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Centromere chromatin structure – Lessons from neocentromeres

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ABSTRACT

Centromeres are highly specialized genomic loci that function during mitosis to maintain genome stability. Formed primarily on repetitive α -satellite DNA sequence characterisation of native centromeric chromatin structure has remained challenging. Fortuitously, neocentromeres are formed on a unique DNA sequence and represent an excellent model to interrogate centromeric chromatin structure. This review uncovers the specific findings from independent neocentromere studies that have advanced our understanding of canonical centromere chromatin structure.

Centromeres are highly specialized genomic loci, that function during mitosis to ensure accurate chromosome segregation and maintain genome stability. Cytogenetically centromeres can be identified as the primary constriction of metaphase chromosomes where they serve as the assembly site for the kinetochore, a multiprotein structure that fvdorms attachments to the microtubules of the mitotic and meiotic spindles. Whilst kinetochore architecture and microtubule interactions have been described in detail [1] less is known about their underlying chromatin organisation. Our interest in centromeres arises from a desire to understand their chromatin architecture and determine how important this is for function. This information could prove therapeutically useful as centromeric breaks and rearrangements are commonly associated with human diseases such as cancer [2].

Chromatin is a complex of DNA and proteins within the nucleus of mammalian cells. It functions to package and compress the large amounts of DNA into the nucleus and to regulate gene expression. Nucleosomes represent the primary level of chromatin folding where by double-stranded DNA is wrapped around eight core histone proteins, these nucleosomes are then folded into a higher-order fibre which is further packaged into the large-scale chromatin structures observed in interphase cells [3–6]. We and others have shown that within these packaging levels, chromatin structure regulates gene expression by controlling accessibility of the transcriptional machinery and transcription factors to DNA. For instance, transcription start sites are often depleted of nucleosomes [7], forming open or disrupted 30-nm chromatin fibres [8,9] whilst large-scale chromatin fibres can be decompact in a transcription dependent manner [9–11]. Interphase chromatin is characterised as either ‘euchromatin’ or ‘heterochromatin’: euchromatin often corresponds to gene-rich regions; in contrast, heterochromatin is transcriptionally silent. Whilst early cytological

experiments revealed the heterochromatic nature of centromeres [12], the underlying structure and organisation of this chromatin has remained obscure. This is primarily due to centromeres being located on highly repetitive DNA sequences and the difficulties associated with analysing these loci.

Human centromeres are composed of tandem arrays of α -satellite DNA, a repeat based on a 171bp monomer which are 50–70% identical, arranged to create a higher order repeat (HOR) unit [13]. Due to the challenge in assembling these near-identical DNA sequences the reference human genome lacked sequence information for all centromeres until its most recent build (GRCh38/hg38) which contains sequence representation for all human centromeres although these do not yet completely represent the linear DNA structure [14,15]. This repetitive property of centromeres hinders the use of many chromatin structural assays where the readout often requires microarray, or more frequently nowadays next generation sequencing. However a recent advance in understanding centromere organisation comes from using Nanopore long DNA sequence reads to assemble the centromeric sequence of the human Y chromosome [16]. This study examined the haploid satellite array present on the Y centromere and using BACs spanning from the proximal p-arm to proximal q-arm determined the DNA sequence underpinning the functional Y centromere and packaged as chromatin enriched in CENP-A. New sequencing methodologies will make these repetitive centromeric regions easier to structurally characterise in the future. However, one method we previously used to interrogate chromatin structure was sucrose gradient sedimentation. Using this approach we demonstrated that repetitive satellite-containing chromatin fibres have a more compact structure compared to bulk chromatin fibres, indicating that canonical centromeric domains might have distinct properties [17].

