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Genetics, epigenetics and back again: Lessons learned from neocentromeres

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A B S T R A C T

The duplication and segregation of the genome during cell division is crucial to maintain cell identity, development of organisms and tissue maintenance. Centromeres are at the basis of accurate chromosome segregation as they define the site of assembly of the kinetochore, a large complex of proteins that attaches to spindle microtubules driving chromosome movement during cell division. Here we summarize nearly 40 years of research focussed on centromere specification and the role of local cis elements in creating a stable centromere. Initial discoveries in budding yeast in the 1980s opened up the field and revealed essential DNA sequence elements that define centromere position and function. Further work in humans discovered a centromeric DNA sequence-specific binding protein and centromeric α-satellite DNA was found to have the capacity to seed centromeres *de novo*. Despite the early indication of genetic elements as drivers of centromere specification, the discovery in the nineties of neocentromeres that form on unrelated DNA sequences, shifted the focus to epigenetic mechanisms. While specific sequence elements appeared non-essential, the histone H3 variant CENP-A was identified as a crucial component in centromere specification. Neocentromeres, occurring naturally or induced experimentally, have become an insightful tool to understand the mechanisms for centromere specification and will be the focus of this review. They have helped to define the strong epigenetic chromatin-based component underlying centromere inheritance but also provide new opportunities to understand the enigmatic, yet crucial role that DNA sequence elements play in centromere function and inheritance.

1. Genetic elements instructing centromere formation

Initial studies were performed in the budding yeast *Saccharomyces cerevisiae*, where the site of microtubule attachment became known as a "point centromere". Pioneering studies showed a mere 125 bp DNA sequence containing three conserved DNA elements (CDE I, II and III) to be key for active centromere formation [1–3] (Fig. 1A). The finding that the centromeric DNA sequence placed onto a plasmid carrying a yeast replication origin is all that is required for the plasmid to behave as a chromosome in mitosis and meiosis, set the stage for the development of linear artificial yeast chromosomes and offered definitive evidence for the genetic specification of the centromere. While some elements in the yeast centromere are universal, e.g. an AT-rich DNA sequence core, other features such as its size turned out to be unique to budding yeast. Most other eukaryotic centromeres expand across larger regions of chromosomes, so called regional centromeres that comprise several tens to hundreds of kilobases of DNA, underlying the core kinetochore forming region which is typically flanked by repetitive DNA packaged into heterochromatin [4]. The fission yeast centromere is a well characterised example of a small regional locus featuring a kinetochore-seeding central domain region that is non-repetitive DNA (Cnt) flanked by inverted innermost repeats (imr) containing clusters of tRNA genes. These are further flanked by outer-repeat sequences (otr) that form heterochromatin in an RNAi-dependent manner [5,6] (Fig. 1B). Unlike the fission yeast central core, human centromeres are associated with highly repetitive AT-rich DNA sequences, known as α-satellite or alphoid DNA, structured as an array of simple tandem repeats of a 171 bp monomer building up to complex higher order repeat (HOR) patterns [7,8] (Fig. 1C). Within these HORs (except on chromosome Y), some monomers contain a conserved 17bp motif sequence. The isolation of the initial centromere proteins in humans [9] revealed the existence of CENP-B, a centromeric protein that binds directly to those motifs (CENP-box) [10–12]. This data suggested a broader and more general genetic specification of the centromere, not only restricted to budding yeast, pointing to a structural and possibly functional role for satellite DNA in the mammalian centromere. However, it was clear early on that there is no simple linear relationship between alphoid DNA and centromere specification. A case in point is a chromosomal rearrangement leading to a stable dicentric chromosome where one of two centromeres becomes inactivated [13,14] (Fig. 2A). This observation demonstrated that centromeres can be silenced and that alphoid DNA is not sufficient for centromere determination. The peripheral involvement of satellite DNA in centromere function became acutely apparent in the early 1990ies with the discovery of human neocentromeres that vacated their canonical position at α-satellite repeats and repositioned at a naïve locus, not previously associated with centromere function (Fig. 2B). Often these centromeres form on acentric fragments resulting from breakage or translocation but in some cases the original α-satellite repeats are retained on the same chromosome but are no longer active as a centromere [15–18]. Combined, these observations led to the notion

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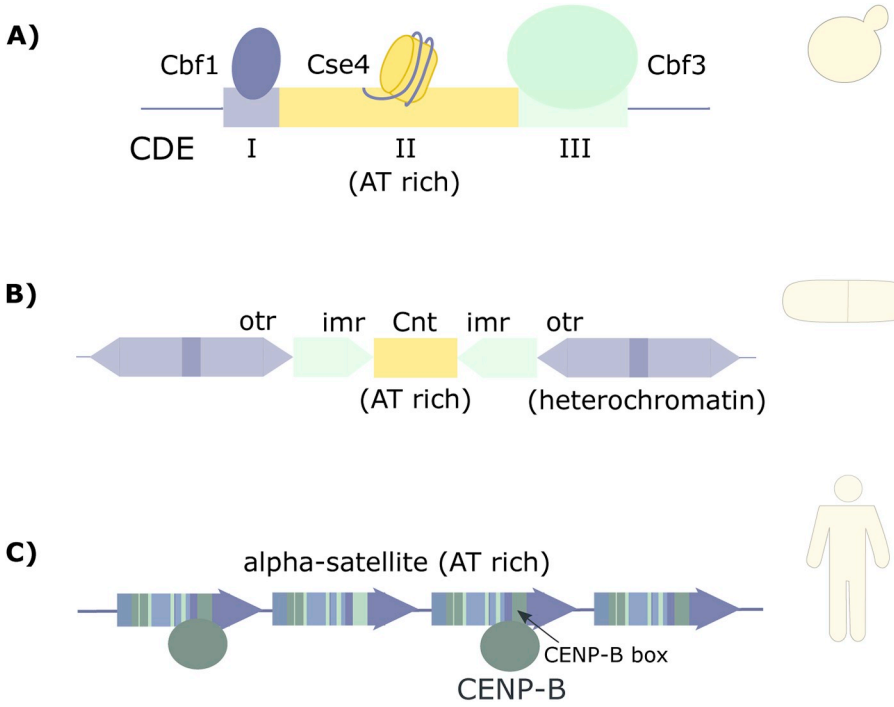

