Structure and regulation of centromeres in mono- and holocentric chromosomes

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Table of content

1. Introduction 1
1.1 Centromere, the locus of kinetochore assembly 1
1.2 The centromere-specific histone H3 variant cenH3 2
1.3 Regulation of cenH3 3
1.3.1 Loading of cenH3 4
1.3.2 Transcriptional regulation of *cenH3* 4
1.3.3 Post-translational regulation of cenH3 5
1.4 Holocentric chromosomes 6
1.4.1 Holocentric species: Chromosome structure and genome organization 6
1.4.2 Meiosis in monocentric chromosome species 9
1.4.3 Holocentric chromosomes and meiosis 10
1.4.4 Meiosis in the holocentric plant species *Luzula elegans* 12
2. Aim of this study 14
3. Results and Conclusion 15
3.1 Regulation of cenH3 in *Arabidopsis thaliana* 15
3.1.1 Transcriptional regulation of *cenH3* in *A. thaliana* 15
3.1.2 Post-translational regulation of cenH3 in *A. thaliana* 16
3.2 Genome organization, mitosis and meiosis of *Luzula elegans* 20
3.2.1 Genome and higher order chromatin organization of *L. elegans* 20
3.2.2 Mitosis in *L. elegans* 25
3.2.3 Meiosis in *L. elegans* 27
4. Summary 33
5. Zusammenfassung 35
6. References 37
7. *Curriculum vitae* 43
8. Eidesstattliche Erklärung 48
9. Publications on which this thesis is based and Declaration on the contribution to these publications 49
9.1 Heckmann et al. (2011) Cytogenetics and Genome Research 134: 220-228
9.2 Heckmann et al. (2011) The Plant Journal 68: 646-656
9.3 Heckmann et al. (2013) The Plant Journal 73: 555-565
9.4 Heckmann and Houben (2013) Holokinetic Centromeres (pp. 83-94) In: Jiang, J and Birchler, J (Eds.): Plant Centromere Biology, Vol. 1, John Wiley & Sons, Inc.: in press.

1. Introduction

Chromosomes consist of DNA, associated proteins and RNA together forming chromatin. Chromatin is traditionally subdivided into hetero- and euchromatin, according staining intensities with DNA-dyes (Heitz, 1929). Heterochromatin is enriched with repetitive and non-coding DNA and strongly condensed, while euchromatin is less condensed and characterized by marks typical for ‘open’ or ‘active’ chromatin (Grewal and Elgin, 2002).

DNA is highly compacted during nuclear divisions visible as defined chromosomes during metaphase. Histones play a decisive role for this DNA condensation. Typically, two molecules each of the major histones H2A, H2B, H3 and H4 form an octameric nucleosome core around which DNA is wrapped. Further DNA condensation is mediated by association of the ‘linker histone’ H1 to DNA between nucleosomes. Moreover, histones are crucial for diverse epigenetic mechanisms. They are manifold post-translationally modified (methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deimination und proline-isomerisation) mainly in their variable N-terminal part (Kouzarides, 2007).

Additionally, there is a variety of histone variants involved in diverse processes; e.g. H2AZ functions as ‘border element’ between eu- and heterochromatin in yeast (Raisner et al., 2005) or mediates the thermosensory response on the global chromatin level in *Arabidopsis thaliana* (Kumar and Wigge, 2010), H2AX is involved in DNA double strand break repair (Lowndes and Toh, 2005), H3.3 is frequently associated with actively transcribed genes (Ahmad and Henikoff, 2002). The centromeric histone H3 variant cenH3 is found in centromeric nucleosomes (Allshire and Karpen, 2008).

1.1 Centromere, the locus of kinetochore assembly

The centromere, a specialized chromosomal locus, is traditionally defined as the primary constriction of a condensed metaphase chromosome. Transiently a multi-protein complex (kinetochore) assembles at centromeres that interacts with spindle microtubules mediating faithful transmission of the genetic material during mitosis

and meiosis. Kinetochore proteins are responsible for sister chromatid cohesion, chromosome movement and cell cycle regulation (Dorn and Maddox, 2012).

Establishment and maintenance of active centromeres is primarily based on the presence of cenH3, originally termed CENP-A (mammalian centromere protein A; Earnshaw and Rothfield, 1985). CenH3 replaces H3 in centromeric nucleosomes and thus marks centromeres epigenetically initiating kinetochore formation (Kalitsis and Choo, 2012). Although centromere function is conserved in eukaryotes (Houben and Schubert, 2003), the centromeric DNA composition is highly variable and except for budding yeast (Clarke and Carbon, 1985) centromeric DNA sequences are neither required nor sufficient for centromere identity (Kalitsis and Choo, 2012). Nevertheless, often plant centromeres contain distinct satellite DNA sequences and families of long terminal repeat (LTR) retrotransposons (Ty3/gypsy elements of the CRM clade) (Houben and Schubert, 2003; Neumann et al., 2011).

1.2 The centromere-specific histone H3 variant cenH3

CenH3 has a rather conserved C-terminus known as histone fold domain including the loop1 region (also known as CAT-(CENP-A targeting) domain), while the N-terminus is more variable in size and amino acid composition between species (Henikoff and Dalal, 2005) (Fig 1).

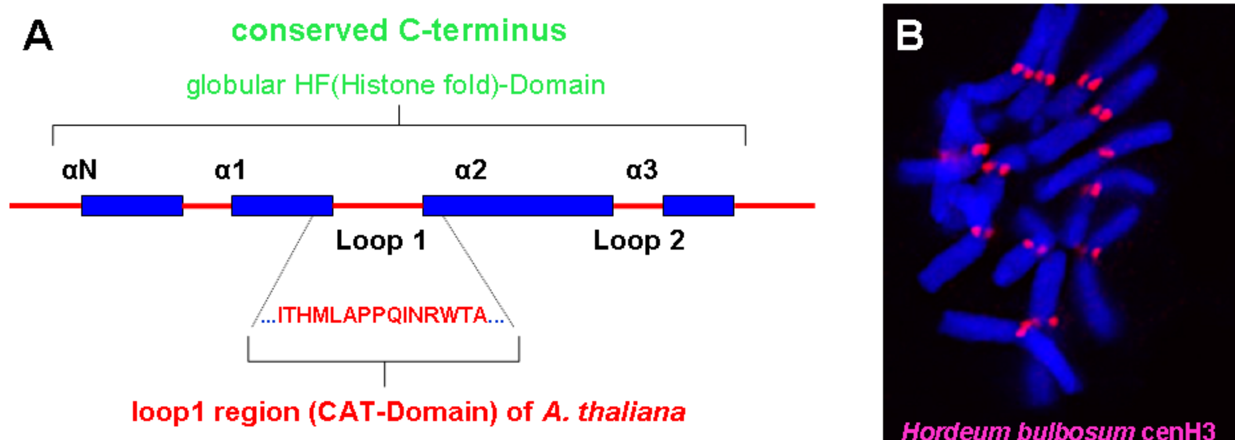


Figure 1 The centromere-specific histone H3 variant cenH3: The N-terminus is highly variable in terms of amino acid composition and length, while the C-terminus is highly conserved except the loop1 region (also known as CAT-Domain). **A**) Schematic structure of the conserved C-terminus known as

histone fold domain. Highlighted the variable loop1 region; exemplified amino acid sequence of the loop1 region of *A. thaliana* cenH3 shown. **B**) Immunolocalization of cenH3 at the monocentric centromeres of *Hordeum bulbosum* (modified according to Sanei et al., 2011). CenH3 (red) and DNA (blue).

The loop1 region is required for cenH3 centromere targeting (Black et al., 2004; Lermontova et al., 2006) while the N-terminus is required for interaction with kinetochore components in yeast (Chen et al., 2000). Absence of cenH3's N-terminus allows its targeting, recruitment of kinetochore proteins, and does not affect severely mitosis in *A. thaliana* (Lermontova et al., 2006; Ravi et al., 2010; Lermontova et al., 2011). However, the N-terminal part is essential for meiotic cenH3 loading in plants (Lermontova et al., 2011; Ravi et al., 2011).

1.3 Regulation of cenH3

As a key player in active centromeres, cenH3 expression and its centromeric turnover need to be tightly regulated at the transcriptional and post-translational level. Any error in this hierarchy can have deleterious effects; i.e. centromere inactivation, dicentric chromosome formation, neocentromere formation, ectopic kinetochore sites, etc. (Allshire and Karpen, 2008; Torras-Llort et al., 2009; Kalitsis and Choo, 2012).

CenH3 overexpression in various species (Van Hooser et al., 2001; Vermaak et al., 2002; Tomonaga et al., 2003; Heun et al., 2006; Lam et al., 2006) may have harmful effects due to ectopic non-centromeric incorporation. Mice and *A. thaliana* heterozygous *cenH3* mutants are viable and fertile, whereas homozygous mutants are embryo-lethal (Howman et al., 2000; Ravi and Chan, 2010). *Drosophila* homozygous *cenH3* embryo mutants show severe mitotic defects and die during embryogenesis (Blower et al., 2006). RNAi-mediated depletion of cenH3 in *Caenorhabditis elegans* embryos led to severe mitotic defects, while meiosis was unaffected (Monen et al., 2005). Thus, apparently cenH3 is not required for meiotic segregation in *C. elegans* (Monen et al., 2005). *A. thaliana* cenH3 RNAi transformants showed a reduced number of mitotic divisions and reduced fertility due to meiotic segregation errors inversely correlating with the remaining endogenous cenH3 amount (Lermontova et al., 2011). No T-DNA insertion lines within the *cenH3*

coding region are available for *A. thaliana* indicating that *cenH3* is an essential gene needed for viability and any knock-out of *cenH3* is lethal.

