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Diversity in the organization of centromeric chromatin

Florian A Steiner[1] and Steven Henikoff

Centromeric chromatin is distinguished primarily by nucleosomes containing the histone variant cenH3, which organizes the kinetochore that links the chromosome to the spindle apparatus. Whereas budding yeast have simple 'point' centromeres with single cenH3 nucleosomes, and fission yeast have 'regional' centromeres without obvious sequence specificity, the centromeres of most organisms are embedded in highly repetitive 'satellite' DNA. Recent studies have revealed a remarkable diversity in centromere chromatin organization among different lineages, including some that have lost cenH3 altogether. We review recent progress in understanding point, regional and satellite centromeres, as well as less well-studied centromere types, such as holocentromeres. We also discuss the formation of neocentromeres, the role of pericentric heterochromatin, and the structure and composition of the cenH3 nucleosome.

Address
Basic Sciences Division and Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

Corresponding author: Henikoff, Steven (steveh@fhcrc.org)
[1] Present address: Department of Molecular Biology, University of Geneva, 1211 Geneva 4, Switzerland.

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Introduction

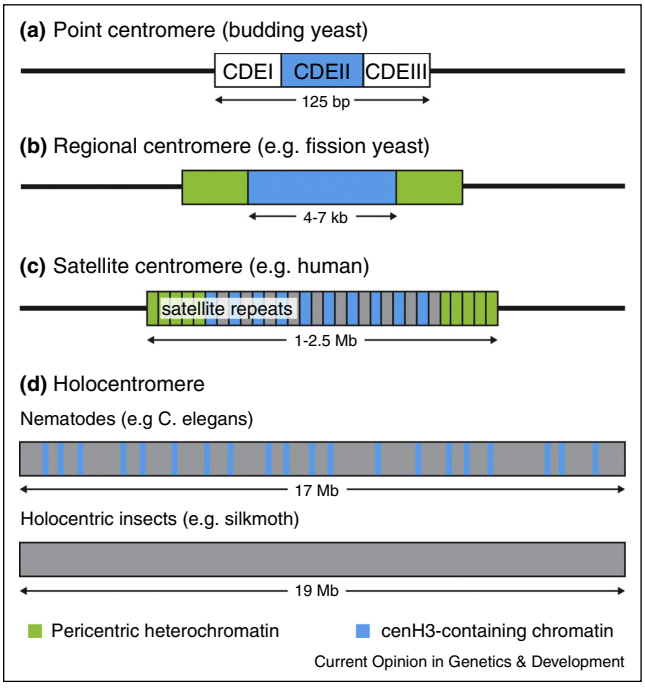
Classical cytological studies revealed the presence of a single region on each chromosome that is required to attach the spindle apparatus for segregating the chromosome during mitosis and meiosis [1]. This region, called the centromere, is bound by a large multi-protein complex called the kinetochore that links it to the microtubules of the spindle apparatus. In most eukaryotic lineages, the variant histone cenH3 (also called CENP-A) is an essential component of centromeres and serves as a marker for centromeric chromatin [2,3]. Despite the realization that cenH3 nucleosomes are essential for centromere identity and function, insight into the organization of centromeric chromatin has been largely lacking. In most species, cenH3 nucleosomes assemble on particular families of tandemly repetitive satellite DNA, and the intractability of long satellite repeat arrays has greatly hindered the analysis of centromeric chromatin. Recent studies have begun to shed light on these enigmatic regions of chromosomes, in part through improvements in sequencing technologies and in part through analysis of model systems that circumvent the challenges presented by the repeat-richness of most higher eukaryotic centromeres. The emerging picture shows that cenH3 is incorporated into well-positioned nucleosomes at centromeres that are distinct from canonical nucleosomes. The specialized chromatin formed at centromeres is important for function, as cenH3 also incorporates at low levels away from the centromere.

