Neocentromeres and epigenetically inherited features of centromeres

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Abstract

Neocentromeres are ectopic sites where new functional kinetochores assemble and permit chromosome segregation. Neocentromeres usually form following genomic alterations that remove or disrupt centromere function. The ability to form neocentromeres is conserved in eukaryotes ranging from fungi to mammals. Neocentromeres that rescue chromosome fragments in cells with gross chromosomal rearrangements are found in several types of human cancers, and in patients with developmental disabilities. In this review, we discuss the importance of neocentromeres to human health and evaluate recently developed model systems to study neocentromere formation, maintenance, and function in chromosome segregation. Additionally, studies of neocentromeres provide insight into native centromeres; analysis of neocentromeres found in human clinical samples and induced in model organisms distinguishes features of centromeres that are dependent on centromere DNA from features that are epigenetically inherited together with the formation of a functional kinetochore.

Keywords

neocentromere; centromere; kinetochore; chromosome segregation

Centromeres, the regions on each chromosome where kinetochores assemble and promote the attachment of spindle microtubules, are essential for chromosome segregation and genome integrity. In humans and almost all other eukaryotes, kinetochore assembly is not dependent on primary sequence, and centromeres are inherited epigenetically (Allshire and Karpen, 2008). The kinetochore protein CenH3/CENP-A, a histone H3 variant that replaces canonical H3 at centromeric chromatin, is a defining feature of centromeres (Buscaino et al., 2010). At least 100 kinetochore proteins have been associated with centromeres in humans (Liu et al., 2006) and many of these are conserved among eukaryotes. CENP-A is at or near the top of the kinetochore assembly dependency pathway (Liu et al., 2006, Mendiburo et al., 2011) and is critically important for proper centromere formation and function.

Neocentromeres are new sites of assembly of functional kinetochores at ectopic loci that rescue chromosome fragments in cells with gross chromosomal rearrangements (Marshall et al., 2008). Centromere positions are generally stable, but genomic alterations can remove or disrupt centromere function; neocentromeres that form elsewhere on the remaining chromosome restore the ability of that chromosome to segregate efficiently (Blom et al., 2010). In rare cases, neocentromeres form on otherwise normal chromosomes, without physical deletion of the native centromere, presumably following inactivation of the native centromere through unknown mechanisms (Amor et al., 2004, Liehr et al., 2010).

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"Evolutionary new centromeres" are important steps in speciation that involve centromere repositioning events that become fixed in the population (Ventura et al., 2007, Capozzi et al., 2009). In this review, we will discuss the importance of neocentromeres to human health and recently developed model systems that are used to study neocentromere formation and function.

Analysis of neocentromeres from clinical samples has revealed some characteristics of human neocentromeres, but this is necessarily limited to retrospective analysis. Insights into the mechanisms of neocentromere formation and function are more readily achieved from direct experiments using model organisms. Recently, induced neocentromere formation has been reported in *Drosophila melanogaster* (fruit flies), the fission yeast *Schizosaccharomyces pombe*, and *Candida albicans*, a multimorphic and pathogenic yeast. We will discuss the strengths of each model organism for understanding neocentromere formation and function as well as how they help us identify which centromere features are dependent on the native context and which features are epigenetically inherited together with kinetochore assembly. Finally, we will highlight some of the important open questions in the field.

Neocentromeres in human health

The majority of neocentromeres reported in clinical samples rescue acentric chromosome fragments associated with duplications or chromosomal rearrangements found in patients with developmental disabilities (Marshall et al., 2008). Such neocentromeres are more likely to be observed clinically because of phenotypes associated with the accompanying amplifications, deletions, or gene disruptions (Marshall et al., 2008). Neocentromeres stabilize genomic rearrangements that form small supernumerary marker chromosomes. Marker chromosomes are abnormal chromosomes formed by rearrangements or amplifications of specific genomic regions. Phenotypes associated with marker chromosomes stabilized by neocentromeres include: facial dysmorphisms, renal defects, short stature, and developmental delays (Mascarenhas et al., 2008, Burnside et al., 2011). Prenatal identification of neocentric marker chromosomes can be achieved by cytogenetic analysis of chromosomes from amniotic fluid and fetal blood cells (Mascarenhas et al., 2008).