Fig. 1. Basic centromere structure in different organisms. Centromeres of three common model organisms are highly diverse in DNA element composition, size and chromatin organization. **A)** In budding yeast, small specific protein binding elements are critical for centromere function. **B)** In fission yeast the centromere core is surrounded by heterochromatin. **C)** Larger regional centromeres, in most animals and plants, associate with particular repetitive sequences although they are non-essential for centromere function.

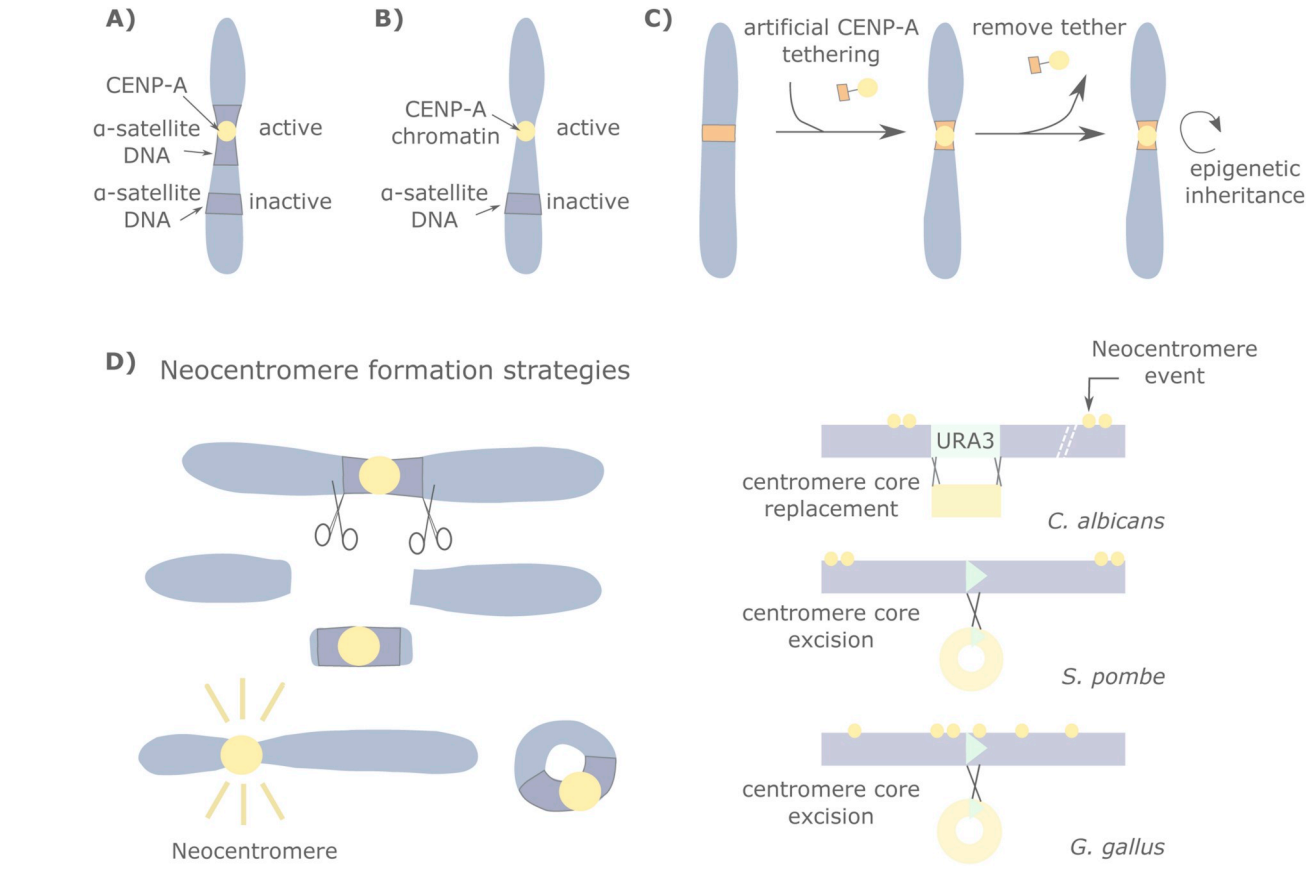

Fig. 2. Shifting centromere research focus from DNA cis elements towards epigenetic specification. A) The discovery of a chromosomal rearrangement leading to a stable dicentric chromosome due to the inactivation of one of the centromeres demonstrated that alphoid DNA is not sufficient for centromere determination. **B)** The discovery of neocentromeres indicated the non-essential nature of centromere DNA and shifted research focus to chromatin defined by CENP-A nucleosomes (yellow). **C)** Artificial tethering of CENP-A at a defined location (orange region) resulted in the formation of an active centromere and kinetochore that remained stable and heritable even without the initial seed. **D)** General strategy for neocentromere formation based on the rescue of an acentric chromosome following deletion of an endogenous centromere. On the right, specific strategies developed in different organisms resulted in preferred neocentromere formation sites (yellow circles).

that centromeric DNA is neither necessary nor sufficient for centromere function and this strong evidence resulted in a shift in focus on epigenetic regulation of centromere assembly and maintenance [19].