Ravi and Chan (2010) complemented a heterozygous *A. thaliana cenH3* null mutant (*cenh3-1*) with various *cenH3* gene constructs (Ravi et al., 2010) allowing functional studies of cenH3 in segregating progenies without endogenous cenH3.

1.3.1 Loading of cenH3

After DNA replication, the amount of histones is diluted by half. Canonical histones are loaded parallel to DNA synthesis, while some histone variants are integrated in a replication-independent manner (Ransom et al., 2010). Centromeric deposition of cenH3 occurs during anaphase/telophase to mid-G1 in metazoans (Jansen et al., 2007; Schuh et al., 2007), during G2 in plants, protozoans and fission yeast (Lermontova et al., 2006; Dubin et al., 2010; Lando et al., 2012) and during S phase in budding yeast (Pearson et al., 2004).

1.3.2 Transcriptional regulation of *cenH3*

Various studies showed a strict regulation of cenH3 expression and its centromeric loading. *CenH3* overexpression (Van Hooser et al., 2001; Vermaak et al., 2002; Tomonaga et al., 2003; Heun et al., 2006; Lam et al., 2006) and *cenH3* expression driven by the S phase-specific histone *H3* promoter (Shelby et al., 1997) resulted in its ectopic non-centromeric incorporation and can lead to severe segregation errors. Proteolytic cenH3 regulation contributes to centromere-restricted incorporation (Moreno-Moreno et al., 2006; Ranjitkar et al., 2010). Specific chaperones such as HJURP (Dunleavy et al., 2009; Shuaib et al., 2010), loading and maintenance factors (Stellfox et al., 2012) and a distinct (peri-)centromeric chromatin environment (Bergmann et al., 2012) are important for centromere activity. Hence, the interplay of cell cycle stage-specific expression regulation together with proteolytic degradation and interaction with chaperones and loading/maintenance factors of cenH3 likely determines establishment and maintenance of kinetochores at functional centromeres.

In silico studies of the upstream region of the putative *A. thaliana cenH3* promoter (*cenH3*pro) indicated two potential E2F transcription factors (TFs) binding sites (GCGGGAAA: -163 bp and -115 bp upstream of ATG) (Lermontova et al., 2006).

In *A. thaliana* eight E2F/DP TFs members are found; classified into three typical E2Fs (E2Fa,b,c), two dimerization proteins (DPa,b), and three atypical E2Fs (E2Fd/DEL2, E2Fe/DEL1, E2Ff/DEL3) (Mariconti et al., 2002). Typical E2Fs are involved in the G1-to-S and G2-to-M transition (Sabelli and Larkins, 2009). E2Fa and E2Fb transcriptionally activate genes related to cell division (De Veylder et al., 2002; Magyar et al., 2005; Sozzani et al., 2006), while E2Fc represses genes related to cell proliferation and mediates the interplay between cell division and endoreduplication (del Pozo et al., 2002; del Pozo et al., 2006). Typical E2Fs form heterodimers (with DPa or DPb) in a cell cycle stage-dependent manner for target binding. Retinoblastoma-related (RBR) protein binds transiently in a hypophosphorylated state typical E2Fs thus inhibiting their TF activity. CYCD3;1 in complex with CDKA;1 regulates cell-cycle entry by RBR phosphorylation (Nakagami et al., 2002). RBR-phosphorylation releases RBR-bound E2Fs that in turn can now regulate their targets. Also, E2F-RBR complexes recruit chromatin remodelling repressive enzymes to E2F-responsive promoters (Luo et al., 1998; van den Heuvel and Dyson, 2008). On the contrary, atypical E2Fs act in a DP- and RBR-independent manner and repress transcription (Mariconti et al., 2002). However, whether they compete with typical E2Fs for binding sites or whether they actively repress E2F targets genes is unclear (Berckmans and De Veylder, 2009). Atypical E2Fs play a role in regulation of endocycle, cell size, proliferation and DNA-damage response (Berckmans and De Veylder, 2009).

1.3.3 Post-translational regulation of cenH3

Data concerning post-translational modifications of cenH3 are limited. In *Saccharomyces cerevisiae* cenH3 methylation of arginine 37 (R37) is required for kinetochore integrity and chromosome segregation (Samel et al., 2012). Recently, acetylation during G1/S of lysine 124 of human cenH3 (K124) was found (Bui et al., 2012). In conjunction with histone H4 K79 acetylation it may play a structural role; i.e. acetylation of nucleosomal cenH3 might loosen the DNA-histone association and

therefore may increase accessibility of cenH3 nucleosomes to modifying or interacting proteins (Bui et al., 2012).

The best studied epigenetic cenH3 modification is phosphorylation of serine 7 of human cenH3. Various studies showed cell cycle-dependent S7 phosphorylation during mitosis by Aurora kinase A (Kunitoku et al., 2003), B (Zeitlin et al., 2001b; Zeitlin et al., 2001a), and C (Slattery et al., 2008), which is essential for kinetochore function and correct chromosome alignment. Cell cycle-dependent phosphorylation of maize cenH3 at serine 50 was described and it is thought to play a similar role as S7 phosphorylation of human cenH3 (Zhang et al., 2005). However, further post-translational modifications of plant cenH3s are not yet found.

1.4 Holocentric chromosomes

Most studied organisms possess one single size-restricted centromere per chromosome, the primary constriction. Such chromosomes are referred to as monocentric. However, in some green algae, protozoans, invertebrates, and plants so-called holocentric chromosomes with dispersed centromeres along chromosomes occur, suggesting that holocentricity has arisen independently several times *via* convergent evolution (Dernburg, 2001; Guerra et al., 2010; Melters et al., 2012). Holocentricity is possibly more common than so far reported. Surprisingly, so far no vertebrates with holocentric chromosomes are described.

In flowering plants (angiosperms), holocentric chromosomes are found among the monocots *Cyperaceae* (sedges), *Juncaceae* (rushes) (Malheiros et al., 1947; Hakansson, 1958), and *Chionographis* (Tanaka, 1977) as well as in dicot genera such as *Cuscuta* subgenus Cuscuta (Pazy and Plitmann, 1995), *Drosera* (Sheikh et al., 1995), or in the nutmeg tree *Myristica fragrans* (Flach, 1966).

1.4.1 Holocentric species: Chromosome structure and genome organization

In case of holocentric chromosomes, microtubules attach to almost the entire (Greek: *holo*-) poleward surface of chromatids and no distinct primary constriction is visible at

metaphase. During anaphase, this holokinetic attachment leads to sister chromatid migration to opposite poles parallel to each other as linear bars. On the contrary, in case of monokinetic attachment to a distinct kinetochore, sister chromatids move to the poles at anaphase, with the centromere leading, as V-shaped structures (Fig 2).

Besides observation of a distinct chromosome appearance, only few studies have been conducted on holocentric plants. Most of these described chromosome morphology and kinetics during mitosis and meiosis as well as DNA and chromatin properties (Lima-De-Faria, 1949; Mola and Papeschi, 2006; Guerra et al., 2010). A holokinetic chromosome organization is thought to enable a greater genomic plasticity; i.e. after DNA double strand breaks resulting chromosome fragments may be maintained and reintegrated elsewhere.

Despite the strikingly different centromere distribution between mono- and holocentric species, similar kinetochore components are found in both active centromere types and centromere functions are mainly conserved (Maddox et al., 2004; Nagaki et al., 2005; d'Alencon et al., 2011). However, for holocentrics centromere-specific DNA sequences have not yet been reported even in the genome-sequenced holocentric animals *C. elegans* (Consortium, 1998; Gassmann et al., 2012) and *Bombyx mori* (Xia et al., 2008; d'Alencon et al., 2010). In nematodes cenH3 is sequence-independent loaded (Gassmann et al., 2012) and centromeres are formed sequence independent (Howe et al., 2001). It is thought that the role of DNA sequences at centromeres is rather to provide a structural basis than sequence specificity.

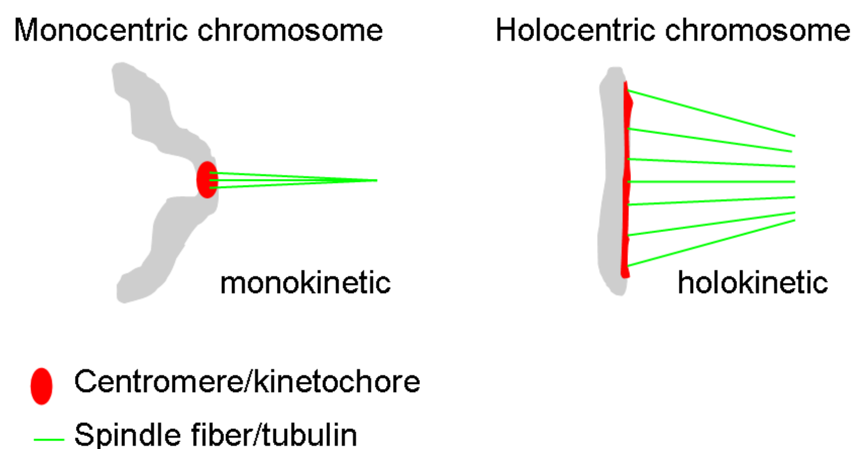


Figure 2 Mitotic anaphase behaviour of mono- and holocentric chromatids (modified according to Heckmann and Houben, 2013). Positions of centromeres and tubulin fibers are shown in red and green, respectively.