In several types of human cancers, neocentromeres have rescued chromosome fragments that arose via gross chromosomal rearrangements (Sirvent et al., 2000, Blom et al., 2010). Solid tumors in cases of well-differentiated liposarcomas are cytogenetically characterized by the presence of a supernumerary ring or giant chromosome with amplified material from the 12q14–21 region combined with DNA from other chromosomes and a neocentromere (Italiano et al., 2009). Other types of human cancers associated with neocentromeres include: retinoblastoma (Morrissette et al., 2001), non-Hodgkin's lymphoma (Blom et al., 2010), acute myeloid leukemia (de Figueiredo et al., 2009), and lung cancer (Italiano et al., 2006). Interestingly, CENP-A is overexpressed in several types of cancer cells (Tomonaga et al., 2003, Amato et al., 2009) and high CENP-A expression correlated with shorter survival times in lung cancer patients (Wu et al., 2012). While overexpression of CENP-A does not appear to be sufficient for ectopic kinetochore assembly in the presence of native centromeres (Van Hooser et al., 2001), excess CENP-A may enhance extracentromeric CENP-A incorporation and thereby facilitate neocentromere formation on acentric supernumerary chromosomes. We suggest that neocentromeres may be underappreciated as a mechanism of stabilizing genomic alterations in cancer cells as only a small proportion of tumor samples are analyzed by cytogenetic techniques capable of detecting neocentromeres (Fletcher, 2005).

Some neocentromeres form in the absence of obvious chromosome rearrangements, and these have very little direct impact on human health. Importantly, these centromere repositioning events are stable through meiosis, as they have been inherited from one generation to the next. In two documented cases, the new centromere position was maintained through at least three generations (Tyler-Smith et al., 1999, Capozzi et al., 2009). While such centromere repositioning is rarely detected (Amor et al., 2004, Ventura et al., 2004, Capozzi et al., 2009, Hasson et al., 2011), it is likely that the rate of centromere repositioning events are underreported, since they are only identified by cytogenetic screening and cause no significant phenotypes.

"Evolutionary new centromeres" – centromere repositioning on an evolutionary scale

Evolutionary new centromeres are essentially neocentromeres that are inherited through many generations and become fixed in the population. Centromere repositioning via ectopic kinetochore assembly is a key step in the formation of evolutionary new centromeres. Evolutionary new centromeres often arise in gene deserts (Lomiento et al., 2008) and accumulate repetitive sequences (Ventura et al., 2007). These evolutionary events, detected via altered synteny of centromere-associated satellite DNA as well as through karyotype analysis, require that kinetochore proteins assemble at a new locus and no longer assemble at the old locus. Six human chromosomes have evolutionary new centromeres (Rocchi et al., 2012). For example, human centromere 6 repositioned from an ancestral location to its current location in a common ancestor of hominids (Capozzi et al., 2009). Comparison of the human and macaque genome reveals that 9 of 20 macaque chromosomes have evolutionary new centromeres that accumulated over the comparatively short time period of approximately 14 million years (Ventura et al., 2007). The large fraction of chromosomes with evolutionary new centromeres in humans and macaques indicates that, on an evolutionary time scale, centromere repositioning is a relatively frequent event. Evolutionary new centromeres are also found in other mammals (Rocchi et al., 2012) as well as in plants (e.g., rice (Nagaki et al., 2004) and cucurbits (Han et al., 2009)).

Retrospective analysis of human clinical neocentromere samples

Most of our current knowledge regarding neocentromere formation and function in chromosome segregation is derived from analysis of neocentromeres isolated from human clinical samples. More than 100 neocentromeres have been characterized in humans (Marshall et al., 2008, Alonso et al., 2010, Klein et al., 2012) and these neocentromeres show remarkable diversity in chromosome position and associated DNA sequences. Neocentromeres have been identified on 21 of 22 autosomic chromosomes as well as on the X and Y sex chromosomes (Marshall et al., 2008, Liehr et al., 2010). Neocentromeres are particularly common on specific chromosome regions including 3q, 13q and 15q (Marshall et al., 2008, Liehr et al., 2010). However, even among neocentromeres found in the same chromosomal band, the exact DNA sequences bound by CENP-A are unique in each case examined to date (Alonso et al., 2003, Marshall et al., 2008, Hasson et al., 2011). Sequences from the characteristic amplification of 12q14–21 in four supernumerary ring chromosomes from well-differentiated liposarcoma cases are not the sites of neocentromere formation, rather neocentromeres formed on amplified sequences from other chromosomes that are also part of the ring chromosome. The neocentromere sequences differed in each patient analyzed (Italiano et al., 2009).

Human centromeres are always found in highly repetitive regions containing alpha-satellite DNA (Vafa and Sullivan, 1997), yet neocentromeres form on very diverse DNA sequences and are not associated with alpha-satellite DNA. Some neocentromeres formed in repetitive

DNA regions (Hasson et al., 2011), but many neocentromeres are not associated with a significant amount of repetitive DNA (Alonso et al., 2010). Human neocentromeres form either in gene deserts or in regions that include actively transcribed genes (Marshall et al., 2008, Alonso et al., 2010). LINE retrotransposon sequences appear to be important for maintaining neocentric chromatin at some neocentromeres, as knockdown of transposon transcript levels impaired neocentromere function during mitosis (Chueh et al., 2009), but this has only been tested for one neocentromere thus far. In at least one instance, a neocentromere formed very close to the breakpoint of a chromosomal rearrangement (Hasson et al., 2011). This result is intriguing as CENP-A has been shown to bind transiently to sites of double-stranded DNA breaks (Zeitlin et al., 2009), suggesting that sites of DNA damage bound by CENP-A may initiate neocentromere formation.