2. CENP-A, an epigenetic centromere mark

Contemporaneous with the discovery of neocentromeres, it became apparent that centromeres feature an unusual chromatin structure. One of the initial set of centromere proteins discovered along with CENP-B and CENP-C was CENP-A, a histone-like component tightly associated with chromatin at centromeres, suggesting that it may function as a centromere-specific core histone [13,20–22], a notion confirmed by its cloning that revealed it to be a novel histone H3 variant [23].

CENP-A-based chromatin has now been identified in a broad range of eukaryotes such as *S. cerevisiae* (Cse4) [24], *S. pombe* (Cnp1) [25], *Caenorhabditis elegans* (HCP-3) [26], *Drosophila melanogaster* (CID) [27,28], *Arabidopsis thaliana* (CENH3) [29], *Xenopus laevis* [30], *Gallus gallus* [31] and *Homo sapiens* (CENP-A), although it is lacking in kinetoplasts, holocentric insects and some fungi [32–35]. CENP-A forms a functional octameric nucleosome replacing histone H3 within its core [20–23,36–38]. These nucleosomes form a platform for the binding of the constitutive centromere-associated network (CCAN) and this interaction is critical for the formation of the centromere-kinetochore complex [39–42]. There are 16 CCAN components. Two of them, CENP-C and CENP-N, directly bind to CENP-A nucleosomes and are critical for the recruitment of other components [43–45]. Of this group, CENP-C and CENP-T are DNA interacting proteins and recruit the KMN network, a group of protein complexes that connect to spindle microtubules [46–50].

The epigenetic behaviour of centromere position and inheritance along with the discovery of the unique chromatin structure that forms the foundation for the entire centromere and kinetochore complex, led to the hypothesis that CENP-A is a central self-replicating element in the epigenetic specification of the centromere [51].

Determining whether CENP-A is key to centromere specification did not result in convincing findings initially. Overexpression in human cells lead to CENP-A mislocalization elsewhere in the nucleus and a partial recruitment of centromere components but no neocentromere activity was observed [52]. In contrast, strong overexpression of CID (*Drosophila* CENP-A) in *Drosophila* S2 cells generated CENP-A domains that nucleated ectopic kinetochores with an ability to bind to microtubules [53,54]. In a more directed way, tethering a CENP-A^{CID}- LacI fusion protein to stably integrated LacO arrays at a defined location away from endogenous centromeres, also led to the formation of functional ectopic centromeres (Fig. 2C). Importantly, following the initial forced recruitment of CENP-A^{CID}, nascent untagged CENP-A^{CID} (without LacI) was also assembled at the ectopic site, maintaining a functional kinetochore even without the need of the initial seed [55]. A similar approach was performed in human U2OS cells by tethering the CENP-A specific chaperone, the Holliday Junction Recognition Protein (HJURP) [56,57]. Targeted HJURP resulted in the stable recruitment of CENP-A to the ectopic site and functional kinetochore formation [58]. In these tethering experiments, the presence of two centromeres per chromosome led to mitotic failure, indicative of a functional centromere but preventing a long-term analysis of ectopic centromeres. This was overcome by using a system engineered on the Z chromosome in chicken DT40 cells [59], where different kinetochore proteins were targeted to a non-centromeric LacO array, while the endogenous centromere was conditionally removed, generating unique ectopic kinetochores that were stably propagated [60]. Therefore, following a long established correlation between the epigenetic behaviour of centromere inheritance and the presence of the histone variant CENP-A, evidence was obtained establishing a causal link where CENP-A is both necessary and sufficient to seed a heritable functional centromere [61].

The central role of CENP-A and its associated proteins in maintaining centromere identity suggests CENP-A is constitutively present at centromeres and is subject to tight control to ensure replication and inheritance of the mark, in synchrony with the cell cycle.

CENP-A chromatin assembly is uncoupled from DNA replication and bulk histone nucleosome assembly [62,63]. Experiments in *Drosophila* embryos using fluorescent CENP-A^{CID} photo-bleaching suggested that nascent CENP-A is loaded into chromatin during a brief window following mitotic exit [64]. Direct evidence for this timing came from experiments in human cells using SNAP-tag technology, a method that allowed the visualization of a specific newly synthesized pool of CENP-A by fluorescent pulse labelling [63]. Consistent with the fly experiments, CENP-A was targeted to centromeres only following exit from mitosis, in early G1 phase. Assembly at mitotic exit appears to be the preferred mode in many animals including chicken cells and frog embryos [65–67], while loading occurs in G2 phase in other organisms such as plants and fission yeast [68–70]. While timing varies, the post-replicative assembly of CENP-A appears to be near universal.