Heterochromatin-forming repeats are typically found in monocentric chromosomes at centromeres, telomeres, nucleolar organizers, subterminal, and interstitial regions (Schmidt and Heslop-Harrison, 1998). The genome organization is mirrored by the distribution of epigenetic marks; i.e. post-translational histone modifications or DNA methylation. Different histone marks, particularly distinct methylated isoforms of histone H3, are associated with either eu- or heterochromatin (Fuchs et al., 2006). For instance, H3K9me2 is uniformly distributed in monocentric chromosomes of plant species with large genomes (1C > 500 Mbp) while smaller genomes as that of *A. thaliana* show enrichment of H3K9me2 at preferentially pericentromeric hetero-chromatin. Independent of genome size, H3K4me2 is associated with euchromatin along chromosome arms. In monocentric chromosomes the genome size significantly influences the distribution of histone methylation marks associated with regions being transcriptionally less active (Houben et al., 2003; Fuchs et al., 2006). Interestingly, entire mitotic chromosomes of *Luzula* (Gernand et al., 2003; Nagaki et al., 2005) and of *Rhynchospora tenuis* (Guerra et al., 2006) display phosphorylated histone H3S10/S28 in a cell cycle-dependent manner, indicating a chromosome wide ‘pericentromere-like’ structure (Houben et al., 2007). However, there are no studies dealing with typical eu- and heterochromatin histone marks and their chromosomal distribution for holocentric plant chromosomes.

In *C. elegans* and *Luzula nivea* cenH3 is distributed along mitotic chromosomes correlating with active centromeres similar as in species with monocentric chromosomes (Buchwitz et al., 1999; Nagaki et al., 2005). During mitotic metaphase, holokinetic centromeres are light microscopically visible as continuous axial line along each sister chromatid, except for the cenH3-negative chromosome termini (Buchwitz et al., 1999; Moore et al., 1999; Nagaki et al., 2005). CenH3 signals are dispersed in interphase nuclei and are visible as various small foci along early prophase chromosomes (Moore et al., 1999; Nagaki et al., 2005). In *L. nivea* the chromosome regions associated with cenH3 appear as a groove-like structure during metaphase (Nagaki et al., 2005). It was proposed that centromere extension may have caused an outer centromeric groove along each sister chromatid (Nagaki et al., 2005).

1.4.2 Meiosis in monocentric chromosome species

Meiosis is a key event for stable sexual reproduction. A single round of DNA replication (meiotic S phase) is followed by two rounds of chromosome segregation (meiosis I and II) generating haploid gametes.

In meiosis I, homologous chromosomes are separated (reductional division), whereas in meiosis II, sister chromatids are separated (equational division). Thus, four haploid gametes are generated. The significance of meiosis is two-fold: First, it facilitates genetic variation (independent assortment of homologous chromosomes and regulated genetic exchange by homologous recombination). Second, it reduces the chromosome number, which is needed to maintain somatic diploidy after fusion of male and female haploid gametes.

Prophase I consists of lepto-, zygo-, pachy- and diplotene during which various crucial events take place. Replicated homologs undergo synapsis mediated by a transient proteinaceous structure, the synaptonemal complex (SC). Along homologs a linear protein axis, the axial element (AE), is formed, to which chromatin loops are attached (Dobson et al., 1994). One typical AE-associated protein is Asy1 (Sanchez-Moran et al., 2008). Parallel to homolog recognition during lepto- to zygotene, the AEs now called lateral elements (LEs), become physically closely connected by polymerization of specific protein(s), named transverse filaments (TF), that span the gap between chromosome axes. A typical TF protein is Zyp1 (Higgins et al., 2005). Synapsis, finished at pachytene, leads finally to the fully formed tripartite SC that is specifically disassembled during diplotene/zygotene. At diakinesis the SC is fully degraded and bivalents start to separate, except at cross-over sites that physically connect the homologs (Page and Hawley, 2004).

During prophase I, when homologs are tightly connected, homologous recombination occurs initiated by DNA double strand breaks (Osman et al., 2011). Meiotic DNA double strand break repair can result in exchange of genetic material between non-homologous sisters (cross-over, CO), cytologically detectable as chiasmata (inter-homolog connection) after pachytene. Transition from pro- to metaphase I is defined as diakinesis. After prophase I, bivalents condense and align at metaphase I plate. Alignment and separation of homologs at anaphase I depend on COs and sister

chromatid cohesion. During anaphase I, arm cohesion is lost while centromeric cohesion is maintained, thus still linking sister chromatids.

Meiosis I is followed by a typically rather short interphase, the interkinesis. However, there are species with either prolonged or lacking interkinesis. Some plants skip telophase I and interkinesis and proceed immediately into meiosis II. The second meiotic division is cytologically similar to a mitotic division. Centromeric cohesion is released allowing the sisters to be pulled to opposite poles, during a so-called equational division, forming tetrads of four haploid spores.

1.4.3 Holocentric chromosomes and meiosis

During meiosis, cohesion between monocentric sister chromatids is released in two steps: i) along chromosome arms during meiosis I, ii) at sister centromeres during meiosis II. Defined regional centromeres provide a basis for co-orientation of sister chromatids at meiosis I and protect cohesins from degradation before anaphase II (Sakuno and Watanabe, 2009).

Holocentrics are challenged by various problems during meiosis that need to be differently solved as in monocentrics. If spindle fibers would attach all along holocentric chromosomes, a recombined chromatid would be pulled to opposite poles on both sides of a persisting CO (Fig 3).

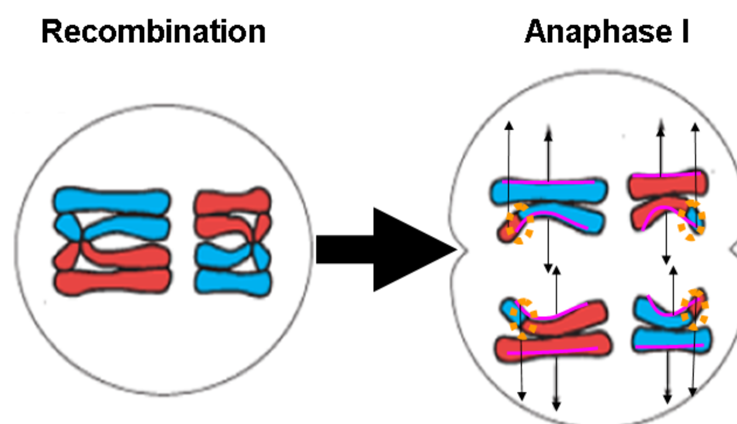


Figure 3 The problem of meiotic recombination within holocentric chromosomes (modified according to d'Erfurth et al., 2009). Spindle fibers (arrowed) from different poles would attach on either side of a cross-over and pull a recombined chromatid to opposite poles during the first meiotic division.

Thus, holocentrics cannot rely on a single predefined centromere site to regulate sister chromatid co-orientation and the two-step loss of cohesion during meiosis. As one adaption holocentrics restrict CO frequency per homolog to one or two distal to terminal CO (Nokkala et al., 2004).

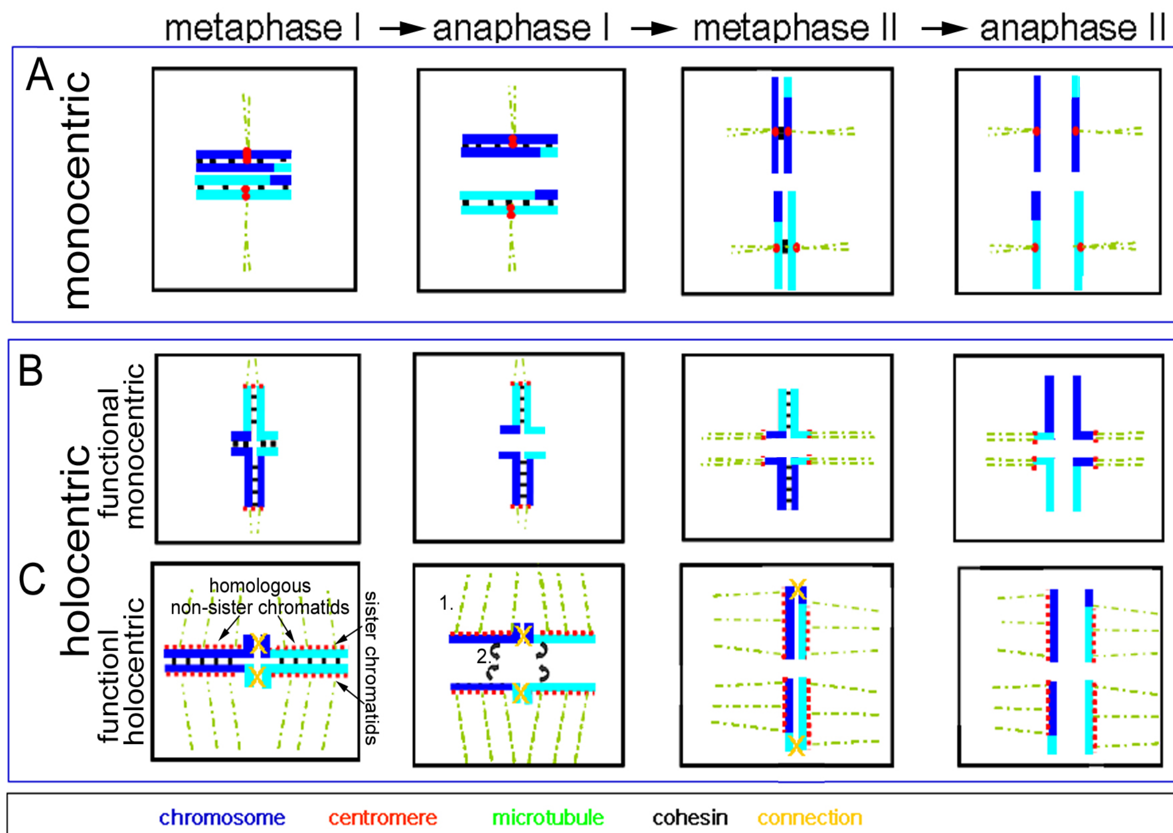


Figure 4 Schematic model of meiosis in species with (**A**) monocentric or (**B**, **C**) holocentric chromosomes (according to Heckmann and Houben, 2013). **B**) Meiosis in holocentrics with functional monocentric chromosomes (*C. elegans*; Maddox et al., 2004; Monen et al., 2005) or **C**) functional holocentric chromosomes (e.g. *Luzula* (Nordenskiold, 1962; Kusanagi, 1973) or mealybugs (Bongiorni et al., 2004)).