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Neocentromeres are a special class of centromere that form in an ectopic location on a chromosome [18]. In stark contrast to canonical centromeres, neocentromeres form on a unique DNA sequence, an important feature that can be exploited to investigate centromere chromatin structure. Significantly, neocentromeres bind all known essential centromere proteins and form a fully functional kinetochore making this a useful model to understand native centromere function [19,20]. How neocentromeres emerge is still unclear but they appear in the case of centromere inactivation or loss, and are a feature of certain cancers where they seem to rescue acentric fragments [2]. Intriguingly in a few rare cases neocentromeres have been discovered for seemingly un-rearranged chromosomes, including human chromosome 3, 4, 7 and Y [21–24]. These are often identified through cytogenetic screening, which observed a shift in the primary constriction away from the canonical block of original α -satellite centromeric DNA. Concomitantly, the original centromere is functionally inactivated creating a pseudo-dicentric chromosome but with one functioning centromere in an ectopic site. Indeed, we actually have no clear understanding of the frequency of these centromere repositioning events as they have only been detected serendipitously and can be phenotypically silent. Another type of centromere are the evolutionary new centromeres (ENCs) and like neocentromeres these may result from the seeding of a new centromere at an ectopic location which have since been inherited through generations and become fixed in the population [25]. This review will uncover lessons we have learned to date from neocentromeres and ENCs, it will explore what is known about their underlying chromatin structure, and it will discuss how this informs native centromere identity and specification.

1. Centromeres are specified by sequence independent epigenetic mechanisms

Andy Choo's discovery of a human neocentromere in 1993 demonstrated that α -satellite sequence did not specify centromere location [18]. This first lesson learnt from neocentromeres remains one of the most striking, as centromeres of higher eukaryotes generally contain large amounts of tandem repeat DNA, often thought necessary for centromere function. This neocentromere, called mar del(10), was identified through routine cytogenetic diagnostic analysis of a patient with developmental delay; it was located at genomic position 10q25 on a marker chromosome which was completely devoid of α -satellite sequences [26,27]. This observation implied that α -satellite DNA sequences are not required for human centromere specification. Indeed, earlier studies of human dicentric chromosomes had hinted towards an epigenetic, rather than a genetic component for centromere specification [28]. Dicentric X chromosomes are a feature of Turner syndrome where partial X chromosome loss and concomitant X chromosome fusion results in a dicentric X chromosome. However this dicentric X chromosome behaves functionally as a monocentric chromosome as one of the two endogenous α -satellite containing centromeres is inactivated, demonstrating that α -satellite sequences alone are insufficient to determine centromere function, supporting an epigenetic or chromatin-related component [28].

Since 1993 over 100 neocentromeres have been described in the human population on every chromosome, except 19, and usually on a unique DNA sequence (Fig. 1) [29]. Whilst certain chromosomes and genomic regions seem to be 'hotspots' for neocentromere formation, sequence analysis has determined that these are formed on different underlying DNA sequences, albeit with a preference for AT rich regions [30,31]. Model systems, such as *Drosophila melanogaster* [32], *Schizosaccharomyces pombe* [33], *Candida albicans* [34] and most recently chicken DT40 cells [35], have all been exploited to understand the underlying DNA and chromatin requirements of centromeres. This latter study used chromosome engineering techniques in a neocentromere formation assay to specifically excise the unique DNA containing Z centromere of chicken DT40 cells and subsequently screened

for the emergence of neocentromeres [35]. Importantly, this experiment did not identify any specific DNA sequence or motif associated with neocentromere formation, supporting the requirement for an epigenetic component or feature for centromere locus specification.

Although the mechanisms for centromere locus specification are unclear, CENP-A, a variant of histone H3 [36], was long considered to be a key component for defining centromere identity. First identified in 1980 as a protein detected by one of a number of anti-centromere antibodies found in patients suffering from the autoimmune disorder scleroderma [37], CENP-A was the smallest antigen detected, the larger two were termed CENP-B and CENP-C [38]. Since then CENP-A has been the focus of biochemical and molecular research identifying it as the undeniable epigenetic component necessary for centromere identity [39–41] and an essential component in kinetochore formation and centromere function (reviewed in Ref. [42]).