Once assembled at the centromere, CENP-A nucleosomes are stably maintained throughout the cell cycle without any appreciable turnover, fatefully redistributed onto sister chromatids during S phase [63,71–73]. The accurate transfer of parental CENP-A nucleosomes onto nascent DNA during DNA replication appears to be restricted to the CENP-A pool at the centromere [74] and may be key to their role in heritably maintaining centromere identity. Active mechanisms have been described to promote CENP-A stability, such as interactions with CENP-C, CENP-N and HJURP, the latter of which may be important during CENP-A recycling at the replication fork [72,75,76]. Intriguingly, emerging evidence suggests that the stable maintenance of CENP-A chromatin, is itself under rather dynamic control involving transcription-mediated chromatin recycling and the SUMOylation status of multiple CCAN components [77–80]. These results point to the existence of multiple dynamic components in order to maintain the integrity of centromeric chromatin.

3. Neocentromeres, origins of a centromere paradigm shift

The first clear indication for the epigenetic regulation of the centromere came from the discovery of human neocentromeres, the first of which was described in 1993. This neocentromere was derived from a rearrangement of chromosome 10, resulting in a centromere-containing ring chromosome and an acentric linear chromosome lacking any centromeric DNA. The latter acquired centromere proteins at a novel location, constituting a functional centromere that rescued mitotic maintenance of the chromosome [15]. Following this initial discovery, around 100 neocentromeres that are isolated from human patients and found scattered throughout the genome have been described. The majority of these are associated with genomics rearrangements, suggesting that the necessity to maintain acentric DNA fragments is the driving force selecting neocentromere seeding events [81]. Indeed, the first attempts to experimentally induce neocentromeres in order to understand their formation and specification were based on the generation of chromosome breaks by global induced mutagenesis. In barley, induced chromosome breaks led to the formation of a neocentromere on an isochromosome of the short arm of barley chromosome 7H [82]. Similarly, in *D. melanogaster*, neocentromeres were obtained following chromosome breakage by gamma irradiation, appearing on acentric mini-chromosomes derived from the terminal region of the X chromosome [83]. Interestingly, it was later shown that in this species, strong overexpression of CENP-A is sufficient to induce neocentromere formation, occurring predominantly in regions adjacent to heterochromatin domains [54].

Most characterized neocentromeres emerged on acentric chromosomes fragments devoid of centromeric DNA, typically isolated from patients with birth defects or developmental delays. In contrast, a unique pseudodicentric neocentromere on Chromosome 4 (PD-NC4) was discovered that showed no apparent chromosomal rearrangements [18]. A selection event to rescue a genomic fragment is therefore not

apparent in this case. Moreover, while initially discovered in a child with developmental defects; this was a case of a familial neocentromere recovered from several family members who were otherwise healthy. PD-NC4 is an example of a centromere repositioning event as neocentromere formation occurred on a chromosome 4 without any detectable alteration retaining alphoid DNA marking the original locus [18]. These findings in patients prompted more targeted and specific approaches designed to elucidate neocentromere formation mechanisms. This new wave of approaches was based on the targeted deletion of an original centromere and further selection for retention of the remaining acentric chromosome (Fig. 2D).

4. Artificial systems for neocentromere generation

In the pathogenic yeast *Candida albicans*, regional centromeres can expand from 4 to 15 kb and CENP-A is assembled into unique sequences that cover around 3 kb on each chromosome which are surrounded by direct or inverted repeats lacking classical pericentric heterochromatin [84]. By taking advantage of the higher rates of homologous recombination in *Candida*, a selectable marker was used to replace the centromere of chromosome 5. Surviving cells maintained the chromosome by neocentromere formation that occurred either adjacent to the reporter gene insertion site, distal up to 400 kb away [85] or even further out [86] (Fig. 2D).

In *Schizosaccharomyces pombe*, centromeres expand 40–100 kb and are composed of two domains. The central domain where CENP-A nucleosomes are assembled, ranging from 10 to 15 kb that is bordered on either side by inverted repeats composed of heterochromatin [6]. Similar to *Candida*, the centromere I from fission yeast was deleted, in this case by Cre-mediated recombination. This approach directed the formation of an acentric chromosome that acquired neocentromeres at an approximate mean frequency of $6{,}2 \times 10^{-4}$ [87]. Neocentromeres formed adjacent to subtelomeric heterochromatin as mapped by ChIP-chip analysis of centromeric proteins (Fig. 2D). Another system for centromere inactivation in fission yeast is based on mutations of inner kinetochore components that cause pericentromeric heterochromatin spreading into the centromere core. After centromere inactivation, neocentromeres appeared preferentially in the pericentromeric heterochromatin [88]. As the neocentromeres tend to form adjacent to the original site, these repositioning events may be driven by the presence of residual CENP-A^{Cnp1} in that region.

Finally, in chicken DT40 cells, Cre recombinase-based experiments were designed to conditionally remove the centromere from chromosome Z. This is one of the three centromeres in chicken cells lacking tandem repetitive sequences featuring centromere domains of 30–40 kb [59]. The Z chromosome is also structurally distinct from the rest of the chicken genome. It is less gene-dense than any autosome and has a higher density of interspersed (mostly LINE) elements [89]. In this case, the mean frequency of neocentromere formation was $3{,}6 \times 10^{-6}$ and, similar to the yeast system, the majority of the neocentromeres formed in the vicinity of the endogenous centromere, although clones with neocentromeres in all different chromosome regions were also isolated [90] (Fig. 2D).

