Swapping CENP-A at the centromere

Bradley T. French and Aaron F. Straight

Faithful genome segregation depends on the functions of the eukaryotic centromere, which is characterized by the histone variant CENP-A. Gene replacement in human cells and fission yeast has now been used to show how CENP-A biochemically encodes centromere identity, as well as reveal an unexpected role for CENP-B in centromere function.

Centromeres provide the assembly platform for the mitotic kinetochore, the primary interaction site between the chromosome and the mitotic spindle. Centromeres and kinetochores bind spindle microtubules, monitor proper attachment of the chromosomes to the spindle and generate forces for chromosome movement and segregation. Defects in centromere and kinetochore function lead to chromosome instability and disease-associated aneuploidy. Thus, understanding how centromeres are established and maintained has been a major area of research for the past several decades.

Chromatin at eukaryotic centromeres is distinct from bulk chromatin in that it contains nucleosomes in which the histone H3 has been replaced with a centromere-specific histone, CENP-A. This protein is required for centromere function and is sufficient for generating functional kinetochores when targeted to ectopic sites in several different systems[1]. The underlying DNA sequence of centromeres, on the other hand, is poorly conserved and is not required for centromere identity (other than in budding yeast)[1]. Although human centromeres typically occur on repetitive alpha satellite sequences, several instances of 'neocentromeres' assembled on non-alpha satellite sequences have been described[2]. Thus, the prevailing model is that centromeres are epigenetically defined by CENP-A. However, directly testing its sufficiency as an epigenetic mark has been challenging because CENP-A deletion is lethal[3] and siRNA depletion of CENP-A has not been penetrant enough to convincingly test the epigenetic marking model[4]. In this issue, Fachinetti *et al.*[5] have applied a definitive approach in human somatic cells and fission yeast to test whether CENP-A epigenetically marks the centromere and to understand which domains of the CENP-A histone are responsible for assembly and maintenance of functional centromeres.

In several metazoan species, CENP-A histones are retained in chromatin through meiosis so that the site of the centromere is already established at fertilization. To understand the determinants of new centromere formation, several methods have been used by various research groups to generate artificial centromeres in cells and animals. Experiments to engineer human artificial chromosomes (HACs) with synthetic alpha satellite DNA sequences have demonstrated that alphoid DNA and CENP-B binding to it both promote new centromere formation[6]. These artificial centromeres incorporate CENP-A and form functional centromeres. An alternative approach involves fusing CENP-A and other centromere proteins to bacterial repressors such as the *lac*– and *tet*– repressors, and then using *lac* and *tet* operator sequences integrated into the chromosome to tether those fusions to non-centromeric chromosomal loci. Although these types of forced localization studies have yielded substantial insights into centromere formation *in vivo*, they do not report on native centromeres. An alternative approach involves reconstituting chromatin from purified components and assessing its ability to recapitulate centromere function in *Xenopus laevis* egg extracts[7]. The advantage of this approach is the ability to independently manipulate the composition of the histones, DNA and egg extract to assess the contribution of each to centromere and kinetochore assembly. However, this approach also just reports on an artificial centromere that does not capture the full complexity of a natural endogenous centromere.

Facchinetti *et al.*[5] complement this toolkit with a powerful cell biological approach that allows a detailed biochemical analysis of CENP-A at endogenous centromeres. Using an approach similar to classical yeast genetics, the authors use somatic cell genetics in human cells to replace the endogenous CENP-A with H3–CENP-A chimaeras for complementation testing. They were able to assess which domains of CENP-A are required to maintain centromere identity and function in long-term culture experiments. To accomplish this, they generated a cell line in which one CENP-A allele had been deleted and the other flanked by LoxP sites, creating a system in which endogenous CENP-A can be inducibly inactivated by the addition of Cre recombinase. They stably expressed CENP-A chimaeras using this system, and allowed the chimaeras to accumulate before depleting the endogenous protein. They then assessed the function of the chimaeras after 2–6 weeks of culture after Cre addition, well after endogenous CENP-A becomes undetectable (which usually takes 7–9 days). Traditional RNA-interference complementation experiments often suffer from partial depletion phenotypes that lead to ambiguous results, and this has certainly been true in studying centromeres where less than 10% of endogenous CENP-A can support cell viability[8]. The approach by Fachinetti *et al.*[5] bypasses these limitations by completely removing the endogenous protein and assessing mutant complementation without interference from the wild-type protein,

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thereby providing considerable insight into centromere assembly.

