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Neocentrics and Holokinetics (Holocentrics): Chromosomes out of the Centromeric Rules

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Abstract
The centromere appears as a single constriction at mitotic metaphase in most eukaryotic chromosomes. Holokinetic chromosomes are the exception to this rule because they do not show any centromeric constrictions. Holokinetic chromosomes are usually forgotten in most reviews about centromeres, despite their presence in a number of animal and plant species. They are generally linked to very intriguing and unusual mechanisms of mitosis and meiosis. Holokinetic chromosomes differ from monocentric chromosomes not only in the extension of the kinetochore plate, but also in many other peculiar karyological features, which could be understood as the 'holokinetic syndrome' that is reviewed in detail. Together with holokinetic chromosomes we review neocentromeric activity, a similarly intriguing case of regions able to pull chromosomes towards the poles without showing the main components reported to be essential to centromeric function. A neocentromere is a chromosomal region different from the true centromere in structure, DNA sequence and location, but is able to lead chromosomes to the cell poles in special circumstances. Neocentromeres have been reported in plants and animals showing different features. Both in humans and *Drosophila*, neocentric activity appears in somatic cells with defective chromosomes lacking a functional centromere. In most cases in plants, neocentromeres appear in chromosomes which have normal centromeres, but are active only during meiosis. Because of examples such as spontaneous or induced neocentromeres and holokinetic chromosomes, it is becoming less surprising that different structures and DNA sequences of centromeres appear in evolution.

The centromere is the structure responsible for chromosome movement in eukaryotes. Its function is based in 3 processes: sister chromatid cohesion which establishes chromosome polarity at mitosis and meiosis [Dej and Orr-Weaver, 2000]; kinetochore assembly and microtubule anchorage in centromeric heterochromatin [Allshire, 1997; Rieder and Salmon, 1998], and check-point of anaphase starts when all chromosomes are properly joined to microtubules [Rieder et al., 1994].

The present knowledge allows distinguishing various components in the centromeric region: (i) the centromeric DNA, located at the primary constriction and generally containing megabases of repetitive sequences, (ii) the

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modified H3 histones forming special nucleosomes permanently joined to centromeric DNA at the functional centromere, and (iii) the protein-rich kinetochore, joining centromeric chromatin to microtubules, which in turn may be subdivided into inner kinetochore, centromeric chromatin interface, and outer kinetochore as microtubule-binding interface [Santaguida and Musacchio, 2009].

Centromeric proteins and most mechanisms involved in centromeric functions are similar in different organisms, in such a way that centromere function is highly conserved in evolution. However, centromeric DNA shows remarkable sequence variability between species or even amongst different chromosomes of the same species, as for example between A and B chromosomes [Jones et al., 2008]. This makes it remarkably difficult to define and identify the DNA elements responsible for centromere activity. The 'centromere paradox', referring to the lack of evolutionary conservation of centromeric DNA sequences, with strongly conserved centromere function is still unsolved. Panchencko and Black [2009] provide a recent review on the structure of the centromere in the best studied organisms (yeast, maize, *Drosophila* and human). The most important protein determining centromere function is the modified histone CENH3. CENH3 differs in length and composition of the NH2 end from the H3 histone present in the rest of the nucleosomes [Palmer et al., 1991; Talbert, 2002]. Unfortunately, this protein has been named differently in different species: CENP-A in mammals, CID in *Drosophila,* Cse4p in budding yeast and CENH-3 in plants [Henikoff et al., 2000; Zhong et al., 2002; Black and Basset, 2008].

Centromere heterochromatin consists of megabases of highly repetitive sequences [Allshire, 1997]. This fact might allow a tertiary organization of DNA, somehow facilitating the assembly of centromeric proteins. On the other hand, the diversity of DNA sequences and the high conservation of centromeric proteins indicate that certain proteins might be more important in determining centromeric identity than the DNA sequence itself. Houben and Schubert [2003] proposed that the CENH3 histone might play a key role signalling the regions corresponding to kinetochores. It is proposed that the functional centromere region should contain the chromatin region whose nucleosomes are formed by CENH3, although other determinants of centromere identity might be present [Henikoff et al., 2000], as suggested by dicentric chromosomes showing CENH3 in the active centromere only [Warburton et al., 1997]. Interestingly, centromeric nucleosomes containing CENH3 in *Drosophila* consist of a histone tetramer, instead of an octamer as in the rest of the chromosomes [Dalal et al., 2007].

Centromere identity may be also determined by epigenetic modifications such as DNA methylation, histone acetylation and/or phosphorylation, etc. [Karpen and Allshire, 1997; Choo, 2000]. The current view is that the location of the centromere is epigenetically defined and that the combination of the special structure of centromeric heterochromatin with epigenetic modifications determines centromere function [Black and Basset, 2008; Lamb et al., 2008; Panchencko and Black, 2009].

Observed with the light microscope, the centromere appears as a single constriction at mitotic metaphase in most eukaryotic chromosomes due to the special features of the centromeric heterochromatin and the accumulation of centromere proteins. Holokinetic chromosomes are the exception to this general rule because they do not show any centromeric constriction. Holokinetic chromosomes are usually forgotten in most reviews about centromeres, but they are present in a number of animal and plant species generally linked to very intriguing and unusual mechanisms of mitosis and meiosis. Together with holokinetic chromosomes we review in the present paper neocentromeric activity, a similarly intriguing case of regions able to pull chromosomes towards the poles without showing the main components previously described as essential to centromeric function.

Neocentromeres

A neocentromere is a chromosomal region different from the true centromere in structure, DNA sequence and location, but is able to lead chromosomes to the cell poles in special circumstances. Neocentromeres are of interest for structural, functional and evolutionary information, and neocentromeres may be an important tool for identifying the main elements of centromere determination. Establishing the intervening elements in neocentric activation of a non-centromeric chromosome region is a useful approximation for understanding centromere function. For example, the mere existence of neocentromeres demonstrates that centromere DNA sequence itself is not essential for determining centromere function.

Neocentromeres were first described in rye by Katterman [1939], calling them 'T chromosomes' to describe the terminal stretching to the poles. Later on, Rhoades and Vilkomerson [1942] found them in maize using the term 'neocentromeres' with higher success.

Neocentromeres have been reported in plants and animals showing different features. Both in humans and *Drosophila*, neocentric activity appears in somatic cells in defective chromosomes lacking a functional centromere; in this manner the neocentromere substitutes centromeric activity and assures the transmission of these acentric chromosomes during cell division. In contrast, plant neocentromeres generally appear in chromosomes having normal centromeres, being active only during meiosis.