5. Common elements driving the formation of neocentromeres

The accumulated collection of different neocentromeres, as well as the artificial generation of neocentromeres in different species allows us to define shared features that are required for centromere specification. All neocentromeres described up to date are universally marked by CENP-A [17,91–93] (Fig. 3A). While perhaps not surprising, it underscores the critical role of the centromere-specific histone variant in building the structural core of the kinetochore [94]. In addition to CENP-A, many other CCAN components and kinetochore proteins are present at neocentromeres [95,96]. It should however be noted that in holocentric insects and a subset of fungi CENP-A and some other conserved centromere proteins are absent [32,35] and kinetoplasts lack essentially all centromere and kinetochore proteins that are identified in most model organisms [33,34]. Therefore, while CENP-A is crucial for standard models from yeast to humans, its role is by no means universal in the Eukaryotic domain and other mechanisms must exist to define and inherit centromeres.

6. Centromere size

Mapping neocentromere positions along the genome has allowed for a comparison of centromere domains and specific DNA sequences supporting centromere formation. One relevant parameter is centromere size. In *S.pombe*, the CENP-A^{Cnp1} domain expanded to around 20 kb in the artificially generated neocentromeres, a size comparable to the endogenous centromeres (15 kb) [87]. A similar picture emerges from chicken DT40 cells whose endogenous non-repetitive centromeres expand to ~35 kb while experimentally generated neocentromeres extended over 40 kb [90]. However, in *C.albicans*, neocentromeres tend to maintain a smaller CENP-A^{Cse4} domain (1 kb, 1.2 kb or 4 kb in different clones) compared to endogenous centromeres (around 3.3 kb) [85]. Similarly, in humans, neocentromeres appear to cover a smaller area than canonical centromeres with CENP-A domains spanning 80–300 kb [38,97] compared to an estimated area (based on the human X chromosome) of 1 Mbp on an α-satellite containing centromere [98]. Smaller human neocentromeres load less total CENP-A compared to endogenous α-satellite-bearing ones which may account for the instability associated with some neocentric chromosomes [99–101].

CENP-A distribution among centromeres within one cell is generally uniform and changes in CENP-A levels are regulated to ensure that, despite fluctuations, CENP-A remains centromere restricted at comparable levels [101]. Neocentromeres assemble less CENP-A but appear to do so on a smaller genomic locus in a non-random fashion, probably due to the contribution of local sequence or chromatin features [38]. Thus, despite their smaller size, neocentromeres might feature a similar CENP-A nucleosome density comparable to canonical centromeres.

Given the finding that neocentromeres can be significantly smaller, yet largely functional, opens an avenue to define the minimal chromatin size that will support centromere formation.

7. General features of centromeric chromatin

At the primary DNA sequence level, the most conserved feature across neocentromeres is the tendency to form preferentially on AT rich sequences [81,90], although the preference is slight and by no means sufficient to explain centromere formation. Moreover, some human neocentromeres are enriched in repetitive LINE elements and a potential role of the L1 retrotransposons in the regulation of neocentromere activity has been suggested [102]. At the chromatin level, more similarities arise. The centromere core is frequently flanked by heterochromatin enriched in histone modifications such as trimethylation of lysine 9 (H3K9me3) [103] (Fig. 3B). Within the core, CENP-A nucleosomes are interspersed with nucleosomes containing the canonical histone H3 and these H3 nucleosomes show different histone modification marks in different species. Most prominently, in *Drosophila* and human cells, dimethylation of lysine 4 (H3K4me2) is enriched at the centromere core and is an essential mark for its maintenance and function [104,105]. At chicken centromeres, H3K4me2 is present in lower amounts [106]. This modification is typically associated with active genes alongside H3 lysine 36 dimethylation. However, the centromere core does not uniquely correlate with active transcribed chromatin as it was shown to be hypoacetylated (at H3K9 or H4K5, K8, K12 and K16 residues), suggesting a centromere-specific chromatin status [104]. Nevertheless, a critical low level of acetylation, particularly H3K9Ac, is required to maintain centromere function and to prevent heterochromatin spreading into the centromere core causing subsequent kinetochore inactivation of human centromeres [107–109].

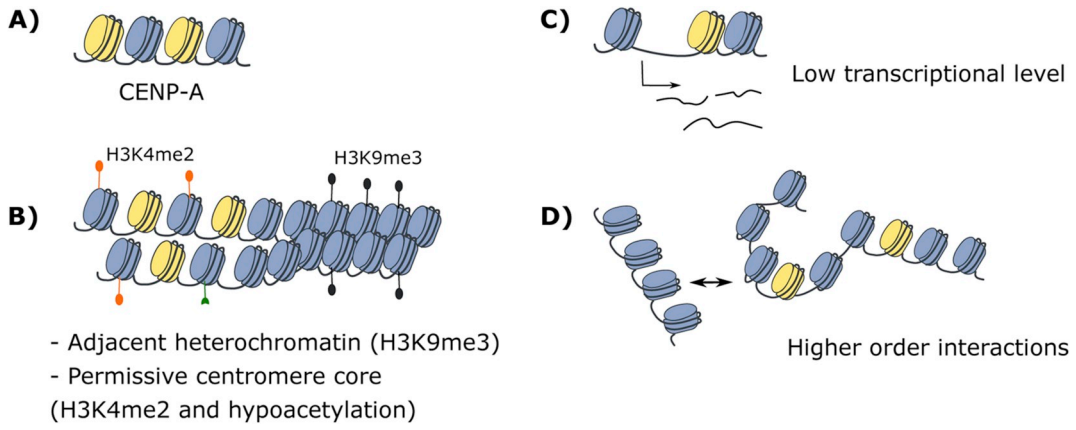

Fig. 3. Principal chromatin features involved in centromere specification. Centromeres are epigenetically defined by the presence of CENP-A chromatin ((A), yellow nucleosomes). Different histone modifications within the centromere core and surrounding areas are important for centromere function (B) and the balance between those modifications and low level transcriptional activity (C) must be finely tuned to maintain centromere identity. Neocentromere contacts with distal chromatin domains (D) also have a role in centromere function.