In principle, two options exist to deal with holocentricity and meiosis: 'Functional monocentricity' during meiosis and 'inverted meiosis' (Fig 4). In the first case, holocentric chromosomes act as monocentric ones; i.e. microtubule attachment to a restricted chromosomal region. Data from *C. elegans* suggest that active meiotic kinetochores are cenH3-independently formed at one chromosome terminus during meiosis I and at the opposite one during meiosis II in a CO-dependent manner (Albertson and Thomson, 1993; Maddox et al., 2004; Monen et al., 2005). Thus, in nematodes with no distinct predefined site for cohesion protection, the site of a single CO determines where active kinetochores are formed as well as where cohesion will

be kept and where released during meiosis (Schvarzstein et al., 2010). Second, holocentric bivalents align at metaphase I in such a manner that non-sister chromatids of homologs rather than homologous chromosomes segregate at anaphase I. Thus, the first meiotic division is equational and the second one is reductional, i.e. cytological an inverted sequence of meiotic events compared to the typical reductional-equational sequence observed in monocentrics. Inverted meiosis is thought to occur e.g. in the grass genus *Luzula* (Nordenskiold, 1962; Kusanagi, 1973) and the mealybug *Planococcus citri* (Bongiorni et al., 2004).

Our knowledge on meiosis of holocentrics is so far mainly based on light-microscopic observations in few plant species, e.g. *L. elegans* (Nordenskiold, 1962; Kusanagi, 1973), *R. tenuis* (Guerra et al., 2010), and different species of the genera *Cuscuta* [e.g. (Pazy and Plitmann, 1987, 1994; Guerra and Garcia, 2004)], *Eleocharis* [e.g. (Hakansson, 1954; da Silva et al., 2005; Vanzela et al., 2008)], *Chionographis* (Tanaka, 1980) and *Carex* [e.g. (Hoshino and Okamura, 1994; Hoshino and Waterway, 1994)], as well as various non-plant species [e.g. (Nokkala et al., 2004, 2006; Viera et al., 2009)]. Only for nematodes with functionally monocentric meiotic chromosomes, molecular data are available (Howe et al., 2001; Monen et al., 2005).

1.4.4 Meiosis in the holocentric plant species *Luzula elegans*

Meiotic studies have been performed in the wood-rush *L. elegans* Lowe (formerly *L. purpurea*) taking advantage of its low number and large size of chromosomes (Malheiros et al., 1947; Kusanagi, 1962; Nordenskiold, 1962). The data suggest an inverted sequence of meiotic events in this species.

After prophase I, interstitial COs are found which are not visible anymore at metaphase I. Due to a single terminal chiasma homologs typically form a rod bivalent at metaphase I. The bivalents lie flat on the equational plate perpendicular to the spindle pole; chromatids are auto-oriented, reminiscent of mitotic chromosomes. During anaphase I, non-sister chromatids of a rod-like bivalent move towards the same pole most likely linked by persisting terminal(ized) chiasma(ta). The association *via* terminal(ized) chiasmata is often termed ‘chiasma terminalisation’ (Nordenskiold, 1962; Bongiorni et al., 2004) based on the idea that an initial chiasma ‘moves’ during

meiosis I from its original location to the end of the homolog. However, 'chiasma terminalisation' has been challenged in monocentric and holocentric meiosis (Viera et al., 2009). Rather terminal chiasmata are later released than interstitial ones. During telophase I, however possibly persisting chiasmata are released in *L. elegans*. Until metaphase II alignment, homologous non-sister chromatids are associated along their length. During metaphase II, chromatids are aligned and segregate to opposite poles. Hence, meiosis seems to be inverted from a cytological point of view in *L. elegans*; displaying an equational first and a reductional second division (Fig 4).

2. Aim of this study

In 2009, at the beginning of the experimental work for this thesis, it was assumed that the molecular composition of kinetochores is conserved between mono- and holocentric organisms (Oegema and Hyman, 2006). Therefore, emphasizing the idea that in addition to studies of monocentric chromosomes studies of holocentric chromosomes can provide understanding both conserved and diverged features of centromere structure and function.

In species with holocentric chromosomes the genome organization as well as the chromosome structure and behaviour during mitosis and meiosis have been extensively investigated only in the nematode *Caenorhabditis elegans*. Similar studies in plants were missing.

Knowledge on the transcriptional regulation including potential transcription factors was not available for *cenH3*. Also, potential post-translational modifications of cenH3 (in plants) and the responsible enzymes were nearly unknown.

Thus, the aim was to study centromere organization in the monocentric plant species *Arabidopsis thaliana* from a molecular point of view, focussing on the regulation of cenH3, and to study the genome organization as well as behaviour of holocentric chromosomes during mitotic and meiotic division of *Luzula elegans.*

3. Results and Conclusions

3.1 Regulation of cenH3 in *Arabidopsis thaliana*

Studies in various organisms revealed that the level of cenH3 needs to be tightly regulated in order to guarantee correct centromere function. At the beginning of this study, data on the transcriptional and post-translational regulation of cenH3, particularly in plants, were limited.

3.1.1 Transcriptional regulation of *cenH3* in *A. thaliana*

It was asked whether there is a correlation between the incorporation in (late) G2 of cenH3 into centromeric nucleosomes and its expression. The fact that E2Fs regulate target genes cell cycle-dependently (Berckmans and De Veylder, 2009) and the close vicinity of potential E2F binding sites upstream the *cenH3* ATG (Lermontova et al., 2006), qualified *cenH3* as a potential E2F-target gene. Initial work on the transcriptional regulation of *cenH3* supported this idea (Heckmann, 2009).

Therefore, three questions were asked: Is *cenH3* transcribed through the entire cell cycle or only shortly prior to its deposition? Is cenH3 expressed only in meristematic and dividing cells or constitutively in all tissues? How is *cenH3* transcriptionally regulated and which transcription factors (TFs) are involved?

To figure out the transcriptional regulation of *A. thaliana cenH3* and its role for functional centromeres, the structure and activity of the *A. thaliana cenH3* promoter (*cenH3*pro) and its regulation by E2F TFs was studied (Heckmann et al., 2011a).

Various *cenH3*pro::*GUS* reporter gene constructs showed that *cenH3*pro is active in dividing tissues, that already 512 bp of *cenH3*pro are sufficient, while longer fragments enhance reporter gene expression, and that intragenic regulatory elements within the second intron confer regular expression in root meristems.

Different approaches showed that *cenH3* is an E2F target; i) ChIP with E2F-specific antibodies, ii) transient coexpression of a *cenH3*pro reporter construct with all E2F TFs, iii) stable overexpression of E2Fs, and iv) mutagenesis of the two E2F binding

sites present in *cenH3*pro. Transient expression and stable overexpression of E2Fs demonstrated that *cenH3* is induced by E2Fa and E2Fb, while E2Fc decreased *cenH3*pro activity *in planta*. Mutation of the two E2F binding sites of the *A. thaliana cenH3*pro led surprisingly to an increased *cenH3*pro activity, indicating E2F-mediated transcriptional repression of *cenH3*pro; particularly depending on the more upstream E2F binding site (E2F2). This repression might be based on the interplay of typical and atypical E2Fs in a cell cycle stage-dependent manner and/or the interaction of typical E2Fs with the retinoblastoma-related (RBR) protein.

The data suggest that E2Fs are likely involved in differential transcriptional regulation of *cenH3 versus H3* (*H3* promoters lack E2F sites). This *cenH3* regulation might be conserved, since E2F-mediated transcriptional regulation is conserved between species, and since E2F sites are also found in human and *Drosophila cenH3*pro regions.

3.1.2 Post-translational regulation of cenH3 in *A. thaliana*

Data on post-translation cenH3 modifications in plants are sketchy. It is known that maize cenH3 undergoes phosphorylation (Zhang et al., 2005). Likely, this mark might play a similar role for kinetochore function and chromosome alignment as the cell cycle-dependent phosphorylation of Serine7 of human cenH3 by Aurora kinases (Zeitlin et al., 2001b; Zeitlin et al., 2001a; Kunitoku et al., 2003).

Initial Bimolecular Fluorescence Complementation (BIFC) experiments (Susann Hesse and Dmitri Demidov, unpublished) showed a physical association of the *Arabidopsis* homologous AtAurora1 and AtcenH3. Therefore, it was asked whether AtcenH3 undergoes phosphorylation; and if so, at which residue(s)? Are Aurora kinases involved in cenH3 phosphorylation in plants? And which physiological role plays this potential mark *in planta*?