2. Centromeres accumulate α -satellite DNA

The observation that centromere specification is sequence independent but yet all human centromeres are associated with α -satellite DNA presents an intriguing paradox that implies some role for the α -satellite DNA sequences in centromere stability. This is also consistent with other eukaryotic species, where centromeres typically contain large amounts of satellite DNA, although the actual repeat sequences are not well conserved [43]. Coupled with this is the apparent requirement for α -satellite DNA in Human Artificial Chromosome formation (HAC) formation [44–47]. However a recent study exploited a CENP-A nucleosome seeding strategy and successfully generated HACs on unique DNA sequence lacking both α -satellite or CENP-B box DNA motifs [48]. The question then remains: if α -satellite DNA is not functionally required at centromeres, does it confer centromere stability? Indeed, this has been postulated for both neocentromeres and ENCs.

ENCs have been observed in birds, primates and other mammals [25,49] but are best described in macaque where 9 of the 20 centromeres are ENCs and contains large blocks of satellite DNA indistinguishable from other macaque centromeres [50]. In contrast two newly emerged ENCs, in orangutan and horse, are not associated with satellite DNA suggesting it is acquired over time as these centromeres mature [51,52]. A similar model has been proposed for the domestic donkey (*Equus asinus*) where 16 of the 31 centromeres lack satellite DNA and satellite DNA recruitment represents a late event in centromere maturation [53]. This then implies that neocentromere formation may be a first step in the emergence of ENCs, which is followed by satellite DNA accumulation and subsequent loss of satellite repetitive DNA at the ancestral centromere. Indeed, partial loss of α -satellite DNA has been observed at the now inactive endogenous centromeres for two reported neocentromeres on human chromosome 4 and 8 [21,54,55]. Similarly, centromere inactivation in some engineered human dicentric chromosomes is also associated with partial loss of α -satellite DNA [56].

One reason neocentromeres acquire α -satellite DNA during maturation could be for centromere stabilisation. Indeed centromere sliding or drift has been reported for both satellite free ENCs and neocentromeres [57,58] and could prove deleterious for cells if it affected the expression of nearby genes. In addition, whilst neocentromeres form fully functional kinetochores and are stably propagated, they are still associated with significantly higher chromosome mis-segregation rates and mitotic errors [59,60]. Thus the consensus α -satellite repetitive DNA may provide a safety buffer against centromeric drift [61] and reduce neocentromere instability [59,60] (Fig. 2).

3. Core centromere domains are associated with heterochromatin

One method used to characterise the genomic features of canonical human centromeric chromatin is high resolution immunofluorescence on extended interphase chromatin fibers. These studies describe a linear

Fig. 1. Neocentromeres reveal that centromere specification is sequence independent. The genomic locations of 106-human constitutional neocentromeres. This figure includes an additional 15 neocentromeres described since 2008 [75]. The known locations of neocentromeres are represented by bars aligned against the chromosome ideograms with specific colours sub-classifying the neocentromeres. This sub-classification is based on the genomic alteration associated with neocentromerization. In most cases neocentromeres form to rescue acentric chromosomal fragments and these marker chromosomes are either class I marker chromosomes (unbalanced karyotype) and are represented by black bars or class II marker chromosomes (balanced karyotype) and are represented by red bars. Green bars represent sites of centromere repositioning for seemingly un-rearranged chromosomes and grey bars represent neocentromere locations due to unknown chromosomal rearrangements.

centromeric chromatin structure distinct from both euchromatin and heterochromatin termed ‘centrochromatin’ [62–65]. During interphase, ‘centrochromatin’ consists of a CENP-A-containing chromatin core decorated with the H3K4me2 mark, that is normally associated with open or permissive chromatin, but lacks other typical euchromatic marks such as H3K4me3 or H3K9ac [63,64]. The core centromeric domain is flanked by pericentromeric heterochromatin consisting of silent chromatin marks such as H3K9me3, H3K9me2 and H3K27me3 [63,66]. Interestingly, transcription arises from both the core centromeric domain and flanking heterochromatic domain during interphase and mitosis, although its role at each stage is still not entirely understood

(reviewed in Refs. [67,68]).