This chromatin structure is likely connected to the transcriptional status of the centromere. In humans, RNA polymerase II (Pol II) as well as transcription factors localize to mitotic centromeres, and RNAPol II disruption generates kinetochore-associated defects [110–112]. Moreover, transcription arising from the centromere has been described in several organisms such as fission yeast, *Drosophila*, mouse and humans [113]. On the other hand, tethering experiments manipulating centromeric transcription directly showed that strong transcription is deleterious to centromere function possibly displacing key centromere proteins. At the other extreme, strong repression leads to heterochromatin acquisition at the centromere core, also inactivating kinetochores [114]. Similarly, affecting the balance of chromatin modifications such as H3K4me2 in the centromere core, leads to altered centromeric transcription and causes loss of H3K9 acetylation and spreading of H3K9me3 heterochromatin into the centromere [108]. Therefore, the balance between histone modifications and low level transcriptional activity must be finely tuned to maintain centromere identity and function (Fig. 3C).

Finally, more recent work revealed that centromeres in chickens and human cells also show increased levels of histone H4 monomethylation in lysine 20 (H4K20me1) [115,116], a modification that has been related to DNA replication and DNA damage response but also to both activation and repression of transcription [117]. At centromeres H4K20me1 enrichment appears mainly on the histone H4 assembled into CENP-A containing nucleosomes once it is incorporated into chromatin, and reducing its levels affect kinetochore function [115].

The recovery of different neocentromeres in different species has allowed for the characterization of the chromatin status of several seeding sites. In *S. pombe* it was shown that neocentromeres preferentially form near telomeric heterochromatin and heterochromatin proteins are necessary for this formation [87]. Similarly, overexpression of CENP-A (CID) in *Drosophila* led to neocentromere formation preferentially near telomeres and pericentric heterochromatin coincident with transcriptionally silent, intergenic chromatin domains [54]. These results suggest a common role for heterochromatin in neocentromere site specification. However, such a role does not appear to be universally required as chicken centromeres on non-repetitive DNA and several human neocentromeres are devoid of typical heterochromatin [90,97]. Therefore, rather than a direct requirement for heterochromatin, the low level of transcription associated with such a chromatin state may be the driving force for neocentromere seeding, perhaps explaining why heterochromatin is not always necessary at neocentromeres [97]. This would be consistent with the fact that repetitive DNA transcription from endogenous centromeres has emerged as a highly conserved property for centromere function [113]. At neocentromeres the link to low transcription is less clear. In *C. albicans* neocentromeres emerged either in transcriptionally active or intergenic regions [85,118]. However, in chicken cells, the majority of neocentromeres appeared in transcriptionally inactive regions, while one was found at an actively transcribed locus. Interestingly, in this latter case, neocentromere formation resulted in a reduction of the transcriptional status of this region [90]. This indicates that neocentromere formation can modulate transcription to achieve an appropriate level, compatible with centromere function and may explain why neocentromeres do not appear in regions containing highly transcribed essential genes.

Therefore, although it is clear that an intermediate transcriptional status is required for maintaining centromere identity, it is not trivial to uncouple whether transcription is required for centromere function in a direct way, due to the production of centromeric RNAs or indirectly due to the chromatin remodelling and histone modifications associated with transcription [108,119,120]. Some of the centromere-derived transcripts have been shown to interact with centromere proteins and may have a direct role in centromeric protein recruitment [112,121–123].

The human neocentromere on chromosome 10 described above, originated in a region enriched in LINE repeats and showed active L1 retrotransposon transcription [102]. Further analysis using hybrid cells revealed that neocentromere activation did not silence the genes that were present in that region [124]. It would be interesting to analyse if experimentally generated neocentromeres that appear in silent regions acquire RNA Pol II binding and induce some level of transcription over time. Moreover, as neocentromeres are associated to unique DNA sequences they are useful tools to determine the enrichment of specific histone modifications.

8. The role of higher order chromatin features in centromere function

From a more recent perspective, analysis performed in *Candida*, revealed that neocentromeres generated at different loci along the chromosome resulted in kinetochores that cluster with active endogenous centromeres within the nucleus [86]. This result suggested that the higher order chromatin organization may be another important feature in defining sites that can support centromere formation. Indeed, 4C analysis performed in chicken DT40 cells revealed that following neocentromere formation, neocentromere sites interact with distal heterochromatin rich domains in a manner dependent on centromeric proteins [125]. Therefore, apart from DNA or chromatin features, the global genome architecture and 3D interactions that may play important roles in centromere function, could also be essential factors determining potential neocentromere sites (Fig. 3D).