Distinct types of centromere organization

Given the essential function of the centromere, it seems surprising that different strategies of centromere organization have evolved (Figure 1). The simplest centromere is that of budding yeast, where a single cenH3/Cse4 nucleosome is assembled on a specific sequence that is sufficient to impart full centromere function on a replicating plasmid. In this organism, centromeric DNA is organized in three domains, Centromere DNA Elements I-II-III (CDEI-II-III), totalling ~120 bp. The cenH3 (Cse4) nucleosome is assembled on CDEII, which is highly AT-rich, whereas CDEI and CDEIII are binding sites for Cbf1 and the CBF3 complex, respectively. Proteins of the CBF3 complex are unique to budding yeast and have recently evolved to carry out specialized centromeric functions. Cbf1 belongs to the family of helix–loop–helix transcription factors, and its homologues are exclusively non-centromeric in other yeast species. Cbf1 and CBF3 bend DNA and constrain the cenH3 nucleosome over CDEII. Because a single cenH3 nucleosome connects to a single microtubule during mitosis [4], this type of centromere is called 'point centromere' (Figure 1a). The simplicity of the point centromere makes it an ideal model to study the nature of the centromeric nucleosome and its attachment to the kinetochore.

Centromeres of other organisms consist of multiple cenH3 nucleosomes that in most cases are restricted to a specific region of the chromosome and are therefore referred to as 'regional centromeres' (Figure 1b). The term was originally coined for fission yeast centromeres, which form on unique or low-copy sequences flanked by heterochromatic regions [5]. 'Satellite centromeres' are also restricted to specific regions that are made up of particular families of tandem repeats, which vary in size

Figure 1



Diverse types of centromeres are found in different organisms. **(a)** The point centromere, found in budding yeast, consists of three DNA elements, CDE I–II–III. A single cenH3 nucleosome assembles on CDE II. **(b)** Regional centromeres, found for example in fission yeast, consist of a centromeric region that is usually flanked by pericentric heterochromatin. **(c)** Satellite centromeres, for example found in humans, are similar to regional centromeres in that they occupy contiguous regions of chromosomes flanked by pericentric heterochromatin, but they are composed of particular families of tandem repeats, occupied by alternating arrays of cenH3 and H3-containing nucleosomes. **(d)** Holocentromeres cover the entire length of the chromosomes. In most lineages, including that of nematodes, cenH3 preferentially incorporates at specific sites, whereas in holocentric insects, cenH3 has been lost.

and sequence between species and even between chromosomes within species (Figure 1c). Nucleosome footprints generated by micrococcal nuclease (MNase) digestion are centered over these repeats, indicating that they help phase and position cenH3 nucleosomes [6,7]. Accordingly, repeat-length often lies between 100 and 200 bp, the approximate length of nucleosomal DNA [8•]. At some centromeres, CENP-B boxes, 17-bp sequences bound by the CENP-B protein, help position centromeric nucleosomes [9]. CENP-B has been domesticated from pogo-like transposase and may play a role analogous to Cbf1 at budding yeast point centromeres. The number of repeats usually greatly exceeds the number of cenH3 nucleosomes, raising the question how individual nucleosomes are deposited. 3D-integrated fluorescence imaging strategies and quantitative immunoblotting revealed that the human centromere contains ~400 cenH3 molecules, only a small fraction of the tens of thousands of alpha-satellite copies present [10••]. However, very recent characterization of the sequences recovered by chromatin immunoprecipitation with sequencing (ChIP-seq) of cenH3 and the inner kinetochore protein CENP-C shows that most of them belong to two classes of highly homogeneous alpha-satellite dimers [11], each of which precisely phases an ~100-bp cenH3 nucleosome on either side of a CENP-B box [12]. These homogeneous dimeric units are of sufficient abundance to account for most of the cenH3 molecules in the genome, which suggests that human centromeres are defined by DNA sequences that precisely position cenH3 nucleosomes. Mapping of cenH3 nucleosomes is limited by the gaps in the genome assemblies at centromeres, as current sequencing technologies cannot assemble highly homogeneous satellite arrays. The development of technologies to sequence long fragments will therefore be crucial to connect satellite evolution to centromere function and to finish the human and other genome projects.