Core centromeric transcription is essential for proper centromere function and identity. In interphase, transcription from this CENP-A core domain has been implicated in the chromatin remodelling necessary for the incorporation of newly synthesised CENP-A in early G1 [69] whilst in mitosis core centromeric transcription is required for accurate chromosome segregation [70,71]. Transcription of pericentromeric heterochromatin appears to be necessary to maintain the heterochromatic state of this region in many organisms and this heterochromatin and associated Heterochromatin Protein 1 (HP1) are required for proper sister chromatid cohesion during mitosis [72]. Furthermore,

Fig. 2. Centromeres accumulate α -satellite DNA. Neocentromeres form at unique DNA sequence devoid of α -satellite (yellow band). CENP-A ChIP (green) defines neocentromere location which can drift locally and potentially disrupt local gene expression. Based on data from ENC [57,94], one model proposed by several in the field is that over time neocentromeres acquire α -satellite sequence, which is simultaneously lost from the original centromere location, to stabilise these centromeres and buffer against centromeric drift.

Fig. 3. Centromere CENP-A core domains are associated with heterochromatin. (A) Schematic representation of the proposed higher-order structure of centromeric chromatin. Compact heterochromatin surrounds the CENP-A defined centromeric core. As this core domain contains active euchromatic marks we predict it may have a less compact chromatin fibre. (B) Schematic representation of the proposed higher-order structure of neocentromeric chromatin. Neocentromeres form in euchromatic chromatin which is less regularly folded but devoid of active and heterochromatic marks. (C) A model of the 3D architecture of neocentromeric chromatin based on the proposed model by Nishimura et al., 2019 [81]. Neocentromere core domains that lack pericentromeric heterochromatin associate in 3D with distant heterochromatin.

pericentromeric heterochromatin is thought to constrain the core CENP-A domain preventing spreading into adjacent euchromatin which could impact on gene expression [73].

In contrast to canonical centromeres, neocentromeres do not share this same linear centromeric chromatin structure as defined by specific histone marks and the role of transcription is not clear. ChIP-chip analysis of three human neocentromeres observed no active histone marks and a lack of pericentromeric heterochromatic features [31]. In addition, high resolution ChIP-seq for specific euchromatic H3 modifications including H3K4me2, H3K9me3, and H3K36me2, show no enrichment within or surrounding the core CENP-A domain at non-repetitive chicken centromeres and chicken neocentromeres [35,74]. Transcription has been reported at neocentromeres during mitosis and interphase, however neocentromeres are not always associated with actively transcribing genes [30,75–79]. Indeed the neocentromere formation assay performed in chicken cells showed no preference for neocentromere formation at transcriptionally active versus inactive genomic regions [35]. Interestingly however for the original mar del (10) neocentromere transcription of the underlying LINE correlates

with its centromeric function [80]. Human neocentromeres also appear to lack pericentromeric heterochromatin and whilst one might predict this to result in major chromosome instability, only slight cohesion defects are reported [21]. How human neocentromeres support the assembly of a functional kinetochore despite lacking these apparently necessary chromatin features is a subject of intense study.

One study reported that like human neocentromeres, heterochromatin regions were not detected around non-repetitive chicken centromeres and neocentromeres, and the authors hypothesised that perhaps neocentromeres form their heterochromatic structures via alternative mechanisms [35]. With this in mind Nishimura et al., used 4C analysis to determine the 3D genomic architecture of three independent neocentromere loci both before and after neocentromerization [81]. Significantly, neocentromeres were commonly associated with two distal heterochromatin regions - at distances of 16 MB and 34 MB - and both regions were highly enriched for H3K9me3, an association that was only evident after neocentromerization. Two canonical centromeres - the native chicken Z centromere deleted in the neocentromere formation assay, and chicken chromosome 1 - also showed 3D

Fig. 4. Centromeric CENP-A core domain may be composed of structural subdomains. (A) CENP-A ChIP across several neocentromeres suggests this core centromeric domain is structurally subdivided. (B) A proposed model of the 3D architecture of neocentromeric chromatin. Limited data from 4C analysis predicts a compact CENP-A core domain which may be further subdivided into two domains.

genomic association with the same heterochromatin regions. These long range interactions were dependent on CENP-H which localises to centromeres and neocentromeres throughout the cell cycle and is essential for the deposition of newly synthesised CENP-A [82,83]. Significantly, these data from neocentromeres highlight the importance of structural heterochromatin in centromere maintenance and function because in absence of pericentromeric heterochromatin neocentromeres associate in 3D with distal heterochromatin (Fig. 3).