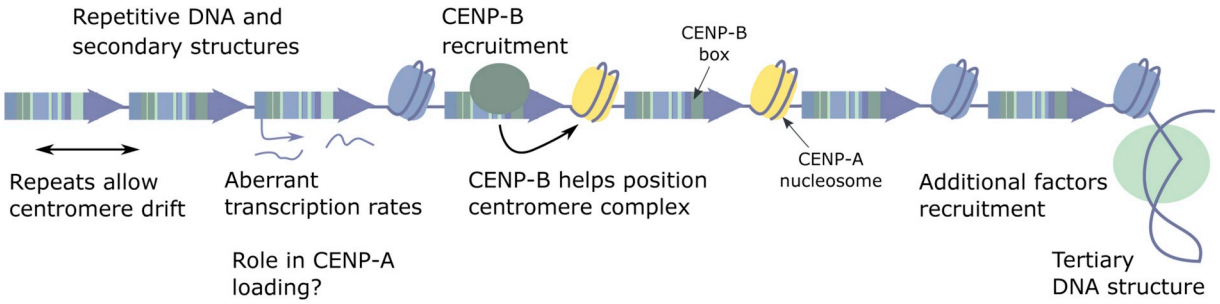

Fig. 4. The emerging role of DNA sequence elements in centromere function. Although centromeres are epigenetically defined there is a renewed focus on the contributing roles of DNA sequence elements. These include, CENP-B binding to alphoid DNA and a possible impact of repetitive arrays and secondary DNA structures unique to repetitive alphoid DNA on transcription and the recruitment of additional factors.

In yeast it has been shown that AT rich isochores tend to have a more extended chromatin conformation and they show more interaction in 3C analysis. Furthermore, AT rich regions show distinct patterns of histone modifications and gene expression profiles, in comparison to GC rich regions [126]. Consistent with this, human transcriptome analysis revealed a clustering of weakly expressed genes in domains (named antiridges) with a high AT content and a high LINE repeat density [127]. Therefore, several features that are associated with centromere formation also correlate with each other. Additionally, neocentromere specification may have different requirements in different species. These constraints make it difficult to arrive at a unique consensus on what are the key factors and what are peripheral contributors to centromere formation. The ability to generate neocentromeres at will in different models will be key to come to a better understanding of site selection for neocentromere seeding.

9. Centromere drifting of evolutionary new centromeres

As we have seen above, there is no universal agreement about what local features are responsible for neocentromere specification, and it is quite likely that random chance plays a major role. It is generally clear that chromatin-based epigenetic identity plays a dominant role. If indeed centromere inheritance is largely uncoupled form the underlying DNA sequence, this would make a clear prediction: Pure chromatin-based replication of centromere identity would be subject to stochastic fluctuations and drift if no DNA sequence-based elements restrict centromere protein position in cis. Indeed, recent evidence has shown that centromeres can migrate into surrounding areas over time. This centromere itinerancy has been described in nature in species containing native non-repetitive centromeres such as horses [128], donkeys [129] or orangutans [130]. These novel centromeres, which arise during evolution through repositioning, are known as evolutionary new centromeres (ENCs). Although many of these ENCs do contain repetitive DNA, they presumably originated in naïve regions and the acquisition of the repeats represents a later step in their evolution [100,131].

In particular, work developed using horse cells demonstrated the existence of different CENP-A domains at the non-repetitive DNA centromere from chromosome 11 among different individuals [132]. Moreover, by analysing heterozygous single nucleotide polymorphisms (SNPs) within the centromeric domains, revealed distinct 'positional epi-alleles', each one extending 80–160 kb across a region of about 500 kb. This indicates that CENP-A domains are mobile and centromere specification is not fixed [132]. Even more remarkable, in donkeys, centromeres are found on unique DNA sequences on either 15 or 16 chromosomes, indicating the existence of a centromere-polymorphism in the population [133]. Moreover, at these loci, CENP-A domains map to different positions within a 600 kb region across different individuals indicating these epi-alleles are relatively dynamic. In crosses between donkeys and horses individual centromere alleles transmitted stably to the hybrids but changes in local centromere position could be detected in one generation. Conversely, mitotic divisions by *in vitro* culturing of cells derived from these animals revealed that centromere position remained unaltered, suggesting a robust but mutable pattern of inheritance [133]. This is in line with previous analysis of the CENP-A domain on the endogenous non-repetitive chromosome Z in chicken cells. Centromere position varies among 21 independent isolates from a chicken laboratory cell stock, suggesting that centromere drift occurs during cell proliferation. However, subclones obtained from short-term culturing of one isolate showed a stable domain position indicating that the centromere drift can occur but centromere position is metastable. In fact, centromere drift was more frequent in CENP-U and CENP-S deficient cells indicating the centromere complex restricts movement [134].

10. From genetic centromere specification to epigenetics and back again

The local drift of CENP-A domains is expected from a self-templating epigenetically defined mechanism for centromere specification. In fact, due to the existence of ENCs in repetitive DNA tracks, it has been hypothesized that a possible role for the repetitive centromeric DNA is to generate a safe space for CENP-A drifting to occur, avoiding migration towards genomic regions containing essential genes [135] (Fig. 4). Thus, the presence of alpha-satellite DNA may promote centromere maintenance at evolutionary time scales by increasing kinetochore plasticity [135]. Apart from this role, the most obvious potential contribution of repetitive α-satellite DNA is the presence of CENP-B boxes, the cognate binding site for CENP-B [10–12] (Fig. 4). Once recruited, CENP-B interacts with CENP-A and also interacts and stabilizes CENP-C, therefore, enhancing the fidelity of centromere function [136]. Moreover, the presence of centromeric DNA has been shown to have a role in phasing CENP-A nucleosomes. This is more pronounced at CENP-B binding sites, indicating that both CENP-B and the satellite DNA itself play a role in nucleosome positioning within the centromere [38].

While dispensable at chromosomally formed neocentromeres, alphoid DNA including CENP-B boxes appears essential for the formation of human artificial chromosomes (HACs) that are initiated from a purified DNA template [107,137–139]. Clearly DNA sequence, in part via CENP-B recruitment has the potential to kick-start a centromere. Exactly why this property can be bypassed at chromosomally formed neocentromeres remains unknown but the dominant positive feedback driving CENP-A chromatin formation has been the principle argument to dismiss the role of genetic elements driving centromere specification.