In *Drosophila melanogaster*, neocentromeres were observed in acentric minichromosomes in euchromatic regions close to the centromere, they were able to join the centromeric protein ZW10 and microtubules, and were transmitted in mitosis and meiosis [Murphy and Karpen, 1995; Williams et al., 1998]. Maggert and Karpen [2001] found other acentric fragments formed by different euchromatic regions unable to display neocentric features, indicating that pericentromeric regions might keep epigenetic determinants necessary for neocentric activity.

Human neocentromeres [Voullaire et al., 1993; du Sart et al., 1997; Choo, 1998; Marshall et al., 2008] are the best characterized. They appear in marker chromosomes (acentric fragments produced after chromosome breakage) conferring the property of being mitotically stable. The most remarkable feature is their lack of alpha satellites, which is considered the key DNA sequence for centromeric function in humans [Choo, 1997]. However, these neocentromeres join a functional kinetochore with more than 20 centromeric proteins including CENP-A, CENP-C and CENP-E [Choo, 1997; Saffery et al., 2000]. More than 60 different neocentromeres have been reported in different locations on all human chromosomes [Amor and Choo, 2002].

Plant neocentromeres have been observed in intact chromosomes at meiosis in 13 species of flowering plants and one in moss [Dawe and Hiatt, 2004]. The best characterized neocentromeres are those of rye and maize. Only 2 cases have been reported in mitotic chromosomes, one in barley [Nasuda et al., 2005] and the other in maize [Topp et al., 2009]. Nasuda et al. [2005] first reported isochromosomes and their derived telosomes in barley, which behaved similarly to human neocentromeres as they lacked most of the features of the barley centromere (C-band, the barley-specific centromere satellite and retroelements) but, interestingly, centromeric proteins localized at the neocentromeric region.

In plants, the canonical centromeres contain 2 types of repetitive sequences: tandem repetitions of short elements (satellites), similar in size and organization to the primate alpha satellite, and disperse repetitions of retrotransposon-like elements. Generally, centromeric retrotransposons are conserved between phylogenetically related species, whereas centromeric satellites are species-specific [Jiang et al., 2003]. In cereals, the retrotransposons belong to the CR family, derived from the Ty3/gypsy retrotransposons [Langdon et al., 2000b]. The CR family has 2 remarkable features: its exclusive location at the centromeric region, and its high degree of evolutionary conservation (up to 80%) amongst cereal species diverged more than 50 million years ago [Langdon et al., 2000a; Nagaki et al., 2003]. Rice and maize centromeres are the best studied, showing a remarkable organizational similarity. Sequencing genomic libraries of both species has shown that the main DNA sequences in rice are the CentO satellite with a 155-bp monomer [Dong et al., 1998], and the centromeric retrotransposon CRR [Cheng et al., 2002]. In maize, the main sequences are the satellite CentC with a 156-bp monomer [Ananiev et al., 1998a] and the centromeric retrotransposon CRM [Nagaki et al., 2003]. Do plant neocentromeres contain sequences similar to the canonical centromere sequences, or is the molecular composition completely different?

Neocentromeres in Maize

In maize, neocentric activity was first described in the terminal heterochromatic knobs at both meiotic divisions [Rhoades and Vilkomerson, 1942]. At the light microscope, neocentromeres are observed as heterochromatin stretched to the poles in such a way that the chromosomes look dicentric or polycentric. The stretching is particularly conspicuous at metaphase II when the true centromeres lay on the equatorial plate and the neocentromeres strongly pull the chromosome ends to the poles. However, chromosome fragments are not produced, even when centromere and neocentromere orientation may be opposite, because neocentromere tension disappears before anaphase ends [Rhoades, 1952; Yu et al., 1997].

Neocentromere activation requires the presence in the same cells of the abnormal chromosome 10 (Ab10), characterized by a large knob in the long arm [Rhoades and Vilkomerson, 1942; Peacock et al., 1981]. The gene *supressor of meiotic drive 1 (smd1)* is located in this region and controls neocentromere activity [Dawe and Cande, 1996].

Two different tandem repetitive DNA sequences have been reported in maize knobs: the 180-bp repetition called Zm4-21 [Peacock et al., 1981], and the 350-bp TR-1 sequence [Ananiev et al., 1998b; González-Sánchez et al., 2007]. Both sequences may be simultaneously present or one may be predominant, interrupted by retrotransposons. This structure is similar to centromeric regions of

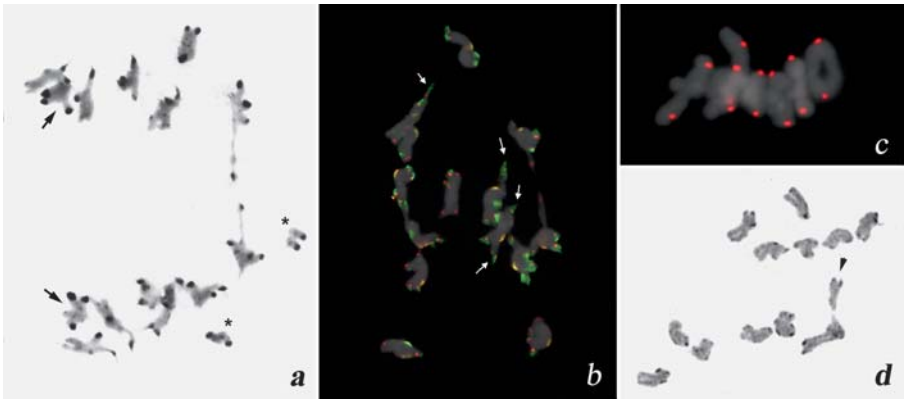

Fig. 1. Rye terminal neocentromeres. **a** C-banded anaphase I showing terminal neocentromeric activity in all chromosomes. The B chromosomes (asterisks) contain subtelomeric heterochromatin, but never form neocentromeres, indicating that not every subtelomeric heterochromatin is able to acquire neocentric activity [Manzanero et al., 2003]. **b** FISH in anaphase I using the subtelomeric sequence pSc200 (green), the centromeric sequence Bilby (orange) and the telomeric sequence pAtT4 (red). Arrows point to examples where the stretched sequence is subtelomeric, but the telomere is not stretched and lies behind the end of the chromosome [González-García et al., 2006]. **c** Immunostaining with anti-CENH3 in a metaphase I cell, showing that this protein is present in the centromeres, but not in the terminal regions of the chromosomes. **d** Ag-impregnation technique in an anaphase I cell shows that protein accumulates at the terminal regions. The arrowhead points to terminal neocentromeres.

many plants and animals [Richards and Dawe, 1998]. However, the main centromeric protein CENH3 is not present in maize meiotic neocentromeres [Dawe et al., 1999; Zhong et al., 2002]. These neocentromeres join microtubules as the true centromeres, but with 2 important differences: neocentromeres do not join microtubules end to end but laterally, and they do not maintain the cohesion between sister chromatids [Yu et al., 1997].