In some taxa, the centromere is spread along the length of the entire chromosomes, a centromere type referred to as holocentromeric (Figure 1d). Holocentromeres have arisen at least 13 times independently, and their organization varies between taxa [13]. Holocentromeres are best-studied in *Caenorhabiditis elegans*. Nucleosome footprinting with MNase and cenH3 and CENP-C ChIP-seq experiments revealed that single cenH3 nucleosomes are preferentially incorporated at dispersed sites of accessible chromatin that coincide with transcription factor hotspots and are flanked by well-positioned canonical nucleosomes [14••]. Additional cenH3 molecules are incorporated at low level into broad regions of low transcriptional activity and nucleosome turnover [14••,15]. Interestingly, holocentricity in insects is correlated with loss of cenH3 and CENP-C, while homologues of many other kinetochore proteins are still present, suggesting that holocentricity allows for alternative ways of linking chromatin to the kinetochore [16••]. Loss of cenH3 is not exclusive to holocentric organisms, because monocentric trypanosomes also function without cenH3 [17••].

Diversity in pericentric heterochromatin

Centromeres of multicellular eukaryotes are typically embedded in heterochromatin, condensed regions of chromosomes marked by H3K9 methylation and non-histone chromatin proteins such as HP1, which facilitates cohesion of the sister strands [18,19]. However, it remains unclear whether heterochromatin also plays a role in kinetochore function. The idea of pericentric heterochromatin as an integral part of the functional centromere stems from the observation that disruption of heterochromatin leads to chromosome segregation defects [20,21]. However, recent findings have challenged that notion, and have indicated that heterochromatin is required for centromere establishment, but not maintenance in fission yeast [22]. Moreover, there are naturally occurring fission

yeast strains that contain chromosomes that lack pericentric heterochromatin [23••]. Neocentromeres that arise from the deletion of the natural centromere can form in actively transcribed regions without requiring heterochromatin [24••]. In chicken cells, the kinetochore covers the span of DNA that is bound by cenH3 nucleosomes, but not the pericentric heterochromatin, as measured by quantitative fluorescence and ChIP-seq experiments [25]. These studies argue that pericentric heterochromatin is non-essential for centromere function.

Pericentric heterochromatin might inhibit spreading of the centromere over neighboring genic regions [26]. Regional centromeres of fission yeast have tRNAs that act as barriers between pericentric heterochromatin and the centromeric core [27]. Notably, budding yeast point centromeres and *C. elegans* holocentromeres do not contain pericentric heterochromatin. However, individual cenH3 nucleosomes are flanked by Cbf1 and CBF3 in budding yeast and well-positioned canonical nucleosomes in *C. elegans*, which may fulfill a 'boundary' function by containing cenH3-nucleosomes within centromeric sites.

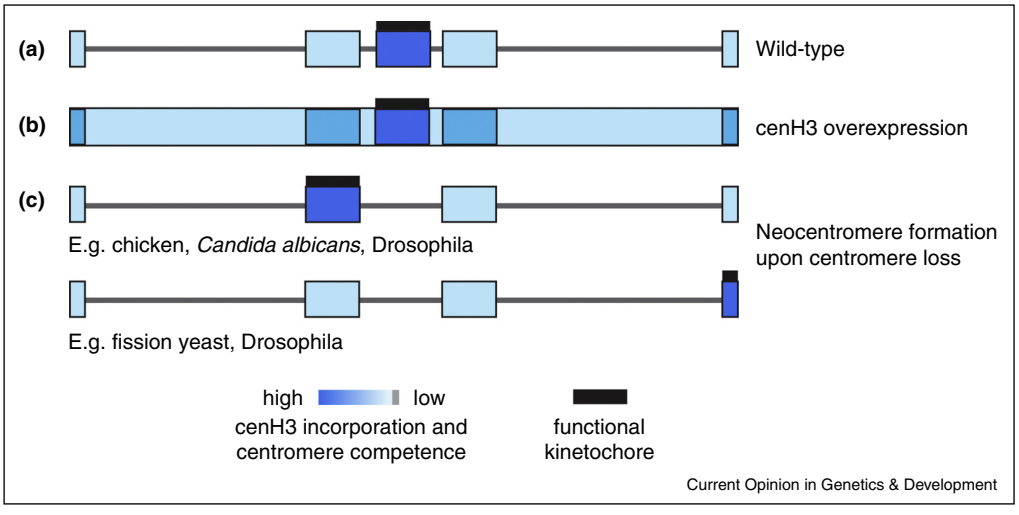
Pericentric chromatin is characterized by the presence of heterochromatin protein 1 and dimethylation or trimethylation at lysines 9 and 27 of histone H3 [28], whereas cenH3 is specifically associated with a histone H4 lysine 20 monomethylated partner [29]. Transcription of pericentric heterochromatin in fission yeast is essential for its maintenance [30,31], and centromeric transcription in vertebrates has been shown to be important for the loading of centromeric proteins [32•,33–36]. These results imply that RNA is an important component of both centromeric and pericentric chromatin.