Chromatin immunoprecipitation (ChIP) analyses have failed to define common features of human neocentromeres (Rocchi et al., 2012). Human centromeres have distinct chromatin domains. Centromeric chromatin is hypoacetylated and contains CENP-A nucleosomes interspersed with nucleosomes containing histone H3 dimethylated at lysine 4. The CENP-A core is surrounded by pericentric heterochromatin (Sullivan and Karpen, 2004). Proximal heterochromatic regions, identified by ChIP of histone H3 methylated at lysine 9 (H3K9me) and heterochromatin protein 1 (HP1), are not required either for neocentromere formation or for function in chromosome segregation (Alonso et al., 2010). However, the authors suggest that heterochromatin may contribute to chromosome cohesion, because neocentromeres with little or no detectable heterochromatin exhibited slight cohesion defects (Alonso et al., 2010).

Some neocentromeres also are more prone to chromosome missegregation than native centromeres. Neocentromere mosaicism (presence of the neocentromere in a subset of somatic cells) suggests that the chromosome carrying the neocentromere was lost in a subpopulation of the cells. This implies that some neocentromeres are less stable than native centromeres *in vivo* (Marshall et al., 2008). Human neocentromeres also have defects in the localization of Aurora B kinase, an essential regulator of kinetochore-microtubule attachments, and in error correction (Bassett et al., 2010). Determinants of the chromosome segregation efficiency of different neocentromere positions remain to be identified. One possible explanation for the reduced function in chromosome segregation is that some neocentromeres in humans have reduced incorporation of CENP-A relative to normal centromeres (Irvine et al., 2004). Additionally, human neocentromeres are frequently smaller than native centromeres with CENP-A binding regions of ~100kb at neocentromeres and CENP-A binding regions of ~200 kb to 1.5 Mb at native centromeres (Sullivan et al., 2011). In human artificial chromosomes, the length of chromosome regions surrounding the alpha-satellite DNA affects chromosome segregation accuracy (Rudd et al., 2003), potentially by contributing to the polar ejection forces necessary for chromosome alignment (Ke et al., 2009). Because neocentromeres are frequently found on chromosomes with short chromosome arms, some of the missegregation of chromosomes with neocentromeres may be attributed to the reduced polar ejection forces acting on these chromosomes.

Overall, chromosome regions that maintain neocentromere function show little similarity, and unlike native human neocentromeres, none of them are associated with alpha-satellite DNA. Currently, identification of common neocentromere features is limited by the low resolution of microscopy approaches and the limited number of neocentromere positions that have been analyzed with molecular biology techniques. High resolution analysis with tiling ChIP-chip or ChIP-seq of CENP-A binding regions in many neocentromere strains together with the use of model systems to develop testable hypothesis that can be tested in human cells should provide additional insight in the future.

Model organisms to understand mechanisms of neocentromere formation

The inability to manipulate neocentromeres in human cells limits the ability to understand the process of neocentromeres formation. The conservation of neocentromere formation from humans to plants to fungi justifies the study of neocentromeres in diverse organisms and suggests that such studies can provide significant insight into neocentromere formation and function.

In plants the term neocentromere is used in two different ways: "rescue" neocentromeres that, like human neocentromeres, appear following centromeric deletions or rearrangements, and neocentromere "knobs" that do not involve canonical kinetochore proteins (Nasuda et al., 2005, Topp et al., 2009). Neocentromere "knobs" drive selection of specific chromosomes during meiosis (Mroczek et al., 2006). Thus, their mechanisms of formation and transient maintenance are not likely to be representative of other types of neocentromeres (Dawe and Cande, 1996). For the purposes of this review, we will focus on plant "rescue" neocentromeres that are more similar to those found in humans. Neocentromeres that rescue chromosomes following inactivation of a native centromere or a chromosomal rearrangement occur in divergent plant species, including barley, maize and rice (Nasuda et al., 2005, Gong et al., 2009, Topp et al., 2009). Plant neocentromeres form either proximal to the native centromere (Nasuda et al., 2005) or at distal loci on the chromosome arms (Topp et al., 2009).