Nowadays, only one maize neocentromere has been described as able to be transmitted to the progeny in mitotic chromosomes [Topp et al., 2009]. This neocentromere arose in an oat-maize addition line after chromosome 3 breakage. The neocentromere was found in a fragment lacking the main centromeric DNA elements, but it contained CENH3 and joined microtubuli, indicating that a new centromere had arisen after chromosome breakage and reorganisation.

Neocentromeres in Rye: Terminal Neocentromeres

Katterman [1939] and Prakken and Müntzing [1942] first described rye chromosomes stretched to the poles by their terminal ends at first and second meiotic divisions, calling them 'T chromosomes'. They observed this activity in 6 of the 7 bivalents in inbred lines, which led them to relate neocentric activity with endogamy. However, Kavander and Viinikka [1987] found them in open pollinated cultivars, concluding that endogamy is not the sole cause producing neocentromeres. As in maize, rye neocentromeres appear during meiosis only, and coexist with an active centromere in the same chromosome.

Terminal neocentromeres are observed when the ends of the chromosomes are closer to the poles than the true centromeres and are conspicuously stretched (fig. 1), revealing tension from spindle microtubules [Viinikka, 1985], which is best observed at anaphase I [Manzanero and Puertas, 2003]. This activity may be present in all chromosomes of the complement, even 2 neocentromeres in the same chromosome. Nevertheless, the tension on neocentromeres ends before producing chromosome breakage, and the centromeres finally direct the chromosomes to the appropriate cell pole.

Neocentric activation in rye is a quantitative character, in such a way that the number of chromosomes per cell showing neocentromeres varies amongst plants of the same line and anthers of the same plant [Rees, 1955; Viinikka, 1985; Viinikka and Kavander, 1986]. Hayward [1962] crossed inbred lines and obtained their F1 and F2 progeny and backcrosses to determine the genetic basis of neocentric activation. Most F2 showed a 3:1 pattern, but not all progenies corresponded to single locus segregation, deducing a polygenic determination. Puertas et al. [2005] obtained progenies which could be adjusted to the segregation of 2 loci, in such a way that individuals may have 0–4 active alleles resulting in no neocentric activity

to the maximum frequency. Individuals with even extreme neocentric activity are fully fertile, indicating that neocentromeres do not affect normal meiotic segregation.

In any case, neocentromeres are more frequent in chromosome ends with large blocks of heterochromatin observed with C-banding (fig. 1a) [Manzanero and Puertas, 2003]. It is remarkable that terminal neocentromeres are associated with subtelomeric heterochromatin blocks in rye and maize, revealing a relationship between both features.

DNA sequences present in rye neocentromeres have been detected by in situ hybridization [Manzanero and Puertas, 2003]; they consist of the subtelomeric sequences pSc200 and pSc250 (also called pSc74 and pSc34, respectively) [Bedbrook et al., 1980; Vershinin et al., 1995]. The cereal centromeric sequence CCS1 [Aragón-Alcaide et al., 1996], and the rye centromeric sequence Bilby [Franki, 2001] were not found in neocentromeres, disproving a possible translocation of centromeric sequences to the subtelomeric heterochromatin [Manzanero and Puertas, 2003]. Interestingly, the telomeric sequence pAtT4 [Richards and Ausubel, 1988] is not stretched to the poles, but only the subtelomeric sequences are stretched (fig. 1b), indicating that subtelomeric and not the telomeric sequences are involved in neocentric activity.

Acentric fragments carrying subtelomeric heterochromatin, obtained after gamma-irradiation at early meiosis, never showed neocentric activity [Puertas et al., 2005]. On the contrary, broken chromosomes joined by a thin thread of chromatin to the centromeric region showed neocentric activity, strongly suggesting that subtelomeric sequences need a *cis*-acting centromere to be active as neocentromeres.

Interestingly, B chromosomes show a large block of C-banded subtelomeric heterochromatin in the long arm which is never active as a neocentromere (fig. 1a). This heterochromatin does not contain the DNA sequences pSc200 or pSc250, but does contain the sequences E3900 and D1100 [Sandery et al., 1990; Blunden et al., 1993; Houben et al., 1996; Langdon et al., 2000a], demonstrating that not any terminal heterochromatin is able to undergo neocentric activity.

Data from our laboratory show that the rice centromeric protein CENH3 [Nagaki et al., 2004] is not present in the terminal ends of rye chromosomes (fig. 1c); however, terminal neocentromeres show proteins revealed with the silver-staining technique (fig. 1d), and join spindle microtubules in end-to-end type unions [Östergren and Prakken, 1946; Puertas et al., 2005]. Unfortunately, the nature of these proteins is unknown.

Neocentromeres in Rye: Interstitial Neocentromeres

Rye chromosome 5 (5R) shows the interstitial heterochromatic C-band 5RL1-3 in the long arm [Mukai et al., 1992; Cuadrado et al., 1995]. It appears as a secondary constriction at meiosis in certain genotypes: haploid rye [Levan, 1942; Schlegel, 1987], wheat-rye hybrids, Triticale-wheat hybrids, the monosomic 5R wheat-rye addition line [Schlegel, 1987], inbred lines [Heneen, 1962] and some varieties of diploid rye [Levan 1942]. Occasionally, it appears decondensed and stretched to the poles forming a neocentromere at metaphase I.

The 5R interstitial neocentromere was first described by Schlegel [1987] in haploid rye, wheat-rye hybrids and the monosomic 5R wheat-rye addition line. Manzanero et al. [2000, 2002] found this neocentromere in the monosomic and disomic 5RL wheat-rye addition line, noting that the constriction behaved as a neocentromere because it showed conspicuous stretching to the poles, co-oriented with the centromere at metaphase I, joined spindle microtubules and maintained the cohesion of sister chromatids at anaphase I (fig. 2a, b). Silver staining showed that proteins are permanently joined to the constriction from metaphase I to metaphase II [Manzanero et al., 2002]; but data from our laboratory show that the centromeric protein CENH3 is not present in the interstitial region of the 5R chromosome, but only at the true centromeres (fig. 1c).

Manzanero et al. [2002] reported that the constriction is partially labelled in FISH experiments with the probe pSc119.2 [Bedbrook et al., 1980; McIntyre et al., 1990], which also labels the subtelomeric region of the 5R chromosome and 11 wheat bivalents. The possible translocation of centromeric sequences to the constriction was discarded by FISH experiments with the centromeric probes CCS1 [Aragón-Alcaide et al., 1996], and Bilby [Franki, 2001].