Neocentromeres can form when cenH3 is misincorporated

In most organisms, there is incorporation of cenH3 away from the centromere that is detectable both by ChIP-seq and fluorescence microscopy (Figure 2a). Indeed, about 66% of cenH3 was found to be chromatin-bound outside the centromere in human retinal pigment epithelium cells [10••], and about 30% in chicken cells [24••]. In fission yeast, cenH3 incorporates at low levels into H3-depleted regions, an effect that is enhanced in certain chromatin remodeler mutants [37,38]. In *C. elegans*, a significant portion of cenH3 is localized randomly within domains of low transcription and nucleosome turnover [14••,15]. In budding yeast, cenH3 is also found at low levels at repetitive regions such as Ty-transposable elements, telomeres, and the rDNA repeats [39] or highly expressed genes [40].

This non-centromeric incorporation is increased when cenH3 is over-expressed, with varying effects on centromere function and chromosome segregation (Figure 2b). In budding yeast, mild cenH3 overexpression leads to an ~11-fold excess in chromosome arms over centromeres without any detectable phenotype [41,42]. Deletion of Psh1, involved in cenH3 turnover, leads to an even higher excess in chromosome arms while maintaining viability [43,44]. In humans, cenH3 overexpression increases incorporation at sites of high nucleosome turnover, transcription factor hotspots and subtelomeric regions [45,46], but does not lead to the formation of a functional kinetochore

Figure 2



Distribution of cenH3-chromatin. **(a)** In wild-type cells cenH3 concentrations are highest at the centromere — the only site that assembles a functional kinetochore — but there is non-random cenH3 incorporation away from the centromere, most notably at the regions around the centromeres and at telomeres. **(b)** Upon cenH3-overexpression, the non-centromeric incorporation increases and can extend to all positions in the genome. **(c)** If the centromere is lost, neocentromeres form preferentially at sites that are competent for cenH3 incorporation.

[47,48]. Nevertheless, cenH3 misregulation may play an important role in the development of aneuploidy in cancers [49,50]. In Drosophila, chromosome segregation defects result from cenH3 overexpression and mislocalization, but only a small subset of ectopic sites seem to form functional centromeres [51,52].