Distinct regions of the CENP-A histone confer its centromere-specific function (Fig. 1). The CATD (CENP-A targeting domain) is part of the histone fold, and when transplanted into the analogous region of histone H3 ($H3^{CATD}$) it is sufficient to direct the $H3^{CATD}$ chimaera to centromeres[9]. Direct recognition of the CATD by the CENP-A-specific chaperone HJURP in part determines the centromeric assembly of CENP-A (ref. 10). Furthermore, another centromere protein, CENP-N, directly recognizes the CATD of nucleosomal CENP-A. Although recognition by CENP-N is important for centromere assembly[11], it is not sufficient for kinetochore formation *in vitro*[7]. In gene replacement experiments that swap the endogenous CENP-A for $H3^{CATD}$, the CATD is found to be required for survival in clonogenic survival assays. However, centromeres containing only $H3^{CATD}$ cannot maintain recruitment of CENP-C, -I, -N or -T, resulting in loss of kinetochore function, an increase in aberrant mitoses and decreased long-term survival after depletion of endogenous CENP-A. Despite this, $H3^{CATD}$ continues to be loaded at centromeres in an HJURP-dependent manner. Together these observations indicate that the CATD is sufficient to specify centromere identity but not centromere function.

A second domain of CENP-A important for its centromere-specific function is its hydrophobic C-terminus (CAC). This region mediates the specific association of CENP-C with CENP-A nucleosomes[12,13] which is sufficient for kinetochore formation in frog egg extracts[7]. Consistent with this, Fachinetti *et al.* show that $H3^{CATD+CAC}$ chimaeras properly recruit centromere and kinetochore proteins, and are loaded at centromeres by HJURP *in vivo*, promoting long-term survival. Surprisingly, they also show that chimaeras lacking the CAC but instead containing the CATD and the CENP-A N-terminus ($H3^{NH2+CATD}$) are sufficient to promote long-term survival. Although these chimaeras recruit a low level of CENP-C, they assemble functional kinetochores. Depletion of CENP-C by small interfering RNA (siRNA) further diminishes centromeric CENP-C and impairs mitosis, indicating that CAC-independent CENP-C recruitment is sufficient for kinetochore assembly.

How is CENP-C recruited independently of the CAC, and how does the CENP-A N-terminus support kinetochore function?

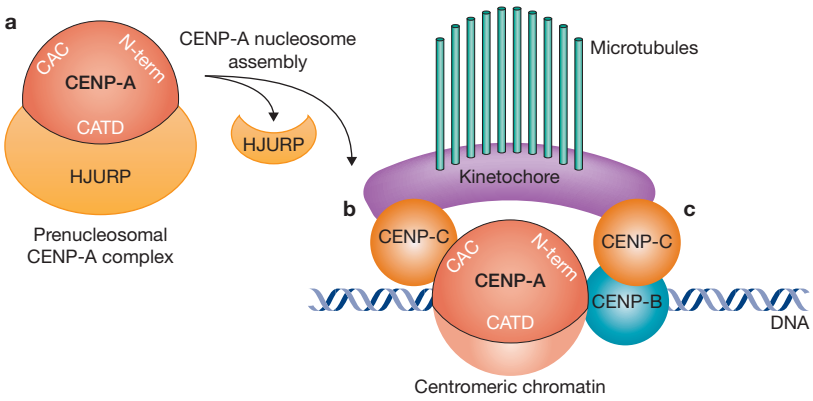

Figure 1 Three functional domains comprise the centromere-specific histone, CENP-A (red). (**a**) Part of the histone fold domain, the CATD (CENP-A targeting domain), is directly bound by the CENP-A chaperone HJURP, and specifies the centromeric localization of CENP-A during CENP-A loading. (**b**) Once incorporated into DNA at centromeric chromatin, the CENP-A C-terminus (CAC) directs kinetochore assembly by binding CENP-C, which makes direct contact with the kinetochore complex. (**c**) A low level of CENP-C is also recruited by the CENP-A N-terminus, possibly through CENP-B, providing an alternative, redundant pathway for kinetochore formation.

The authors find that chimaeras lacking the N-terminus recruit less of the DNA-binding protein CENP-B. Further, $H3^{NH2+CATD}$ chimaeras lacking the CAC (and therefore CAC-dependent kinetochore assembly) require CENP-B to promote long-term survival and proper genome segregation, whereas full-length CENP-A does not. These data suggest CENP-B supports a parallel pathway for kinetochore formation, possibly by recruiting CENP-C (Fig. 1).

This highlights an unexpected role for CENP-B in kinetochore function. Identifying an *in vivo* function for CENP-B has puzzled researchers for decades. CENP-B binds a 17 bp motif (known as the CENP-B box) in alpha satellite repeats[14], suggesting a role for CENP-B in centromere function. However, human neocentromeres that lack alpha satellite DNA (and thus CENP-B association) form functional kinetochores, whereas endogenous centromeres of pseudodicentric chromosomes maintain CENP-B in chromatin, even though they lack CENP-A, and do not form kinetochores[2]. CENP-B homologues have not been identified in several species and CENP-B knockout mice are viable and largely defect-free[15]. Studies of HAC formation have indicated a role for CENP-B and CENP-B-box-containing alpha satellite sequences in *de novo* centromere formation[6] and in heterochromatin formation[16]. However, the mechanism by which CENP-B accomplishes these tasks is still unclear. Much remains to be determined about CENP-B function, but Fachinetti *et al.*[5] have opened an avenue of investigation that may increase our understanding of centromere establishment and its subsequent maintenance.