The cause producing this interstitial neocentromere is unknown. In haploid rye, it appears in about 70% of the cells [Schlegel, 1987], but in other cases, such as wheat-rye addition lines, it appears at variable frequencies, from less than 5% to about 30% of the cells [Manzanero et al., 2000]. It seems that the haploid condition of the 5R chromosome might prompt the neocentric activity in the interstitial heterochromatin, as in haploids or hybrids. However, this explanation is not sufficient in the case of disomic 5RL addition lines, where the 5R may show neocentric activity in up to 30% of metaphases I. However, this frequency is variable from plant to plant and between generations, suggesting an environmental determinant.

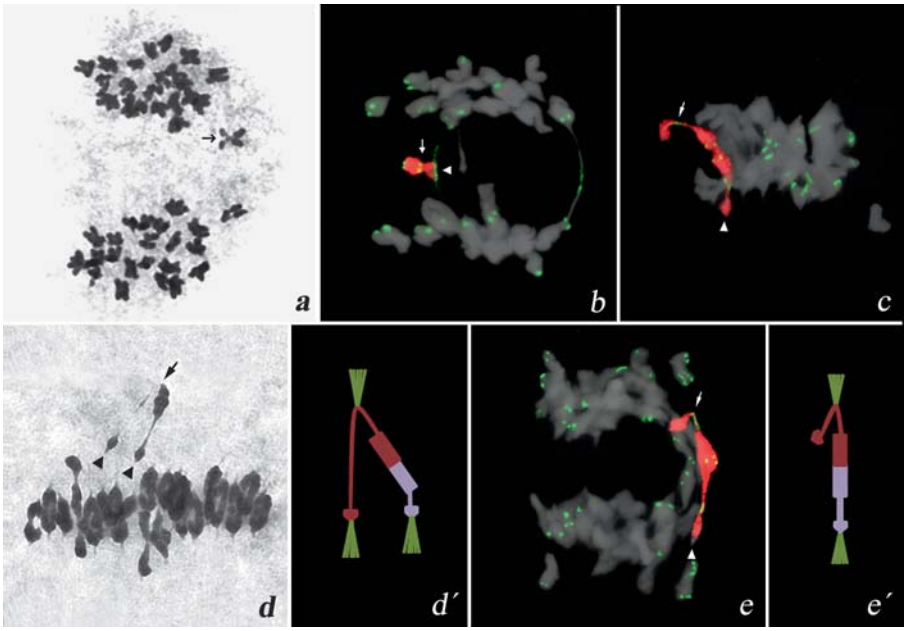

Fig. 2. Interstitial neocentromeres in the 5RL chromosome. **a** Anaphase I of the disomic 5RL wheat-rye addition line showing one 5RL directed to the downward pole and the other (arrowhead) in amphitelic orientation showing chromatid cohesion at the interstitial constriction (acetocarmine staining) [Manzanero et al., 2000]. **b** Anaphase I of the monotelosomic 5RL wheat-rye addition line. FISH with the rye-specific repetitive sequence UCM600 (red), the centromere rye-specific Bilby (green), and pSc119.2 (green) located in the constriction, in a subtelomeric position of 5RL and in subtelomeric regions of 11 wheat chromosome pairs. The centromere is observed in amphitelic orientation stretched to the poles (arrowhead), whereas the 5R chromatids remain cohesed at the constriction (arrow). **c**, **e** Metaphase I and anaphase I of the ditelosomic addition line 5RL, interpreted in **e′**. The 5RL bivalent is oriented to the upward pole by the neocentromere and to the downward pole by the centromere of the homologous chromosome. FISH with the same probes as in **b**. **d** Metaphase I of the 5RL ditelosomic addition line (acetocarmine staining). The 5RL bivalent shows the centromeres towards the same pole (arrowheads) and the interstitial neocentromere towards the opposite pole (arrow), interpreted in **d′** [Manzanero et al., 2000].

Holokinetic Chromosomes

Holokinetic chromosomes lack a localized centromere and have instead a single kinetochore plate extended along their whole or almost the whole length. Although most commonly referred to as holocentric chromosomes, they do not show a proper centromere, in the sense of a primary constriction connecting the 2 sister chromatids, as observed in monocentric chromosomes. At metaphase typical holokinetic chromosomes show the sister chromatids separated from each other by a regular distance. In this way, the term holokinetic chromosome, used by several authors [Hughes-Schrader and Schrader, 1961; Pérez et al., 1997; Mola and Papeschi, 2006], is more appropriate.

Often mistaken as an exception, they are rather a common fundamental chromosome structure found among many protozoan, green algae, several invertebrate phyla, 2 closely related plant families (Cyperaceae and Juncaceae) [Luceño et al., 1998; Roalson et al., 2007], and some plant genera such as *Drosera* and *Cuscuta* [Mola and Papeschi, 2006]. Probably, holokinetics are even more common than they have been reported. As the monocentric condition represents the 'usual' state, it is supposed to occur in many groups including those where direct evidence of a primary constriction has not been clearly demonstrated [Wrensch et al., 1994]. Curiously, they have never been reported in vertebrates.

A few species with holokinetic chromosomes have been intensively analysed, with *Caenorhabditis elegans* the only representative among model organisms. However, the data available from *C. elegans* and a few other species allow a reasonable view about their variation and function.

Holokinetic chromosomes have arisen independently several times during the evolution of plants and animals

[Mola and Papeschi, 2006]. A good example is the genus *Cuscuta*, a highly specialized group of holoparasite plants of the family Convolvulaceae. Within this genus, holokinetics are known from the Old World subgenus *Cuscuta* only, whereas the other 2 subgenera display typical monocentric chromosomes [Pazy and Plitmann, 1995]. Recently, Lozzia et al. [2009] found that *C. parodiana*, an Argentinian species from *Cuscuta* subgenus *Grammica*, also display holokinetic chromosomes, suggesting that the holokinetic state has arisen twice, independently, within the genus.

In spite of their polyphyletic origin, holokinetics from different groups display many common characteristics. Their major feature is the chromatid-size kinetochore. Immunodetection of the key kinetochore protein, the homolog of CENP-A/CENH3, in the mitotic metaphase chromosomes of *C. elegans* [Moore et al., 1999] and the Juncaceae *Luzula nivea* [Nagaki et al., 2005] showed a similar structure: a long kinetochore plate at the external side of each sister chromatid, except at the distal regions. Similar results were previously observed by electron microscopy from different organisms [Comings and Okada, 1972, and references therein].

In monocentric chromosomes the kinetochores are similar in size at metaphase and interphase nuclei [Mole-Bajer et al., 1990]. Contrarily, kinetochores of holokinetic chromosomes are observed as a continuous band in metaphase chromosomes and as numerous, dispersed, small signals at interphase nuclei of both *L. nivea* and *C. elegans* [Moore et al., 1999; Nagaki et al., 2005]. Therefore, holokinetic chromosomes are in this respect somewhat 'polycentric', since the single metaphase kinetochore is actually the result of congregation of small plates dispersed throughout the chromosome.