To figure out, whether AtcenH3 undergoes AtAurora-mediated phosphorylation, recombinant AtcenH3 (Fig 5) and AtAurora1 and 3 (Fig 6) were generated (Heckmann et al., unpublished).

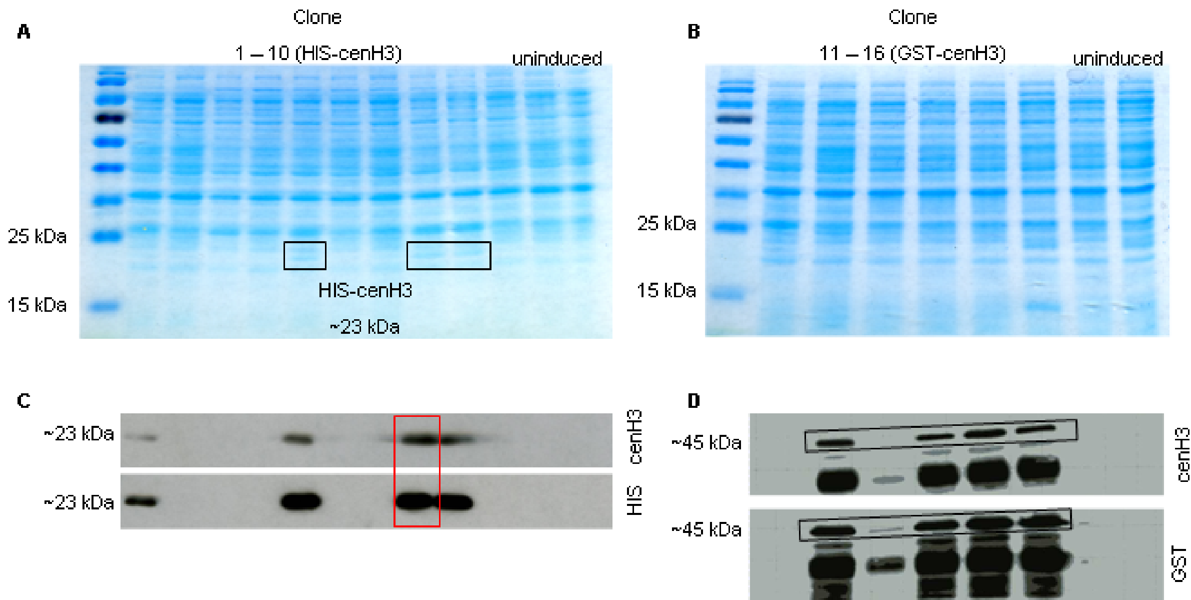


Figure 5 Production of recombinant *A. thaliana* cenH3 in *Escherichia coli*. **A**, **B**) Various bacterial strains and cenH3-expressing plasmids (kindly provided by Dmitri Demidov) were tested for their capacity to express recombinant cenH3 either fused to a 6xHIS- (**A**) or to a GST-tag (**B**) and analyzed on a Coomassie gel. **C**, **D**) Western Blotting: Anti-cenH3 antibody for the HIS- and GST-tagged cenH3 variants (**C**, **D**), and either an anti-HIS (**C**) or anti-GST (**D**) antibody, respectively. Expected size: ~22-23 kDa for HIS-cenH3 (~19 kDa cenH3 and ~3-4 kDa HIS) and ~44-45 kDa for GST-cenH3 (~19 kDa cenH3 and ~25 kDa GST). Note, besides expected size of recombinant GST-cenH3 (delimited by black boxes in **D**) additional bands were detected likely corresponding to degradation products of recombinant cenH3. Thus, after optimization of conditions (induction time, temperature, purification, etc.), only HIS-cenH3 (clone 8; delimited by red box in **C**) was further analyzed (Fig 6).

Both, AtAurora1 and 3, phosphorylate recombinant AtcenH3 *in vitro* (Fig 6, black box). As shown before (Demidov et al., 2009), AtAuroras can undergo auto-phosphorylation *in vitro* (Fig 6, red box).

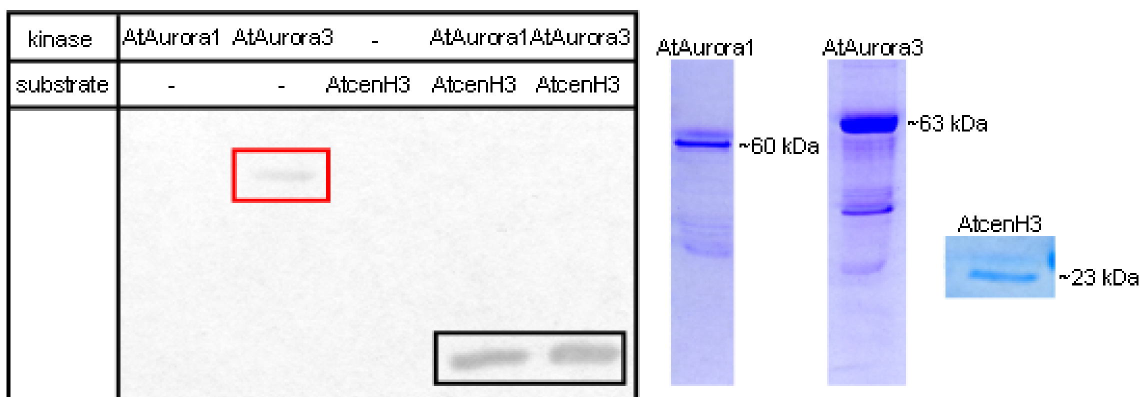


Figure 6 AtcenH3 undergoes AtAurora-mediated phosphorylation *in vitro*. AtAurora kinases phosphorylate recombinant cenH3 (black box) *in vitro*. Purified protein samples used for the kinase

assay are shown (right). Expected size: cenH3 (~23 kDa, see Fig 5), ~60 kDa for GST-AtAurora1 (~35 kDa AtAurora1 and ~25 kDa GST) and ~63 kDa for GST-AtAurora3 (~38 kDa AtAurora3 and ~25 kDa GST). Bacterial strains expressing recombinant AtAurora1 or AtAurora3 were kindly provided by Dmitri Demidov. Note autophosphorylation of AtAurora3 (red box).

To work out which residue(s) of AtcenH3 undergo(es) AtAurora-mediated phosphorylation, recombinant *in vitro* phosphorylated AtcenH3 was supplied to mass spectrometry analysis (kindly performed by Janusz Debski, Warsaw, Poland; and Johanna Lehne, Göttingen, Germany). Unfortunately, both MS-studies failed to determine the residue(s) which undergo(es) phosphorylation. Most likely, the non-radioactively labelled/phosphorylated fraction of recombinant cenH3 protein was not sufficient for MS-based detection.

Next, AtcenH3 was studied *in silico* for potential AtAurora-specific serine (S) or threonine (T) phosphorylation sites (minimal Aurora recognition motif in plants: X B (B) T/S, where X is any low molecular weight (e.g. Alanine) and B is any basic amino acid; personal communication by Dmitri Demidov). Based on *in silico* analysis, 11 peptides allowing the discrimination between all potential sites were used as AtAurora3 substrate in a radioactive kinase assay (assay performed by Dmitri Demidov) (Fig 7). The strongest AtAurora3-mediated phosphorylation signal was found for peptide 4 carrying serine 65 (S65) (Fig 7).

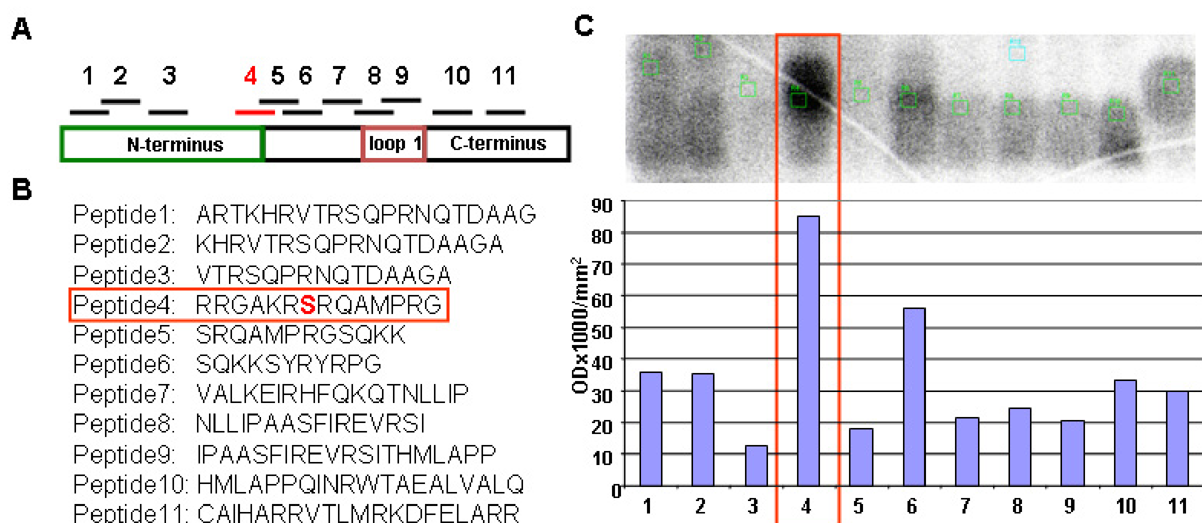


Figure 7 AtAurora3 phosphorylates AtcenH3 at Serine 65 *in vitro*. **A**) AtcenH3-specific peptides according to their position within AtcenH3. **B**) 11 peptides discriminating all potential AtAurora-specific phosphorylation sites of AtcenH3 were used as kinase assay substrate. **C**) AtAurora3 peptide-based

kinase assay: Peptide 4 (red box) carrying serine 65 showed the highest phosphorylation intensity; position of peptide 4 (S65) within AtcenH3 highlighted in red (**A**).

