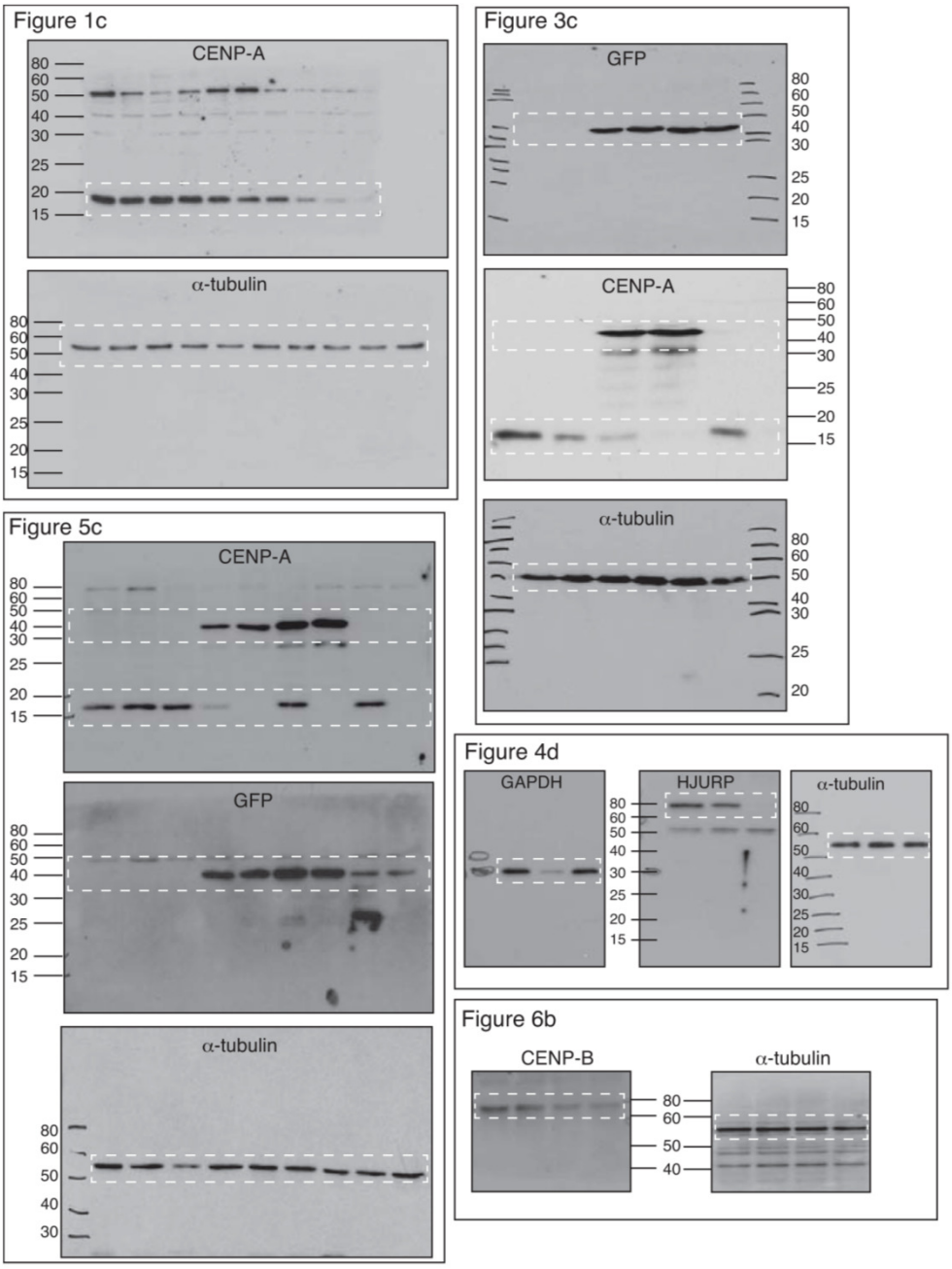


Figure 1c
CENP-A
80
60
50
40
30
25
20
15
α-tubulin
80
60
50
40
30
25
20
15
Figure 3c
GFP
80
60
50
40
30
25
20
15
CENP-A
80
60
50
40
30
25
20
15
α-tubulin
80
60
50
40
30
25
20
Figure 5c
CENP-A
80
60
50
40
30
25
20
15
GFP
80
60
50
40
30
25
20
15
α-tubulin
80
60
50
40
30
Figure 4d
GAPDH
HJURP
α-tubulin
80
60
50
40
30
25
20
15
Figure 6b
CENP-B
α-tubulin
80
60
50
40