Kinetochores assembled at maize neocentromeres exhibit substantial variation in the binding of CENP-A (frequently called CenH3 in plants) among different isolates. Very low levels of CENP-A correlated with decreased chromosome stability and the amount of CENP-A at the neocentromere increased in subsequent generations. Chromosome stability also increased in later generations, suggesting that the neocentromere became more functional as it was inherited (Topp et al., 2009). The observation in plants that neocentromeres stabilize over time suggests that initial CENP-A binding and neocentromere initiation may happen at many locations, and that stable neocentromere formation requires locations where more CENP-A can incorporate so that a neocentromere of substantive size can be maintained.

Fruit flies (*D. melanogaster*) are a classic model for neocentromere formation. Ectopic centromeres can be induced by γ-irradiation-induced chromosome breakage or by overproduction of CENP-A (called CID in *D. melanogaster*) (Williams et al., 1998, Maggert and Karpen, 2001, Olszak et al., 2011) (Figure 1A). γ-irradiation-induced chromosome breakage resulted in formation of neocentromeres in the pericentric region (Maggert and Karpen, 2001). Overproduction of CENP-A resulted in ectopic kinetochore assembly at the boundaries of heterochromatin and euchromatin (Olszak et al., 2011). Additionally, tethering of CENP-A with a LacI/LacO system is sufficient for ectopic kinetochore assembly in fruit flies, and this ectopic kinetochore position is maintained even when the plasmid containing the LacI-CENP-A fusion is lost (Mendiburo et al., 2011). This is a powerful system to direct kinetochore assembly to specific loci in *D. melanogaster*.

Fission yeast (*S. pombe*) is unicellular model for studying neocentromere specification and kinetochore assembly. A useful feature is that it has only 3 chromosomes in its haploid genome. Inducible deletion of centromere DNA via Cre-Lox excision resulted in neocentromere formation adjacent to the telomeres but not at internal loci (Ishii et al., 2008) (Figure 1B). Similar to ectopic centromeres induced by overproduction of CENP-A in *Drosophila*, *S. pombe* neocentromeres are found at the borders of euchromatin and heterochromatin (Ishii et al., 2008). Furthermore, neocentromere formation in *S. pombe* requires functional heterochromatin proteins. In the absence of RNA interference-dependent heterochromatin, the frequency of chromosome rescue via neocentromere formation was

much lower than an alternative mechanism of chromosome rescue (Ishii et al., 2008). Thus, chromatin context strongly influences neocentromere formation in *S. pombe* and *D. melanogaster*. Some human neocentromeres also form at the boundaries of euchromatin and heterochromatin (Wong et al., 2006), however analysis of clinical human neocentromeres indicates that many chromosome contexts may be permissive for human neocentromeres.

Recently, *Candida albicans*, a human fungal pathogen, has emerged as a promising model organism for studying neocentromere formation. *C. albicans* has a diploid genome and small (~3–4 kb/central core) regional centromeres (Baum et al., 2006, Ketel et al., 2009). Neocentromere formation can be induced in *C. albicans* by deleting the native centromere and replacing it with a selectable marker (Ketel et al., 2009) (Figure 1C). Neocentromeres form proximal to the deleted native centromere locus or at distal positions along the chromosome arms (Ketel et al., 2009) as has been observed for human neocentromeres (Marshall et al., 2008, Ketel et al., 2009). Five neocentromere locations on chromosome 5 in *C. albicans* have been described (Ketel et al., 2009). An additional 17 neocentromere positions on chromosome 5 have been identified (unpublished observations). Similar to some human neocentromeres (and unlike native human centromeres), *C. albicans* neocentromeres are not associated with heterochromatin (Alonso et al., 2010). Additionally, neocentromeres in *C. albicans* are associated with larger than average intergenic regions, but can form in both transcriptionally active and in intergenic non-expressed regions (Ketel et al., 2009). Interestingly, both human neocentromeres (Saffery et al., 2003, Wong et al., 2006, Alonso et al., 2010) and plant centromeres can form on transcriptionally active regions (Nagaki et al., 2004). Recent work also suggests that active transcription is important for mammalian centromere function (Chan et al., 2012).

Epigenetically inherited features of centromeres

In both human cells and model systems, neocentromeres are a powerful tool for distinguishing epigenetically inherited features of centromeres from proteins recruited by centromere DNA sequences. A pseudo-dicentric human chromosome (containing a neocentromere with a functional kinetochore, as well as an inactivated native centromere alpha-satellite sequences) has been used to identify features associated with the functional kinetochore, with alpha-satellite DNA sequences or with both (Amor et al., 2004, Bassett et al., 2010). HP1α, a marker of heterochromatin, localized to both the inactivated alpha-satellite sequences and the functional kinetochore (Amor et al., 2004). Further analysis of this pseudo-dicentric chromosome found that all characterized kinetochore proteins, except for CENP-B, associated with the functional kinetochore, rather than with the vestigial alpha-satellite sequences (Bassett et al., 2010). CENP-B is recruited to the centromere in a sequence-dependent manner (Ohzeki et al., 2002) by alpha-satellite sequences not found at neocentromeres, suggesting CENP-B is not necessary for kinetochore assembly. Aurora B kinase is recruited to the functional kinetochore, although its localization pattern is altered. The altered localization pattern results in the reduced ability of Aurora B kinase to detect and correct chromosome-spindle misattachments (Bassett et al., 2010).