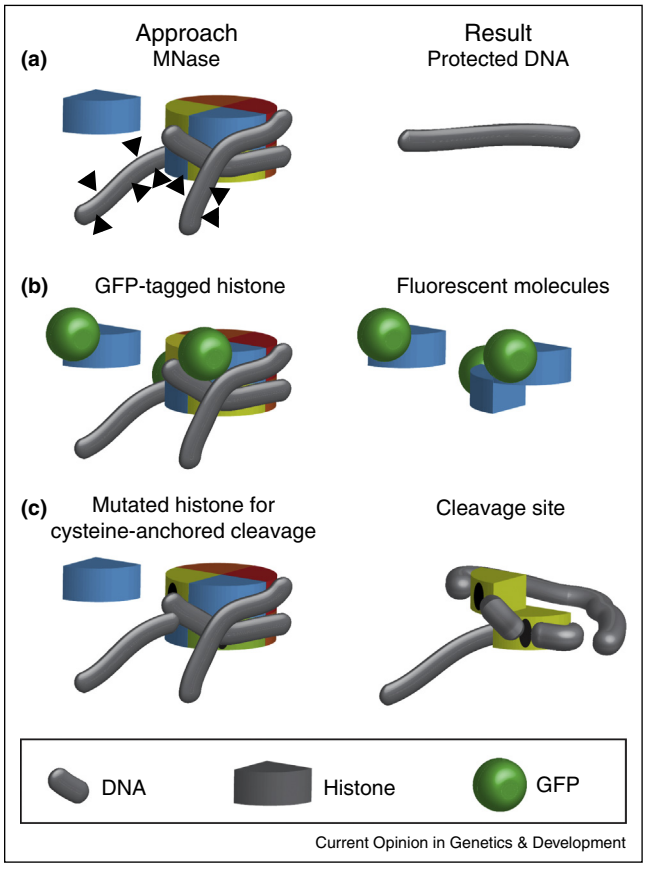
Disruption of the centromere can occasionally lead to activation of a neocentromere [53,54]. Neocentromeres most likely appear at sites that already possessed centromere competence on the intact chromosome [55] (Figure 2c). In species with satellite centromeres they can form on non-repetitive sequence and thus resemble regional centromeres without pericentric heterochromatin. Some nucleosome positions within neocentromeres are occupied preferentially, implying that there are sequences that favor cenH3 nucleosome assembly [10••]. In fission yeast, neocentromeres generally form at telomeres [56]. In other organisms such as chicken, barley and *Candida albicans*, neocentromeres tend to form in close proximity to the deleted centromere [24••,57,58•,59]. In Drosophila, heterochromatin boundaries, either near telomeres or pericentric heterochromatin, serve as preferred sites of de novo centromere formation [60]. CenH3 can be artificially 'seeded' to ectopic sites by tethering centromeric proteins, and once these sites are established, they are epigenetically maintained even in the absence of the original tether [61,62]. These observations support the idea that the level of cenH3 at a particular site determines its centromere competence.

Diverse structural models of cenH3 nucleosomes *in vivo*

CenH3 can be reconstituted with H2A, H2B and H4 and ≥147-bp DNA into octameric nucleosomes with a structure that is very similar to that of H3 nucleosomes [63]. However, reconstitution onto 62–78-bp fragments produces stable 'hemisomes' consisting of one each of the four core histones [64]. What matters of course is the structure of the cenH3 nucleosome *in vivo*, and a variety of mutually exclusive models have been proposed [39,65–67]. The induction of positive supercoils into circular minichromosomes by Cse4 centromeric nucleosomes *in vivo* [68,69] would seem to exclude conventional octameric particles, at least for budding yeast point centromeres, because octamers, but not tetramers, are constrained by contacts between H2A/H2B and H3/H4 or cenH3/H4 to produce only negative supercoils. But because of experimental challenges in determining the precise composition of cenH3 nucleosomes *in vivo*, the issue could not be conclusively resolved, and has long been regarded as controversial [70].

One of the main approaches to distinguish between different models for the cenH3 nucleosome is to estimate the amount of associated DNA (Figure 3a). This approach has indicated that cenH3 nucleosomes contain less DNA than canonical nucleosomes [6,7,14••,41,65]. However, the exact amount of DNA is affected by the extent of digestion, and different interpretations of resulting fragment size distributions have been put forward. For example, two studies examining human and rice cenH3 nucleosomes using similar native ChIP-approaches observed similar MNase fragment size distributions, but came to opposite conclusions about the nature of the particle, namely a partially unwrapped octameric [6] versus a single-wrap tetrameric particle [7]. Therefore, this approach does not conclusively reveal the protein composition of the particle, because only DNA is analyzed, although a comparison of fragment sizes between mononucleosomes and dinucleosomes provides estimates of linker lengths and upper limits on the length of DNA wrapping adjacent particles [14••].