The Holokinetic Syndrome

Holokinetic chromosomes differ from monocentric ones not only in the extension of the kinetochore plate, but also in many other peculiar karyological features, which could be understood as the 'holokinetic syndrome', probably triggered by the large kinetochore plate visible on mitotic chromosomes.

Localized centromeres are observed at metaphase as partially decondensed chromosome regions, forming a primary constriction, typically flanked by heterochromatin [Morris and Moazed, 2007]. On the contrary, holokinetic chromosomes are homogenously condensed having neither primary constrictions nor heterochromatin-rich pericentromere organization. The amount and distribution of heterochromatin in holokinetic chromosomes, identified by C-banding or the base-specific fluorochromes chromomycin A3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI), are quite variable, as they are in monocentric chromosomes [see, e.g., Sheikh and Kondo, 1995; Vanzela and Guerra, 2000; Guerra and García, 2004]. In *L. nivea*, a 178-bp tandemly repeated sequence, isolated by homology with the 155-bp rice centromeric sequence, was localized by FISH in up to 5 major clusters on each chromosome [Haizel et al., 2005]. However, CENH3 displayed an even distribution on *L. nivea* chromosomes and was not associated with flanking heterochromatin [Nagaki et al., 2005].

During mitosis, the most visible structural characteristics of holokinetic chromosomes are the absence of a primary constriction, the existence of a long kinetochore plate, and the spindle fibers attachment along the chromatid length (fig. 3a–c). Holokinetics also display characteristic behaviours at mitosis, having their sister chromatids oriented parallel to the equatorial plate at metaphase and moving to the opposite poles parallel to each other during anaphase [Guerra et al., 2006; Mola and Papeschi, 2006]. In *Rhynchospora tenuis,* a Cyperaceae species with only 2n = 4, this kind of migration results in the formation of duplicated images of anaphase plates (fig. 3d), suggesting that the metaphase plate was equatorially split into 2 identical halves. As the sister chromatids reach the poles pulled by spindle microtubules acting over their length, no Rabl-orientation is expected at interphase or prophase nuclei of these species. Noteworthy, at very early anaphase, the chromosome termini are slightly curved toward the pole, although the microtubules are more concentrated at the middle of the chromosomes (fig. 3a–c). This morphological detail had not been previously observed [Guerra et al., 2006]. A further peculiarity of plant mitotic holokinetic chromosomes is that they appear completely phosphorylated at serine 10 of histone H3 from prophase to anaphase, whereas mitotic monocentric chromosome of plants show phosphorylated H3S10 at a restricted pericentromeric region only [Guerra et al., 2006; Houben et al., 2007].

However, it is during meiosis when holokinetic chromosomes become really intriguing. Firstly, it seems that their kinetic activity changes from a dispersed to a localized distribution. This change was first reported in *Luzula purpurea* by Malheiros et al. [1947] who found that at anaphase I and II the kinetic activity was restricted to both chromosomal termini. Other studies in different groups of plant and animals confirmed the change from dispersed/mitotic to localized/meiotic kinetic activity, although the location of kinetic activity may vary among

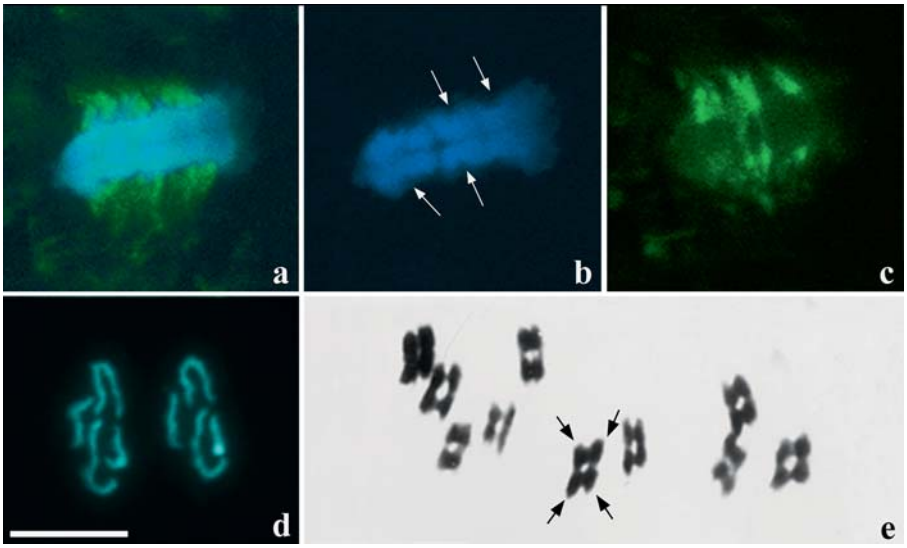

Fig. 3. Holokinetic chromosomes in mitosis of *Rhynchospora tenuis* (2n = 4) and meiosis of *Cuscuta approximata* (n = 14). **a–c** Distribution of microtubules at early mitotic anaphase of *R. tenuis,* showing an overlay projection (**a**) and selected focal planes of slightly curved chromatids (arrows in **b**), and microtubules concentrated on the middle of each chromatid (**c**). **d** Late mitotic anaphase of *R. tenuis* exhibiting duplicated images. **e** Partial metaphase I of *C. approximata* showing chromosomes in a box configuration [modified from Guerra and García, 2004]. Arrows highlight regions of kinetic tension. Bar in **d** corresponds to 10 μm.

organisms. In *C. elegans*, the kinetic activity is localized at the chromosome end opposite to the chiasma [Albertson et al., 1997], whereas in the bug *Triatoma infestans* (Heteroptera) the kinetic activity may occur alternatively in one of the 2 chromosome ends (differentiated by the presence/absence of a terminal C-band), irrespective of the chiasma position [Pérez et al., 1997]. More interestingly, in both species the kinetic activity changes its location from one chromosome end at first meiotic division to the opposite end at the second meiotic division [Albertson and Thomson, 1993; Pérez et al., 2000]. Similarly, in different somatic cell types of the worm *Parascaris univalens*, the kinetic activity can be associated with heterochromatin or euchromatin [Goday and Pimpinelli, 1989]. This means that kinetic activity in holokinetic chromosomes is not dependent on a specific nucleotide sequence but rather is epigenetically controlled [Sullivan et al., 2001; Panchenko and Black, 2009], as in the case observed in neocentromeres.