In fungi, cohesin binding is enriched near centromeres in *S. cerevisiae* (Weber et al., 2004). Pericentric cohesin binding is dependent on the presence of a functional kinetochore and has been proposed to enhance chromosome segregation by stabilizing a looped DNA structure around the centromere (Yeh et al., 2008). In *S. pombe* cohesin binds to pericentric regions and this binding is dependent on heterochromatin proteins (Nonaka et al., 2002). Pericentric cohesin binding in *C. albicans* appears to be linked with the functional epigenetic neocentromere rather than the native centromere (unpublished observations), but the roles of cohesin in neocentromere formation and function are not yet fully characterized.

Neocentromeres have provided particularly valuable insight into the link between DNA replication and centromeres. A specific replication timing pattern for centromeres is an appealing mechanism for facilitating the maintenance of centromere specification. Initial studies in flies and humans did not detect a specific timing pattern for centromeres (Shelby et al., 2000, Ahmad and Henikoff, 2001, Lo et al., 2001, Sullivan and Karpen, 2001), however these experiments were limited by the low resolution of microscopy experiments. Using higher resolution techniques, such as microarrays, fungal centromeres have been shown to replicate early in *S. cerevisiae* (Raghuraman et al., 2001), *C. albicans* and *S. pombe* (Koren et al., 2010) (Figure 2). Centromeres in *C. albicans* were the earliest and most efficient origins in the genome (Koren et al., 2010), and they contain evolutionarily conserved sequence biases (e.g., GC-skew (Sernova and Gelfand, 2008)) indicative of constitutive origin firing (Figure 2B and (Koren et al., 2010)). To determine if efficient replication was determined by the chromosomal context or by the presence of a functional neocentromere, replication timing was measured in a *C. albicans* strain with a homozygous neocentromere. Neocentromere formation dramatically shifted the replication timing pattern on the chromosome such that the neocentromere region now contained the earliest and most efficient origin (Koren et al., 2010) (Figure 2D). Origin recognition complex (ORC) did not bind the locus prior to neocentromere formation, but was recruited following neocentromere formation (Koren et al., 2010). Increasing CENP-A (called Cse4 in *C. albicans*) levels results in increased recruitment of kinetochore proteins (Burrack et al., 2011) and ORC (Koren et al., 2010) to centromere DNA, further suggesting that a kinetochore component recruits ORC and enhances replication efficiency of centromeres and neocentromeres.

Early replication of centromeres in fungi provides a compelling model for the coordination of CENP-A incorporation and DNA replication (Figure 2E). CENP-A is incorporated in S-phase in *S. cerevisiae* (Pearson et al., 2004) and in S- and G2-phase in *S. pombe* (Takayama et al., 2008). Coordinated replication of centromeres and incorporation of CENP-A early in S-phase may help limit extracentromeric incorporation of CENP-A and enhance the inheritance of centromeres in these fungi. CENP-A is incorporated during mitosis in *D. melanogaster* cells (Mellone et al., 2011) and in G1 in human cells (Dunleavy et al., 2009, Foltz et al., 2009). The lack of apparent coordination of replication timing among centromeres in humans and flies may be due to insufficient resolution or may be attributed to differences in the timing of CENP-A incorporation in metazoans. Further high-resolution studies of metazoan centromeres, especially using neocentromeres where repetitive sequences are not as prevalent, will help clarify whether specific replication timing is also a feature of metazoan centromeres.

Open questions and future directions

While recent studies have begun to explore the mechanisms of neocentromere formation, inheritance and function, many open questions remain. A better understanding of neocentromeres will inform our general understanding of centromere and kinetochore assembly and maintenance as well as potentially open up new diagnostic and/or treatment options for patients with congenital neocentromeres or cancers involving neocentromeres.