Serine 65 of AtcenH3 might functionally correspond to S50 phosphorylation of maize cenH3. Also, S65 is in the N-terminal cenH3 part, which varies from other H3 forms. As the N-terminal region is essential for the meiotic process (Lermontova et al., 2011; Ravi et al., 2011), a functional role of S65 phosphorylation particularly during meiosis is likely. Currently, generation of a S65ph-specific peptide antibody of cenH3 is in progress for studying the dynamics and occurrence of this mark *in planta*.

To study the physiological role of AtcenH3 phosphorylation *in planta*, various constructs were generated mimicking hypo- or hyperphosphorylation of S65; i.e. S65 was modified to alanine (hypo-phosphorylation) or aspartate (hyper-phosphorylation). Plasmids kindly provided by Simon Chan (plasmid '100' containing the endogenous *cenH3* locus used successfully to complement *cenh3-1* (Ravi et al., 2010)) and by Inna Lermontova (plasmid 'EYFP-cenH3' containing a 35S-driven EYFP-cenH3 expression cassette (Lermontova et al., 2006)) were the source to generate S65-mutated versions of cenH3.

Localization and dynamics of S65-mutated cenH3s will be studied in *A. thaliana* plants which were transformed with constructs constitutively overexpressing S65ph-mimicking cenH3 variants fused to EYFP.

The physiological role of cenH3S65ph *in planta* will be studied in transformed *cenh3-1* plants (kindly provided by Simon Chan). Heterozygous c*enh3-1* plants, screened before transformation *via* cleaved amplified polymorphic sequence (CAPS) analysis for heterozygous according to Ravi et al. (2010), have been transformed with constructs containing the endogenous *cenH3* locus expressing S65ph-mimicking *cenH3* variants, to point out whether S65ph-mimicking cenH3s complement *cenh3-1*.

Currently, analysis of this transgenics is in progress.

3.2 Genome organization, mitosis and meiosis of *Luzula elegans*

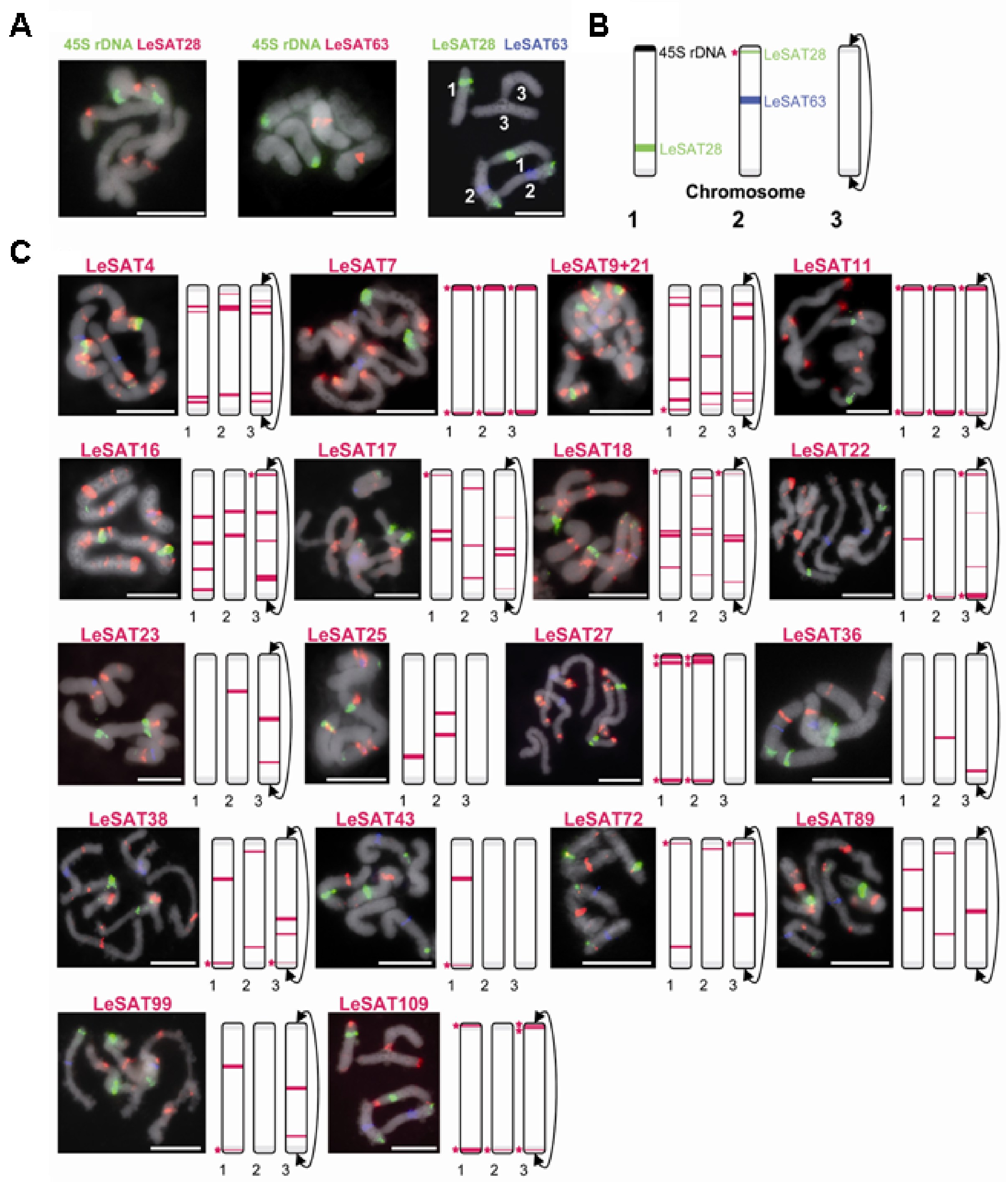
Kinetochore functions and its molecular compositions are conserved among mono- and holocentric species (Oegema and Hyman, 2006), stressing the idea that studies of holocentric chromosomes can help to understand chromosome structure and function as well as centromere biology. There are no detailed studies on holocentric chromosomes in plants with regard to genome organization and mitotic and meiotic chromosome behaviour. Thus, *L. elegans* (*Juncaceae*) was selected for the study of holocentric species' due to its low number of large chromosomes.

3.2.1 Genome and higher order chromatin organization of *L. elegans*

In general, the structure of monocentric chromosomes of higher plants is characterized by distinct eu- and heterochromatic regions. Distribution of heterochromatin depends on organism's genome size.

It was asked whether the composition of holocentric chromosomes displays similar characteristics as monocentric ones (Heckmann et al., 2013). In particular: Does interplay between centromere and large-scale genome organization exist? How are repetitive sequences and epigenetic marks distributed in holocentric chromosomes? Do centromere-specific sequences exist in holocentric chromosomes?

To study the repetitive DNA fraction and epigenetic marks in *L. elegans*, Illumina sequencing combined with bioinformatic and cytogenetic approaches was applied. The genome (3.81 Gbp/1C) contains 61% highly repetitive DNA (Fig 9a). There was a high diversity of satellite DNA; i.e. thirty seven distinct sequence families were identified. By far the most dominant repeat fraction (~33% of the genome) was the Angela clade of Ty1/copia LTR-retrotransposons. An expansion of Ty1/copia (Angela clade) and of satellite sequences most likely resulted in the exceptionally large genome of *L. elegans* compared to other members of the genus with a nuclear DNA content ranging from 0.26-1.99 Gbp/1C (Bennett and Leitch, 2010).



D

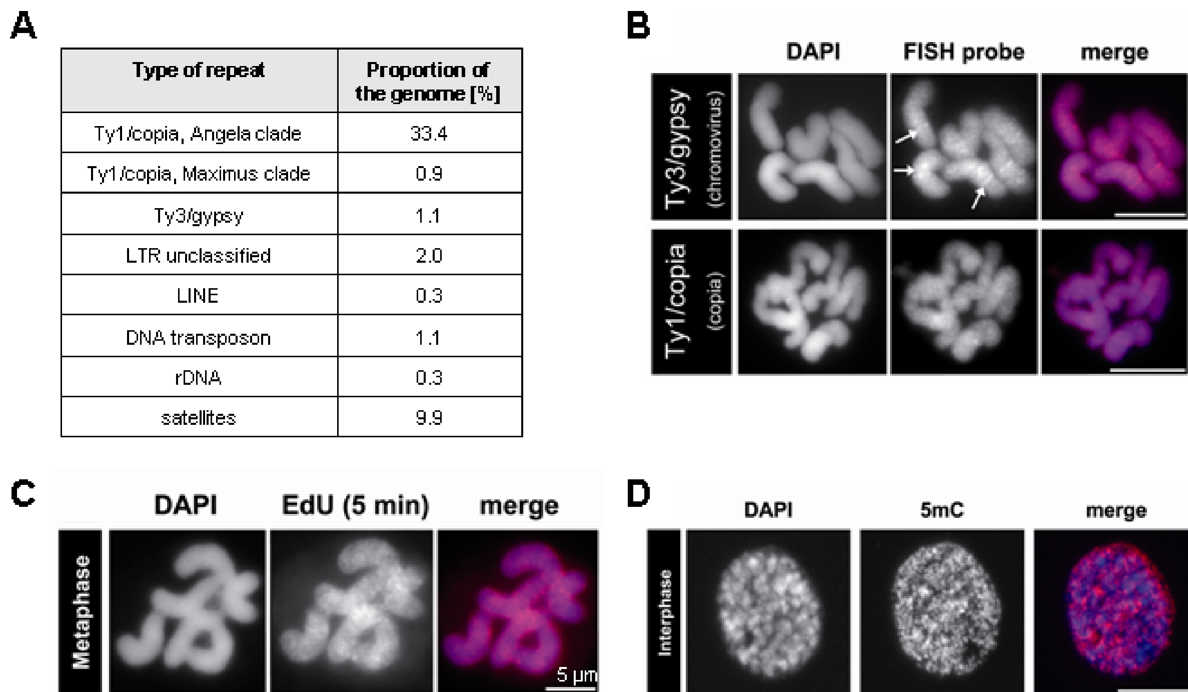
	Chromosome 1	Chromosome 2	Chromosome 3	All chromosomes
No. of cluster	42	37	43	122
No. of centromeric cluster (%)	26 (61.9)	27 (73)	30 (69.8)	83 (68)
No. of non-centromeric cluster (%)	16 (38.1)	10 (27)	13 (30.2)	39 (32)
Ratio of the relative cluster abundance in non-centromeric *vs.* centromeric regions*	5.5 : 1	3.3 : 1	3.9 : 1	4.2 : 1