The experimental approach used by Facchinetti *et al.*[5] provides a valuable method to probe the epigenetic basis for centromere identity. Yet it also highlights the question of whether centromere identity is truly sequence-independent. Neocentromere formation argues against a role for the underlying sequence, but CENP-B-box-containing alpha satellite sequences support *de novo* centromere formation and may also confer long-term stability of the centromeric locus by providing a redundant mechanism for kinetochore formation. Thus, whereas CENP-A self-propagates in an HJURP-dependent manner that does not strictly require CENP-B, CENP-B itself might promote the establishment and positioning of the CENP-A locus.

Complementation testing in human cells by complete replacement of an endogenous protein with a mutant will be indispensible for studying complex cell biological phenomena. This is not new to anyone working with a variety of model systems where balancer chromosomes, counter-selectable markers and other powerful complementation testing methods have provided key insight into the functions of many proteins. As tools for engineering human genomes using site-specific nucleases continue to develop at a breakneck pace[17], these somatic cell genetic approaches should develop rapidly and bring studies in human cells closer to being research tools that are as powerful as yeasts and other model organisms.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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CENP-E hangs on at dynamic microtubule ends

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During mitosis, kinetochores attach to microtubule plus ends, thus allowing dynamic microtubules to properly segregate chromosomes. How this type of 'end-on' attachment between microtubule plus ends and kinetochores is formed and maintained is unclear. CENP-E, a kinesin-7 family member, is now shown to have a role in associating kinetochores with dynamic microtubule plus ends.

During mitosis in vertebrate cells, multiple dynamic microtubules make attachments to large multi-protein complexes termed kinetochores. These are the essential linkers between the dynamic microtubules that align and ultimately segregate duplicated chromosomes, and the chromosomes themselves[1]. To achieve the correct alignment and segregation of duplicated chromosomes during mitosis, it is important that the kinetochore complex forms 'end-on' attachments with the dynamic plus ends of kinetochore microtubules[2]. This architecture allows the microtubule plus ends to align chromosomes at the centre of the mitotic spindle during metaphase, achieving chromosome congression, and then to mechanically segregate the chromosomes during anaphase through microtubule plus-end depolymerization and sliding[3]. How the cell is able to make and maintain an end-on configuration between microtubule plus ends and kinetochores is a major question in mitosis, especially given that the plus end of each individual kinetochore microtubule remains dynamic throughout mitosis: the plus ends stochastically grow and shorten (polymerize and depolymerize) through the rapid addition and loss of many individual tubulin subunits[4]. These kinetochore microtubule dynamics are likely to contribute to the chromosome oscillations that are observed in many cell types[5]. However, correctly oriented kinetochores appear to remain stably attached to a population of dynamic microtubule plus ends despite the chromosome oscillations.

In this issue, Gudimchuk *et al.*[6] examine the role of the kinesin-7 CENP-E in associating kinetochores with dynamic microtubule plus ends during metaphase. The authors first ascertained that CENP-E was present at the kinetochore during metaphase in mammalian cells, as it co-localized with the kinetochore marker CENP-A in metaphase-arrested cells. They then treated cells with a small-molecule inhibitor of CENP-E which locks the protein in a non-moving microtubule-bound state and observed that a small number of metaphase chromosomes in each cell were severely misaligned, suggesting that motile CENP-E may have a role in maintaining proper end-on kinetochore attachment to microtubules during metaphase. To investigate how motile CENP-E motors could interact with the plus ends of dynamic microtubules to maintain end-on kinetochore attachment, Gudimchuk *et al.* performed *in vitro* studies in which they demonstrated the interaction of purified full-length CENP-E–GFP with dynamic microtubules. As expected, full-length CENP-E dimers processively walked to dynamic microtubule plus ends[7]. However, once at the microtubule plus ends, CENP-E dimers remained associated with both growing and shortening microtubule plus ends for many seconds, with average tracking durations of 11.6 ± 1.4 s for shortening and 17.9 ± 1.3 s for growing microtubules. Therefore, CENP-E has the unique property of being both a processive plus-end-directed microtubule motor and also a plus-end tip tracker for both polymerizing and depolymerizing microtubules. Furthermore, beads coated with full-length CENP-E could also follow depolymerizing microtubule plus ends, suggesting that CENP-E may be able to couple microtubule dynamics to cargo motion.

The authors used *in vitro* experiments to discern a possible molecular mechanism for CENP-E's unexpected double function of plus-end-directed motility and microtubule plus-end tip tracking. Specifically, truncated CENP-E motor constructs, comprising the motor domain and short stalk segment only, were generated to assess the consequences of motor tail loss for CENP-E motility and tip tracking. Although the plus-end-directed motility of CENP-E on the microtubule lattice was similar between the full-length and the tail-truncated constructs, there was a remarkable difference in their ability to track depolymerizing microtubule plus ends. Whereas ~70% of the full-length CENP-E motors tracked depolymerizing plus ends, almost none of the tail-truncated CENP-E motors were successful in tracking the shortening microtubule ends, providing strong evidence that the motor tail

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