The mechanism that initiates neocentromere formation is a particularly interesting topic for future study. CENP-A is primarily found at centromere regions, but CENP-A also binds ectopic locations in response to DNA damage (Zeitlin et al., 2009) or upon overexpression of CENP-A (Van Hooser et al., 2001, Heun et al., 2006). One current hypothesis for neocentromere formation is that it is may be initiated by small extracentromeric binding of CENP-A. Overexpression of CENP-A in *S. pombe* (Chen et al., 2003, Joglekar et al., 2008) and *C. albicans* (Burrack et al., 2011) results in increased binding of CENP-A to centromere DNA sequences. While overexpression of CENP-A in the absence of disruption to the native

centromere does not result in significant changes in extracentromeric incorporation of CENP-A in *S. pombe* (Song et al., 2008) or in *C. albicans* ((Burrack et al., 2011)), small amounts of CENP-A binding may be sufficient in the absence of a native centromere. The three-dimensional organization of the genome within the nucleus influences diverse biological processes including genome rearrangements (Zhang et al., 2012) and gene expression (Gheldof et al., 2010). Chromosome conformation, looping, and position within the nucleus potentially influence neocentromere site selection, as neocentromeres may preferentially form in close physical proximity to CENP-A loading regions. *C. albicans* and *S. pombe* may be excellent model systems to test the hypothesis that excess CENP-A incorporation facilitates neocentromere formation when the native centromere is inactivated and to explore the relationship between genome organization and neocentromere formation.

Another important question is whether the control of neocentromere formation occurs at the level of neocentromere maintenance. This is based on the idea that potential neocentromere initiation occurs constantly by CENP-A incorporation at ectopic sites, but that establishment and maintenance of neocentromere formation is normally inhibited through a dominant negative effect exerted by the presence of a functional native centromere elsewhere on the chromosome (Dalal et al., 2007). Dicentric chromosomes, especially those with centromeres far apart from each other, are more prone to breakage during segregation than monocentric chromosomes (Koshland et al., 1987). Human dicentric chromosomes can be stably maintained during mitosis, but inactivation of one centromere is observed both *in vivo* and *in vitro,* suggesting that functionally dicentric chromosomes can be detected and inactivated (Stimpson et al., 2010). Alternatively, does neocentromere formation initiate only after a native centromere has been inactivated? Several model systems for understanding the inactivation of dicentric chromosomes may provide insight into the mechanisms that result in the formation of one, and only one neocentromere per chromosome. In addition to providing insight into regions favorable for neocentromere formation, CENP-A overproduction in *Drosophila* may be a particularly advantageous model for understanding how dicentric centromeres are selected (Olszak et al., 2011). Recent studies in *S. pombe* (Sato et al., 2012) and humans (Stimpson et al., 2010) have also provided insight into mechanisms that inactivate dicentric chromosomes. An interesting area of future research will be to induce neocentromere formation while manipulating factors important for centromere inactivation on dicentric chromosomes. This will determine whether the factors that inactivate native centromeres are the same factors preventing the simultaneous formation of multiple neocentromeres.

In fungi, early DNA replication conferred by kinetochore assembly may enhance neocentromere inheritance, but overall, the mechanisms allowing neocentromeres to be maintained once they form at a specific location have not been well characterized. In the model system *C. albicans,* both short-range and long-range neocentromere movements were observed (Ketel et al., 2009), indicating that the inheritance of neocentromere position on the DNA is impaired relative to that of native centromeres that have been maintained at the same loci over millions of years of evolution (Padmanabhan et al., 2008).

Changes in chromatin structure and histone modification patterns can also induce neocentromere movement in humans (Craig et al., 2003). Comparing histone modifications associated with native centromeres to those associated with neocentromeres may provide further insight into the mechanisms promoting the stable inheritance of centromere position. The role of CENP-A assembly by HJURP (also known as Scm3 in fungi), which is required for CENP-A incorporation at native centromeres (Dunleavy et al., 2009, Foltz et al., 2009), in neocentromere assembly and maintenance has not yet been addressed. Another open question is whether HJURP/Scm3 association with neocentromeres is similar to that at

native centromeres. Additionally, are all neocentromeres equally likely to move to new chromosomal positions or are some more positionally stable than others?

Once neocentromeres form, how do they alter the chromosomal locus and what DNA features are associated with the loci at which neocentromeres form? The LacI/LacO tethering system in *D. melanogaster* permits targeting of neocentromere formation to specific chromosomal regions (Mendiburo et al., 2011). This model may be useful for determining how neocentromere formation alters the surrounding chromosomal region. Additionally, *C. albicans* could be an especially useful model to identify factors that determine the location of neocentromere formation on the chromosome because of its small centromere size (~3–4 kb). Native centromeres in *C. albicans* each have a unique sequence and, unlike most regional centromeres, have been sequenced completely (Sanyal et al., 2004), permitting detailed comparison of DNA features at native centromere and neocentromeres and facilitating a comparison of the levels of kinetochore proteins and other biological markers (such as histone modifications) associated with neocentromeres and native centromeres by ChIP.

Transcriptional activity has been detected at some human neocentromeres (Saffery et al., 2003, Wong et al., 2006, Alonso et al., 2010), as well as at some plant centromeres (Nagaki et al., 2004). In *C. albicans*, the ability to compare high resolution transcript profiles at neocentromere loci in isogenic strains with native centromeres and neocentromeres will determine the positive and/or negative effects of transcription on neocentromere formation and function in chromosome segregation.