Figure 8 Distribution of satellite DNA sequences in *L. elegans* (modified according to Heckmann et al., 2013). **A**) FISH: LeSAT28 and 63 (red) localized individually relative to a 45S rDNA probe (green) and relative to each other (LeSAT28: green; LeSAT63: blue). Mitotic metaphase chromosomes are

morphologically not distinguishable (similar size). LeSAT28 and 63 allowed their discrimination, although the minor LeSAT28 signal on chromosome 2 was not always detectable. **B**) Karyogram based on probes used in (**A**). **C**) FISH: Various LeSATs (red) together with LeSAT28 (green) and 63 (blue) and resulting ideograms. Size bar = 10 µm. Chromosome ends (grey-shaded) in schematic ideograms represent defined terminal (non-centromeric) chromosome regions. Terminal counted satellite clusters indicated by asterisks (see **D**). To guarantee a distinction between centromeric and non-centromeric chromosome regions only clusters within terminal ~5% of chromosome ends were counted as non-centromeric, i.e. both terminal regions represent ~10% of total chromosome length. Note, the centromere discontinuing at each subterminal chromosome end represents on average 75% of metaphase chromosome length (Heckmann et al., 2011b; Heckmann et al., 2013). Thus, the percentage of non-centromeric clusters might be underestimated. **D**) FISH signals of satellites classified into centromeric and non-centromeric clusters according to (**C**). Out of 122 identified satellite clusters, 39 localized in terminal non-centromeric chromosome regions while 83 localized in interstitial centromeric regions. Considering that terminal non-centromeric regions represent only ~10% of total chromosome length, abundance of satellite clusters is in these regions on average 4.2-fold higher than in interstitial centromeric regions. *The relative cluster abundance is calculated based on the absolute values of satellite clusters in non-centromeric and centromeric regions considering their different lengths.

Twenty newly-identified satellite sequences were localized *in situ* (Fig 8). A karyogram allowing the discrimination between the 3 equally-sized chromosome pairs based on these probes was established (Fig 8). Most satellite sequences were found on all three pairs of chromosomes. However, also chromosome-specific families labeling two chromosome pairs (LeSAT23, 25, 27, 28, 36 and 99), or one chromosome pair only (LeSAT43 and 63) were detected.

Satellite repeats showed a tendency of clustering towards the centromere-free chromosome ends in *L. elegans* (Fig 8 c,d). Terminal enrichment of repetitive DNA seems to be a common feature of holocentric autosomes (Heckmann and Houben, 2013), possibly indicating a correlation between holocentricity and terminally enriched satellite DNA. A mutual exclusion of heterochromatin and centromere function could be the reason for clustering of repetitive DNA at centromere-free chromosome ends. Alternatively, terminal heterochromatin might be involved in the physical end-to-end association of homologous chromosomes manifested as rod- or ring-bivalents during meiosis (Heckmann and Houben, 2013). Data on meiosis in *L. elegans* support this idea (see 3.2.3).



Type of repeat	Proportion of the genome [%]
Ty1/copia, Angela clade	33.4
Ty1/copia, Maximus clade	0.9
Ty3/gypsy	1.1
LTR unclassified	2.0
LINE	0.3
DNA transposon	1.1
rDNA	0.3
satellites	9.9

Figure 9 Genome organization of *L. elegans* (modified according to Heckmann et al., 2013). **A**) Repetitive DNA composition of the genome. **B**) LTR-retrotransposons are uniformly dispersed. Note interstitial centromere-atypical clustering (arrows) of Ty3/gypsy elements. **C**) No distinguishable large-scale patterns of early and/or late DNA replicating domains and of **D**) DNA methylation detectable. Size bar = 10 µm (or else indicated).

LTR-retrotransposons are uniformly dispersed along chromosomes (Fig 9b). Surprisingly, neither typical centromere-associated LTR retrotransposons (Ty3/gypsy of the CRM clade) were found nor any satellite DNA revealed a distribution pattern reflecting the almost chromosome-wide centromere distribution in *Luzula*. Apparently, similar as in *C. elegans* (Gassmann et al., 2012), no centromere-specific repetitive sequences were found in *L. elegans*. CenH3 may be associated with a centromere-specific chromatin status rather than with typical centromeric sequences in *L. elegans*. Therefore, *L. elegans* chromosomes likely consist of centromeric chromatin interspersed by non-centromeric chromatin. It is tempting to speculate, that the more a centromere expands, the less important is the primary DNA sequence and the more important becomes a centromere-specific chromatin status or organization.

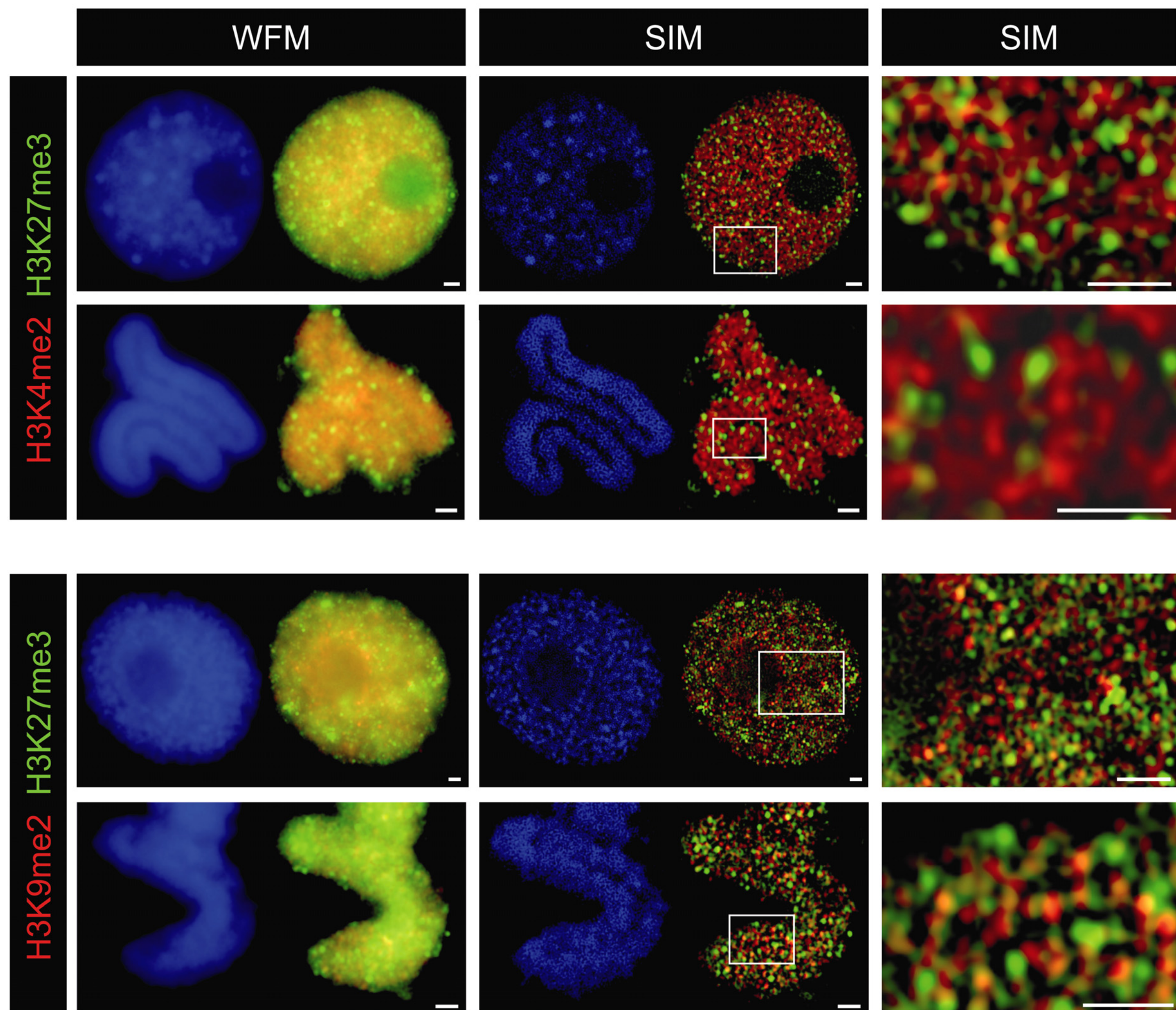


Figure 10 Distribution of typical eu- (H3K4me2) and heterochromatin (H3K9me2 and H3K27me3) associated post-translational histone H3 modifications in *L. elegans* (according to Heckmann et al., 2013). Left: Fluorescence Wide Field Microscopy (WFM). Middle: Structured Illumination Microscopy (SIM). Right: Enlargements of the regions delimited by the white boxes. DNA (blue), histone marks (red and green). Size bar = 1 µm.