Figure 3



Methods for analyzing the structure of nucleosomes *in vivo*. **(a)** MNase (combined with ChIP-seq) tests for nucleosome incorporation and estimates the length of nucleosome-associated DNA, but cannot determine stoichiometry of the particles. **(b)** GFP-tagging of histones allows quantification of the number of molecules present, but cannot determine incorporation. **(c)** H4S47C-anchored cleavage mapping determines the DNA cleavage sites at defined positions within the nucleosome, thus yielding information both on the incorporation of the nucleosome and its position relative to the DNA.

Box 1 CenH3-specific chaperones

In its soluble form, cenH3 and its H4 partner are associated with a specific chaperone, called HJURP in mammals, which facilitates deposition into chromatin [80,81]. CenH3-specific chaperones have also been identified in fungi (Scm3) [67,82–85] and insects (CAL1) [86]. Although essential for incorporating cenH3 at centromeres, it is not known whether they target specific sequences. In humans, HJURP is recruited to the centromere via interaction with the Mis18 complex [87], and phosphorylation of specific residues of HJURP is important for the timing of cenH3 loading [88]. When cenH3 is overexpressed, ectopic incorporation of cenH3 is mediated by the chaperone DAXX, which normally binds H3.3/H4 for replication-independent replacement of histones on chromosome arms [45]. A recent study identified a novel centromeric RNA that associates with HJURP and cenH3 and is required for cenH3 loading [32•]. Whether this RNA is involved in sequence-specific targeting to the centromere or simply plays a structural role remains to be determined.

Several groups have used microscopy to observe the number and localization of cenH3 protein molecules [71–73,74•,75,76] (Figure 3b). As budding yeast centromeres consist of a single cenH3 nucleosome, the number of cenH3 molecules observed should reveal the nature of the cenH3 nucleosome. However, the results have led to estimates of 1–8 molecules per centromere, and interpretations were complicated by the observation of variation during the cell cycle [73] or of cenH3 molecules that are present in proximity to the centromere [72,76]. These experiments also did not address whether the observed cenH3 molecules are incorporated into chromatin. For example, it has been shown that the Cse4-specific chaperone Scm3 (Box 1) is present at the centromere throughout the cell cycle to facilitate incorporation of cenH3 after replication [74•,77]. Because of the binding affinity of Scm3 to cenH3, it is possible that there are two cenH3 molecules present at the budding yeast centromere: one incorporated into a nucleosome, and one bound by Scm3.

To probe *both* the protein and DNA within the particle, our lab adopted an *in vivo* mapping method in which histone H4 is converted into a site-specific cleavage reagent in whole cells [78•] (Figure 3c). As the H4 obligatory partner of all H3 variants, its position within the budding yeast point centromere will unequivocally reveal the composition and orientation of the nucleosome. Two cleavages were observed in the expected positions for canonical nucleosomes, but only a single cleavage for the centromeric nucleosome, that is, one H4 molecule per centromere [78•]. The position of the cleavage by H4 within CDEII further indicated that Cse4 hemisomes are oriented in either direction relative to CDEI–II–III and in two rotational positions along the sequence. Thus the hemisome model is the only one that can account for the observed cleavages at all 16 budding yeast centromeres, whereas the octamer model accounts for the vast majority of cleavages elsewhere. Ectopically incorporated Cse4 nucleosomes appear to form octamers [41], consistent with the observation of human heterotypic tetramers containing CenH3/H4 with H3.3/H4 at ectopic sites [45].

Perspective

Because of its simplicity and the one-to-one relationship between centromeric nucleosomes and the microtubules that attach to them, the budding yeast point centromere has served as an invaluable model for understanding centromeric chromatin. The extent to which the findings in budding yeast directly apply to the centromeres of other organisms continues to be experimentally addressed. Nevertheless, budding yeast Cse4 can complement human CENP-A in human cell culture, where it localizes to centromeres and supports segregation [79], suggesting that the fundamental properties of cenH3 chromatin are conserved in most forms of eukaryotic life.

Acknowledgements

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