As described above, different human neocentromeres have different chromosome segregation efficiencies (Marshall et al., 2008) and error correction abilities (Bassett et al., 2010). While Aurora B localization and cohesion defects contribute to neocentromere chromosome segregation fidelity (Alonso et al., 2010, Bassett et al., 2010), they do not account for the broad range of mosaicism that is observed for human chromosomes with neocentromere. Thus, a current challenge is to identify the other factors that contribute to the segregation accuracy of chromosomes with neocentromeres. Neocentromeres in *C. albicans* are marked with a counter-selectable marker, allowing selection both for and against the presence of the neocentromere. This makes *C. albicans* a particularly useful model organism for the analysis of the segregation fidelity of chromosomes with neocentromeres.

The study of neocentromeres is advancing very rapidly. Methods to analyze human neocentromeres in greater numbers and at higher resolution will improve our ability to understand the events that gave rise to human neocentromeres in clinical isolates. Additionally, model systems provide the ability to induce and manipulate neocentromeres, permitting direct hypothesis testing and providing mechanistic models that can subsequently be tested in human isolates. This iterative process of testing hypotheses (often inspired by observations in clinical isolates) in model organisms, developing mechanistic models that explain the results, and then developing methods to test these mechanistic models in clinical isolates should greatly increase our understanding of neocentromere formation and maintenance.

Acknowledgments

Abbreviations

CEN	centromere
CENP-A	centromere protein A, a centromere-specific histone H3 variant (also known as CenH3)
CENP-B	centromere protein B
ChIP	chromatin immunoprecipitation
ChIP-chip	chromatin immunoprecipitation with microarray analysis
ChIP-seq	chromatin immunoprecipitation with high-throughput sequencing analysis
H3K9me	methylated lysine 9 on histone H3
HJURP	Holliday junction recognition protein (also known as Scm3 in fungi)
HP1	heterochromatin protein 1
LacI	*lac* (lactose) repressor
LacO	*lac* (lactose) operator
LINE	long interspersed nuclear elements
ORC	origin recognition complexl
Scm3	suppressor of chromosome missegregation 3 (also known as HJURP in metazoans)