4. The CENP-A centromeric domain is composed of structural subdomains

Whilst human centromeric α-satellite arrays range from 200 kb to 5Mb the CENP-A core domain is formed only on a portion of this array with CENP-A levels being regulated by mass action [84,85]. Indeed CENP-A core domains are thought to be similarly sized between chicken repetitive centromeres (that span up to 500 kb) and non-repetitive centromeres (such as the chicken Z centromere which spans approximately 40 kb) as similar CENP-A levels are found on both [35,86]. Early immunofluorescence studies of extended human interphase chromatin fibers showed that within the CENP-A containing core chromatin domain, CENP-A nucleosomes are interspersed with H3 nucleosomes in subdomains of between 15 and 40 kilobases (kb) [62,63]. More recently, using several independent quantitative approaches, the actual number of CENP-A nucleosomes within this core domain was determined to be ~200 (each an octameric nucleosome with two molecules of CENP-A) which represents approximately 4% of the total core nucleosomes [85,87]. In mitosis these CENP-A nucleosomes bind the constitutive centromere-associated network (CCAN), a large group of proteins that function as the structural core for kinetochore assembly presumably anchored through the CENP-A nucleosome [88]. Indeed, electron microscopy of mitotic centromeres, both endogenous and the mar del(10) neocentromere, revealed that CENP-A domains are localised at the surface of the primary constriction. These data imply a complex higher order structural folding of mitotic centromeric chromatin such that discontinuous domains of CENP-A chromatin are brought together to form a surface for kinetochore assembly, with intervening H3 chromatin facing inward [89]. Core centromeric domains are much smaller in neocentromeres - 100-400 Kb versus 180 kb-2Mb for human α-satellite centromeres [84] - and contain fewer CENP-A nucleosomes [85], but are nevertheless functionally equivalent to endogenous centromeres. CENP-A distribution across core centromeric domains has been mapped for several neocentromeres

[30,31,35,58,77,79,80,90,91] and some of these revealed a bipartite CENP-A domain organisation [31,54,77,79,80]. Several independent models for centromere folding have been proposed including: solenoid model [62]; layered boustrophedon model [92](Ribeiro et al., 2010); and a looping model [62]. Currently there is no consensus view for the best model (for review see Ref. [93]) but additional methods, such as chromosome conformation capture at neocentromeres might help to distinguish between them.

Indeed the first of the chromosome conformation capture analyses were published earlier this year, and whilst limited to 4C analysis of chicken neocentromeres, these data highlighted centromere interactions within the CENP-A core domain suggestive of a compact interphase chromatin structure [81]. This is in line with ENC in orangutan where the neocentromeric domain was significantly more compact when compared to its wildtype counterpart [94]. Excitingly, these observations suggest that subdomains within the CENP-A core may be necessary for centromeric structure (Fig. 4).

5. Concluding remarks

Here we have described the major lessons we have learned from neocentromeres and ENCs about centromeric chromatin structure. One of the most interesting of these is the association with heterochromatin that, if not present linearly on DNA, appears established in 3D through distal association. In recent years heterochromatin has been shown to organise into phase separated compartments, and therefore it is conceivable that a role for liquid-liquid phase separation could be important for genome organisation at centromeres. Potentially techniques combining widefield microscopy with RNA imaging and correlation with core CENP-A domains could help address this.

In summary neocentromeres are an excellent model to interrogate centromere chromatin structure however a comprehensive analysis of neocentromere chromatin structure is needed and will greatly enhance our understanding of centromeres.

CRediT authorship contribution statement

Catherine Naughton: Conceptualization, Writing - original draft.
Nick Gilbert: Writing - review & editing.

Declaration of competing interest

None.

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