The characteristic trilaminar structure of the kinetochore has never been observed by electron microscopy in meiotic chromosomes of *C. elegans* [Howe et al., 2001], *Luzula* [Braselton, 1981], or Hemiptera species [see, e.g., Comings and Okada, 1972], and the spindle microtubules apparently attach directly to the chromatin. However, most kinetochore proteins investigated have been found on meiotic chromosomes of *C. elegans* [Monen et al., 2005]. Nevertheless, depletion of HCP-3 (CeCENP-A) and CeCENP-C, 2 key proteins for the assembly of mitotic kinetochores, severely disturb mitotic chromosome segregation but do not affect meiosis [Oegema et al., 2001; Monen et al., 2005], suggesting that a typical mitotic kinetochore is dispensable during meiosis.

Monen et al. [2005] observed that at metaphase I CeCENP-A and CeCENP-C were present in the whole chromatin, whereas outer kinetochore proteins were localized on the external surface of the meiotic chromosomes forming a cup-like structure encasing each half bivalent during both meiotic divisions. Accordingly, electron and light microscopy evidence indicates that in *C. elegans* microtubules may attach to the chromosomes by a ribosome-free zone surrounding the bivalent chromatin, apparently corresponding to the location of the outer kinetochore proteins [Howe et al., 2001]. In addition, lateral microtubule interactions might play a role in directing chromosome congregation and segregation, as shown for *C. elegans* female meiosis [Wignall and Villeneuve, 2009]. Curiously, in *Drosophila*, over-expression of its CENP-A homolog protein, CID, results in mitotic chromosomes with mislocalization of CID in almost the whole chromatin, as in the meiosis of *C. elegans*, and assembly of other kinetochore proteins in some regions, forming multicentric chromosomes [Heun et al., 2006].

A further intriguing feature of holokinetic chromosomes is that, in the absence of a centromere, the 4 chromatids of each bivalent lay separated from each other at the end of metaphase I. The 4 individualized distinct chromatids are simultaneously pulled apart generating a single localized region of tension in each chromatid (fig. 3e), characterizing a 'box configuration' [see, e.g., Nordenskiöld, 1962a; Pazy and Plitmann, 1987; Vanzela et al., 2000; Guerra and García, 2004]. Several authors

have claimed that at anaphase I, the sister chromatids segregate to different poles whereas the homologous chromatids fall apart at anaphase II, characterizing the so-called 'inverted meiosis' [reviewed by Mola and Papeschi, 2006]. The segregation of sister chromatids has been well documented on univalent chromosomes, such as the X and Y holokinetic chromosomes of some insects [Hughes-Schrader and Schrader, 1961; Viera et al., 2009] and the B chromosome of *Cuscuta babylonica* [Pazy, 1997]. In both cases the sister chromatids are observed segregating to opposite poles at anaphase I (equational segregation), whereas in monocentric chromosomes the whole univalent migrates to one pole (reductional segregation). However, holokinetic bivalents are apparently not compelled to follow this inverted order. Depending on how sister chromatids are oriented at metaphase I, with the long axis parallel or perpendicular to the cell equator, they may follow an equational or reductional segregation, respectively [Pérez et al., 1997; see also Viera et al., 2009].

From anaphase I to metaphase II of many species with holokinetic chromosomes, the sister chromatids are still closely associated. At least in *C. elegans* and in some Hemiptera, this association is ensured by meiotic cohesins [Suja et al., 2000; de Carvalho et al., 2008]. However, in other organisms, as some Juncaceae and Cyperaceae [Nordenskiöld, 1962b; Da Silva et al., 2005], a diploid number of individualized chromatids is observed at prophase II. At prometaphase II the individualized homologous or sister chromatids must recognize each other and reassociate in order to become correctly oriented to the opposite poles at metaphase II. How they can carry out the proper recognition and reassociation is still an open question. Bongiorni et al. [2004], for example, argue that the homologous chromatids of mealybugs remain joined at anaphase I-prophase II by remnants of sister chromatid cohesion, supposedly conserved between recombined telomeric segments. However, the nature of this cohesion remains to be elucidated. As the chromatids may lie far from each other, this cohesion material should be much more elastic than cohesins rings can be. One possibility is that they are kept connected by thin threads, which are extended at anaphase I-prophase II, and progressively condensed at prometaphase II. Such a connection is independent from chiasmata, which are released at the end of metaphase I or earlier. In *Drosophila* oocytes, achiasmatic homologues display separation and rejoining movements assisted by heterochromatic threads, which ensure the proper segregation during meiosis I [Hughes et al., 2009]. Therefore, chromatin threads could be an alternative way to ensure normal chromosome congregation in the absence of the sister chromatid cohesion.

Actually, anaphase I and II of most plant and animal species with holokinetic chromosomes are often 'sticky', with several bridges or threads connecting the 2 plates, [Mello and Recco-Pimentel, 1987; Pazy and Plitmann, 1987]. The meaning of such sticknesses is usually not considered because it might be an artefact. However, there are data suggesting that the sticky threads may be structural components of the meiotic holokinetic chromosomes: (i) they have been observed by light [Hughes-Schrader and Schrader, 1961] and electron microscopy [Comings and Okada, 1972; Albertson and Thomson, 1993] in many species; (ii) they are not observed in normal mitotic holokinetic chromosomes [Comings and Okada, 1972; Bokhari and Godward, 1980; González-García et al., 1996a]; (iii) they are not found at anaphase I or II of monocentric chromosomes. Stickness in prophase II has been rarely reported, probably because in most species this phase is too short or even absent.

Finally, another peculiar meiotic feature of the holokinetic syndrome is that from metaphase I to the end of meiosis the chromosomes become overcondensed and roundish, looking like small balls or ovals. Even at metaphase II, where monocentric chromosomes are very similar to mitotic ones, holokinetics are globular and smaller than at mitotic metaphase [Goday and Pimpinelli, 1989; Da Silva et al., 2005].

Figures 4 and 5 show meiotic phases of *Rhynchospora pubera* (2n = 10) illustrating several aspects of the holokinetic syndrome. Three of the 5 chromosome pairs of *R. pubera* have a terminal site of 45S rDNA [Vanzela et al., 1998], also differentiated as weak CMA^+ bands [Vanzela and Guerra, 2000], which help to identify the bivalent orientation. The mitotic metaphase (fig. 4a) shows chromosomes without a primary constriction and larger than at metaphase I or II (figs. 4e and 5f). Prophase I seems to be normal (fig. 4b), whereas from metaphase I onwards there are several differences with the monocentric meiosis. From diplotene to early metaphase I, bivalents show one or 2 terminal or subterminal chiasmata (fig. 4c, d). At anaphase I, the 2 chromatids that migrate to opposite poles are not tightly associated and threads connecting some chromatids are observed (fig. 5a, b). At prophase II, 10 individualized chromatids are distinguished at each pole, which are rejoined at metaphase II (fig. 5c–f).