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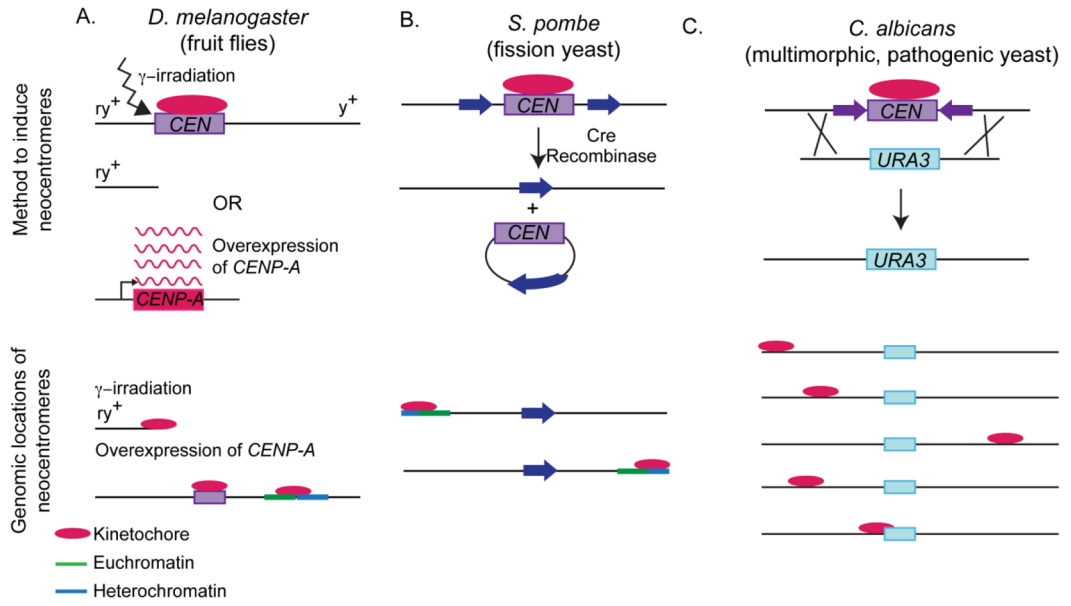
Figure 1. Models to induce neocentromere formation
(A) *D. melanogaster* ectopic kinetochores can be induced by γ-irradiation or overproduction of CENP-A. Following, γ-irradiation, chromosome fragments were stabilized by neocentromeres if the fragment was adjacent to the native centromere. Following CENP-A overexpression, ectopic kinetochores assembled at the border of euchromatin and heterochromatin, resulting in dicentric chromosomes. (B) *S. pombe* neocentromeres can be induced by recombination-mediated deletion of the native centromere. All neocentromeres formed near subtelomeres. (C) *C. albicans* neocentromeres can be induced by replacing the native centromere with a selectable marker through by DNA transformation. Neocentromeres formed proximal to the native centromere or distal to the native centromeres on chromosome arms. Drawings not to scale.
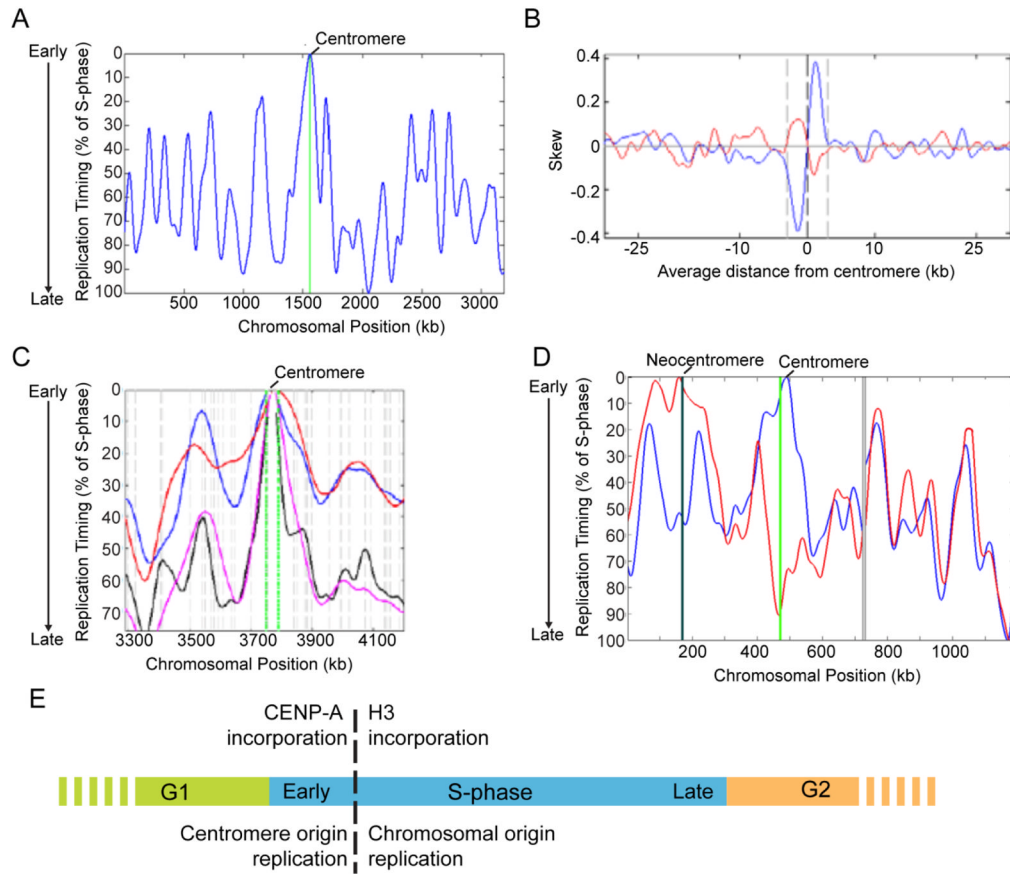
Figure 2. Efficient DNA replication at fungal centromeres
(A) Centromeres are the earliest and most efficient origins on each *C. albicans* chromosome.
The replication timing profile for chromosome in wild-type cells is indicated in blue. The
position of *CEN1* is indicated with a green line. (B) Centromere DNA in *C. albicans* and
related species contains GC-skew, a replication-dependent strand-bias sequence pattern
characteristic of constitutively active replication origins. Mean GC-skew (blue) and AT-
skew (red) skew at the *C. albicans* centromere regions from all chromosomes are shown. (C)
In *S. pombe*, *CEN1* replicates earliest of all loci on chromosome 1. The centromere is
indicated in green, while black, magenta, blue and red lines indicate data from different *S.
pombe* replication timing microarray experiments (Feng et al., 2006, Heichinger et al., 2006,
Mickle et al., 2007). (D) Neocentromere loci become the earliest, most efficient origins
following kinetochore assembly, indicating that the replication pattern is determined by the
presence of a function kinetochore. The native centromere (green line) is the earliest
replicating region in wild-type cells (blue line), while the neocentromere locus
approximately 170kb from the left telomere (black line) is the earliest replicating region in
cells with this homozygous neocentromere (red line). Gray lines indicate a gap in data from
the multiple repeat sequences in *C. albicans* on the right arm of chromosome 5. (E) Model
for coordination of replication timing and maintenance of centromere specification. Adapted
from (Koren et al., 2010).