Finally, the repetitive nature of the DNA sequences may also influence centromere function through its effect on transcription and repair mechanisms. As previously discussed, centromeric transcripts and transcription have been shown to play a role in centromeric chromatin assembly and maintenance [140]. Centromeric transcripts also have an impact on the pericentromeric heterochromatin state

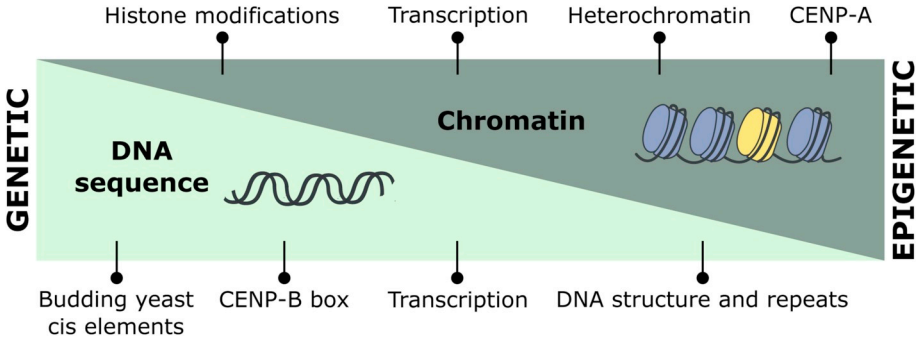

Fig. 5. Relative contributions of genetic and epigenetic factors in centromere specification. Key elements involved in centromere function are highlighted and ranked according to their genetic or epigenetic nature where budding yeast DNA sequences are strong cis elements and chromatin containing CENP-A nucleosomes in higher eukaryotes is strongly epigenetic.

[141]. The tandem repeat structure of centromeric DNA may influence transcription rate. Controlling polymerase speed and maintaining an intermediate level of transcription may be important factors in optimal centromere function [108,142] (Fig. 4). Furthermore, repetitive alpha satellite DNA may increase polymerase stalling and be more prone to the generation of secondary DNA structures, such as stem loops or other more complex DNA topologies that may play a role in the recruitment of other proteins that affect centromere function such as topoisomerases or recombination machinery [143]. In fact, *in vitro* reconstitution in Xenopus extracts of centromeric α-satellite DNA revealed that efficient centromeric DNA replication requires mismatch repair proteins which can bind mismatched hairpins and other DNA secondary structures [144]. In addition, R-loops, three-stranded nucleic acid structures that comprise nascent RNA that remains hybridized to the transcribed DNA strand during transcription, have also been detected at centromeres in mitosis leading to the recruitment of the ATR (ataxia telangiectasia mutated and Rad3-related) kinase. ATR activation stimulates the Aurora B pathway and plays a role in promoting accurate chromosome segregation [145]. Additionally, centromere proteins such as CENP-A, CENP-C, and CENP-T/W maintain centromere integrity and DNA repeat stability by supressing α-satellite rearrangements and centromere recombination [146]. Furthermore, CENP-A, along with CENP-N, CENP-T and CENP-U, have been shown to be recruited to DNA breaks [147], although the relevance of this observation is thus far unknown.

In Sum, while centromere specification and inheritance has a strong epigenetic component that can operate on seemingly any DNA template, there are key contributions of local cis elements beyond direct CENP-B protein recruitment. The relative contribution of genetic and epigenetic factors varies across species where genetic pathways are dominant in budding yeast, despite featuring a centromeric nucleosome. Conversely in humans, CENP-A is dominant despite conserved cis elements in centromeric DNA (Fig. 5). Other genetic and epigenetic features contribute with varying strength, some of which are revealed only in *de novo* centromere formation, when redundancy is minimal.

11. Future perspectives

Through the study of neocentromeres we have discovered several salient features of centromeric chromatin structure that specifies centromere positions. While there is no broad consensus on exactly what are the local DNA sequence and chromatin features favourable for centromeres to form, many advances have been made regarding the elements required for centromere specification.

Artificial systems for neocentromere formation, developed in different model organisms have been particularly powerful and have highlighted the requirement for the presence of heterochromatin and mild transcription levels. However, these features appear not to be universal requirements. Part of the lack of a consensus may be due species-specific differences in centromere organization with distinct requirements for local sequence and chromatin elements. This is why more models for neocentromere formation, particularly in human cells, are desired to detect possible commonalities and better define conditions permissive for centromere formation. So far human neocentromeres have been derived, long after their formation, from patients with only ~100 cases reported world-wide. Development of artificial systems to generate human neocentromeres at will in cell culture would provide larger numbers with better statistical power to tease out the salient features in centromere specification. Moreover, in contrast to established patient-derived neocentromeres, artificially generated cases would allow to time-stamp their formation and witness neocentromere birth, maturation and even evolution in direct comparison with the parent chromatin, prior to centromere formation.

The ability to create human neocentromeres in culture would further allow combination with the targeted or controlled manipulation of specific features, such as modulating transcription, chromatin structure or the presence of specific sequences. Discovering whether neocentromeres formation can be stimulated by specific elements will establish their causal role and facilitate defining the universal requirements for centromere formation. At present, neocentromere seeding frequencies are still very low, indicating we are far from understanding what it takes to make a centromere. Our ability to generate neocentromeres with a probability of “1” will be the ultimate measure of our true understanding of centromere formation.

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