Figure 6 shows some details of *R. pubera* meiosis. At zygotene a bouquet configuration is not recognized, but the 6 chromosome termini bearing 45S rDNA sites are always found associated with each other and with the nu-

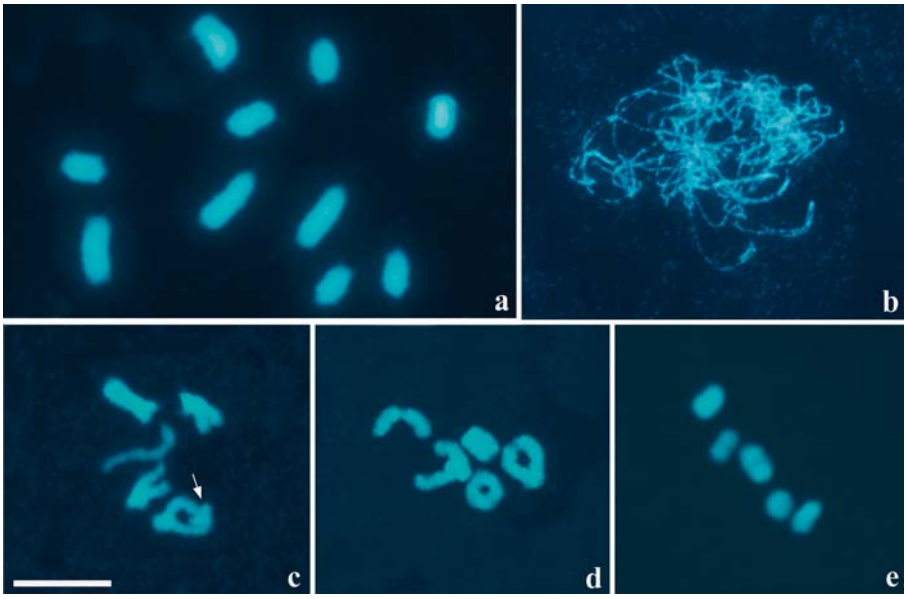

Fig. 4. Mitotic metaphase and early meiotic phases of *Rhynchospora pubera* (n = 5). **a** Mitotic chromosomes. **b–e** Prophase to early metaphase I showing zygotene (**b**), diplotene (**c**), diakinesis (**d**), and early metaphase I (**e**). Note a subterminal chiasma in **c** (arrow) and 2 ring bivalents in **d**. Bar in **c** corresponds to 10 μm.

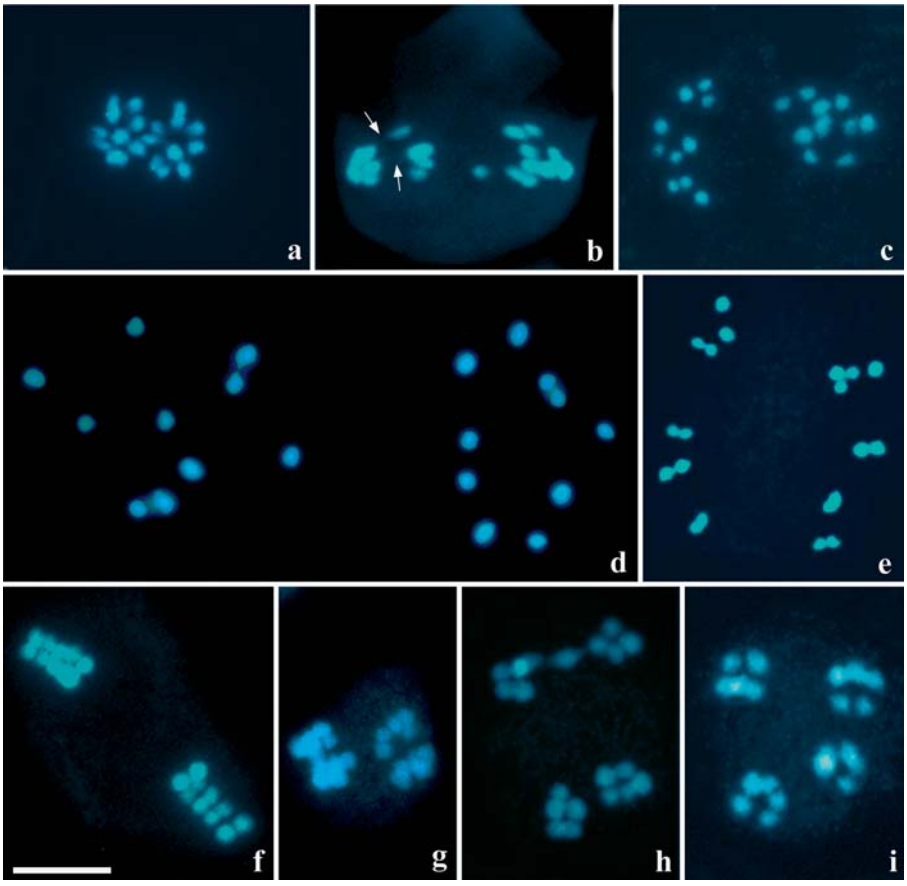

Fig. 5. Late meiotic phases of *Rhynchospora pubera*. **a**, **b** Polar (**a**) and equatorial (**b**) views of anaphase I (arrows in **b** point to threads connecting chromatids); **c**, **d** prophase II, displaying a diploid number of chromatids; **e** prometaphase II with 8 rejoined and 2 nearly rejoined chromatid pair chromosomes; **f** metaphase II; **g** early anaphase II; **h** late anaphase II with a laggard chromatid; **i** telophase II. Bar in **f** corresponds to 10 μm.

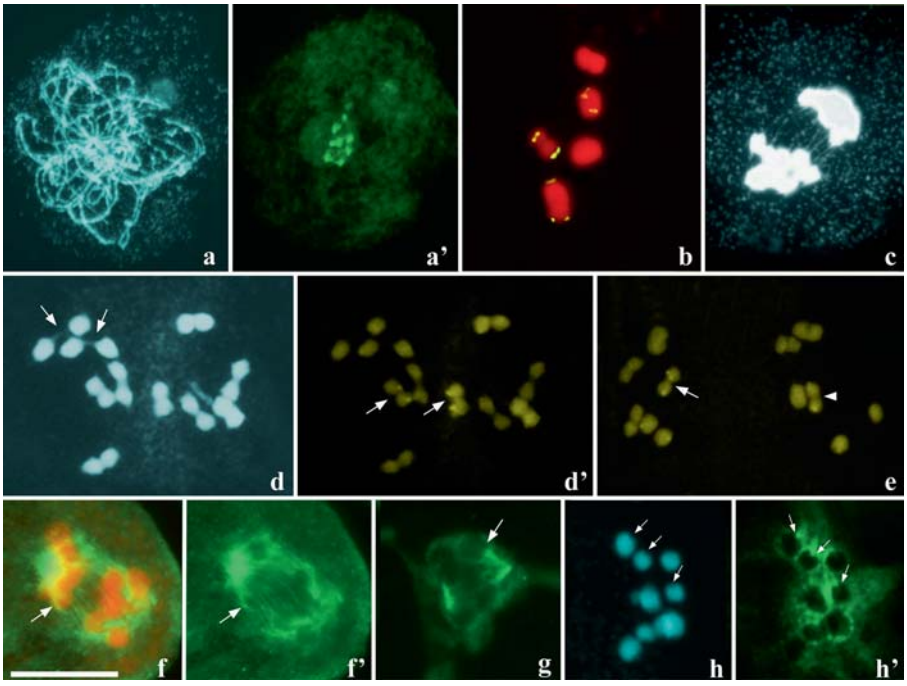

Fig. 6. Additional aspects of meiosis in *Rhynchospora pubera*. **a**, **a′** Zygotene showing all 45S rDNA sites associated with the nucleolus. **b** Distribution of 45S rDNA sites on 3 bivalents at metaphase I. **c** Overexposed anaphase I showing thin threads between the plates. **d** Prophase II with threads (arrows) connecting chromatids. **d′**, **e** Prophase II cell stained with CMA showing laterally (arrow) and terminally (arrowhead) associated chromatids. **f–h′** Microtubule attachments on chromosomes at anaphase I (**f**, **f′**) and II (**g–h′**). Arrows in **f** and **f′** point to a chromatid with dispersed microtubule attachments and in **g–h′** they indicate the chromatin region where microtubules are concentrated. Chromosomes were stained with DAPI (blue), CMA (yellow) or propidium iodide (red) and rDNA sites and microtubules with FITC (green). Bar in **f** corresponds to 10 μm.

cleolus (fig. 6a, a′). In the 3 bivalents having rDNA sites, their chiasmata are located at the opposite side of these sites (fig. 6b). Therefore, the chromosome termini bearing rDNA sites may also acquire kinetic activity. Threads were sometimes observed at anaphase I between the migrating chromatids (fig. 6c) and at prometaphase II (fig. 6d). At this stage, chromatids having CMA^+ bands are correctly joined to each other, but not necessarily by a determined chromosome region (fig. 6d′, e). Microtubules are observed partially covering one chromosome side (fig. 6f, f′, g) and concentrated at a depression in the chromosomes (fig. 6h, h′). The dispersed microtubule attachment could indicate that meiotic chromosomes of *Rhynchospora* may occasionally be pulled to the poles by non-terminal chromosomal regions, as previously observed in bugs [González-García et al., 1996b; Pérez et al., 1997]. In *P. univalens*, the microtubules are concentrated on one chromosome end, but are also attached to the half of the chromosome surface nearest to a pole [Goday and Pimpinelli, 1989]. Dispersed microtubule attachments were also observed by light and electron microscopy in *C. elegans* [Albertson and Thomson, 1993; Howe et al., 2001; Wignall and Villeneuve, 2009]. However, the dumbbell-like prometaphase II chromosomes of *R. pubera*, with the terminal CMA^+ bands laterally positioned in a non-telokinetic way, suggest that the kinetic activity may also be concentrated on the interstitial regions.

Holokinetic Chromosomes and Neocentromeres: Evolutionary Oddities and Lessons to Learn

From protozoan to more evolved cell divisions present in plants and animals, mitosis has changed essentially in 2 aspects: (i) the breakdown of the nuclear envelope at prometaphase still assuring the proper contact between microtubules and kinetochores, and (ii) the fast, simultaneous and accurate separation of sister chromatids to the poles [Raikov, 1982]. The second in turn depends on the first, because in primitive cell divisions anaphase movement is slow when the contact between microtubules and kinetochores becomes difficult due to the permanence of the nuclear envelope throughout the cell cycle. One can then assume that a fast, irresistible and accurate attraction between microtubules and kinetochores has had great success in evolution.

Our present knowledge of centromere identity allows one to conclude that the evolutionarily conserved proteins of the kinetochore recognize epigenetically modified repetitive DNA and specific forms of the histone H3, rather than DNA sequences that are not conserved. Examples such as neocentromeres and holokinetics illustrate that it is becoming less surprising that very different structures and DNA sequences of centromeres appear either in evolution, as the polyphyletic holokinetic chromosomes, or as spontaneous or induced neocentromeres.

Villasante et al. [2007] proposed that the centromeres originated from telomeres in the transition from prokaryotic to eukaryotic chromosomes, the subtelomeric regions having an important role. In their hypothesis, the transposition of retroelements at chromosome ends allowed the formation of telomeres. Afterward, the modification of the tubulin-based cytoskeleton, which allowed specific subtelomeric repeats to be recognized as new cargo, gave rise to the first centromere. During the transition, unstable pseudodicentric chromosomes generated multiple telocentric chromosomes that eventually evolved into monocentric or holokinetic chromosomes. Under Villasante's hypothesis, it is not surprising that subtelomeric regions may occasionally behave as neocentromeres, or new centromeres arise, provided that they contain large amounts of repetitive DNA able to be epigenetically modified in the way of a centromere. In any case, it seems that holokinetics never gave rise to monocentrics because all data indicate that holokinetics have arisen several times independently in phylogenetic groups where monocentric chromosomes are the rule, whereas the opposite situation has never been demonstrated.

Differently from monocentric chromosomes, neocentromeres and meiotic holokinetic chromosomes have neither a typical trilaminar kinetochore plate nor a centromere-specific DNA sequence, therefore being epigenetically determined. Both have arisen independently in different groups of plants, have kinetic activity preferentially on chromosome ends and may display stretching or stickness. However, contrary to meiotic holokinetics, neocentromeres are not necessary for normal meiotic segregation; they may co-exist with a normal centromere, they have variable expression among meiocytes or among chromosomes of the same cell, and they are more commonly found in chromosomes with terminal heterochromatic blocks. Therefore, in spite of some similarities, they basically differ from each other because the large kinetochore of holokinetic chromosomes is a vital structure and has been subject to natural selection forces in different evolutionary lineages, whereas the neocentromeres seem to be a somewhat similar phenomenon attracting the kinetochore machinery, but have still not been selected for any important role in the chromosome physiology of any organism. These variations of spindle attachment found in neocentromeres and meiotic holokinetics seem to be exclusive to the meiotic division leading to the possible conclusion that during meiosis there is a looser regulation of the kinetochore activity allowing atypical meiotic chromosome connections to the spindle in comparison to mitosis.

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