Epifluorescence microscopy revealed on mitotic chromosomes a uniform distribution of eu- and heterochromatin-typical epigenetic marks and of early and/or late DNA replicating domains (Figs 9c, d and 10). Super-high resolution microscopy revealed distinct sub-units of different chromatin states (Fig 10).

An intermingled arrangement of eu- and heterochromatin throughout holocentric genomes might be favored by the almost chromosome-wide distribution of centromeric chromatin. Apparently, the large-scale organization differs between mono- and holocentric chromosomes (Fig 11).

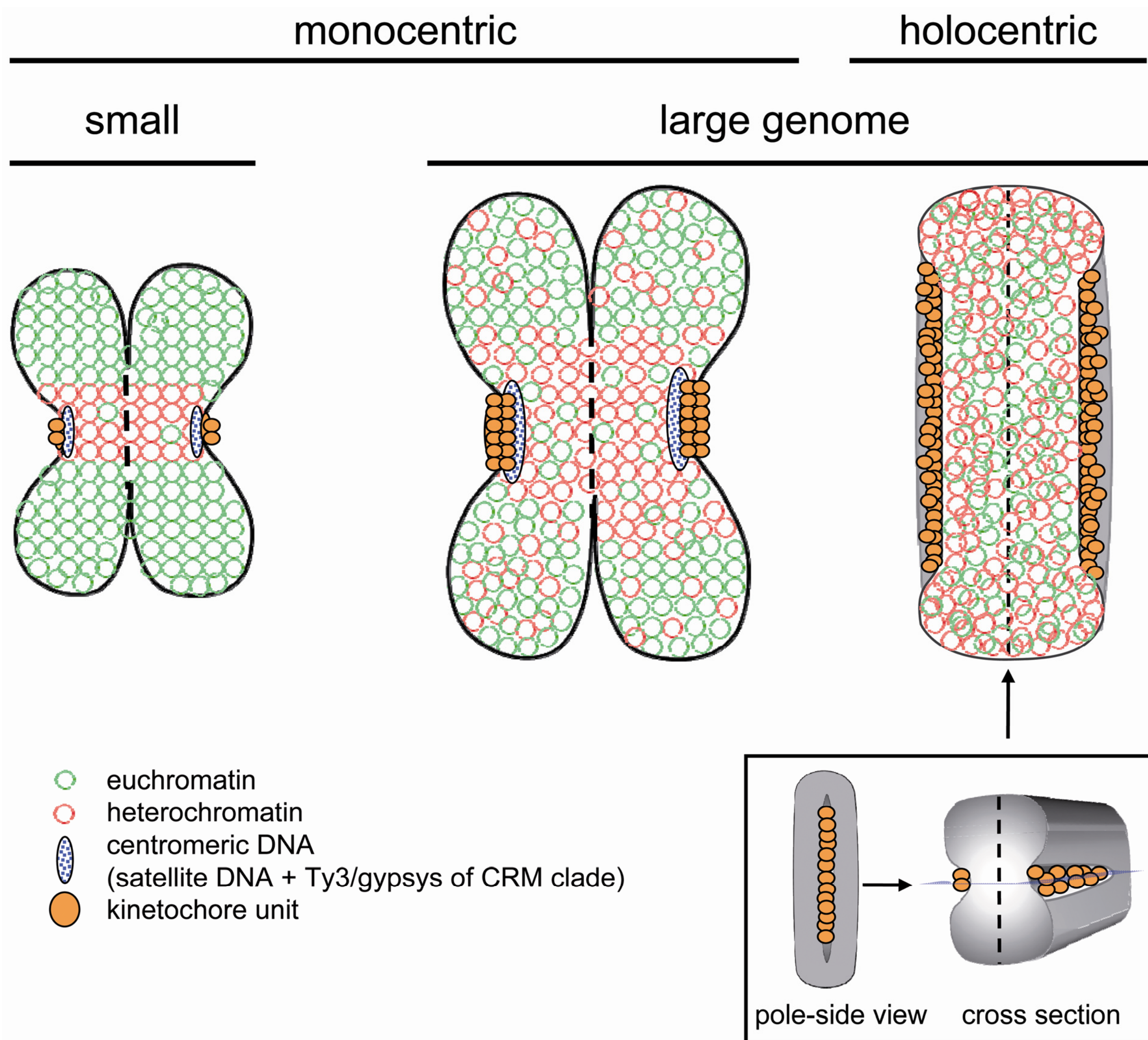


Figure 11 Model of chromatin and centromere arrangement in holocentrics *vs.* monocentrics and according to genome size (according to Heckmann et al., 2013).

3.2.2 Mitosis in *L. elegans*

Initial data obtained for the closely related species *L. nivea* indicated a longitudinal centromere-like groove along each holocentric sister chromatid (Nagaki et al., 2005). To confirm a distinct holocentric chromosome architecture, structure and dynamics of mitotic holocentric *L. elegans* chromosomes were studied. It was asked whether holocentric chromosomes of different origin and of different size are characterized by similar specific features (Heckmann et al., 2011b).

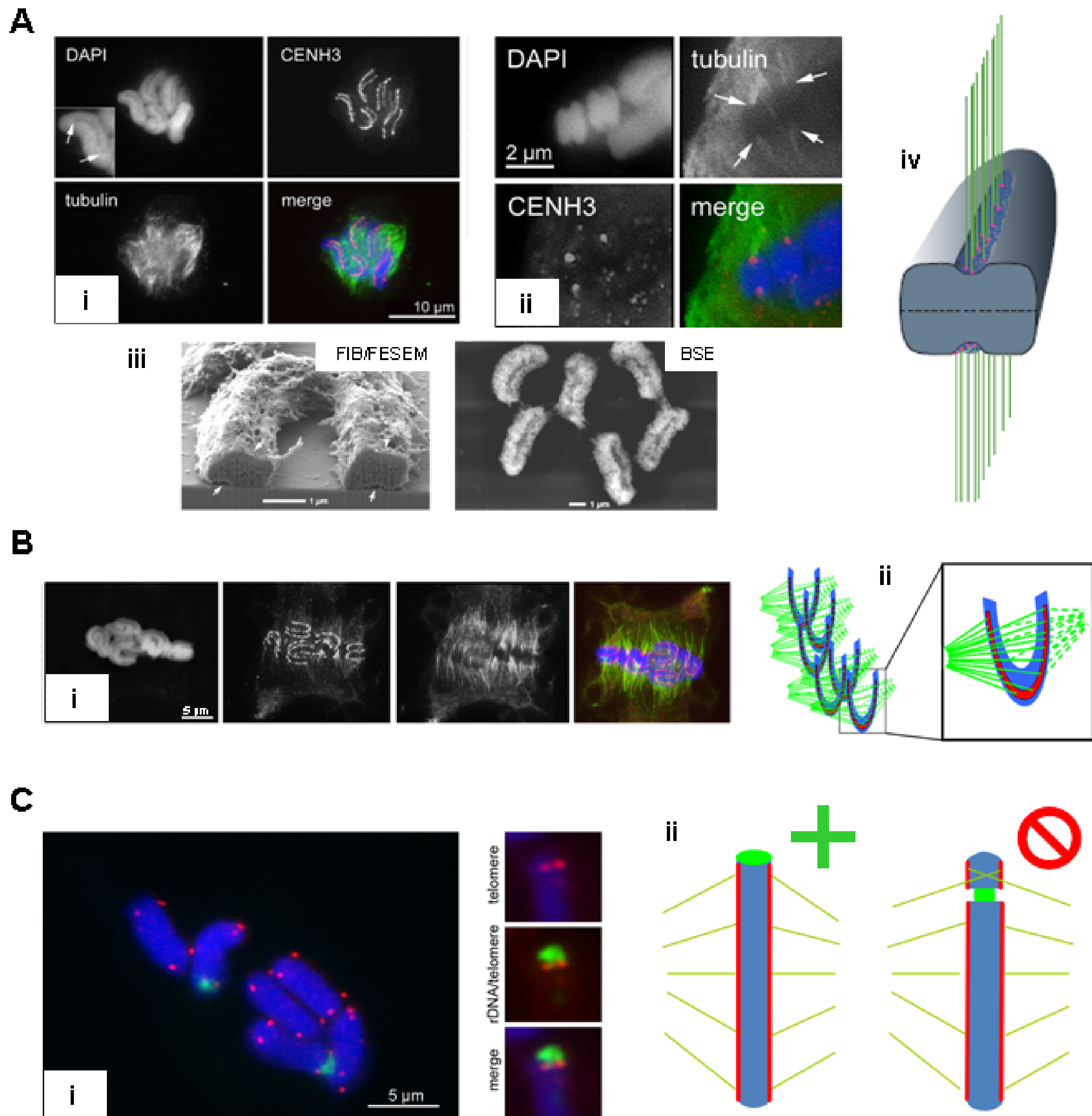


Figure 12 Mitotic chromosomes of *L. elegans* (modified according to Heckmann et al., 2011). **A**) Longitudinal centromeric groove along each sister chromatid except at chromosome ends. i, ii) Immunostaining, iii) scanning electron microscopy (FIB/FESEM: focused ion beam milling/field emission scanning electron microscope; back-scattered electron (BSE) signals) and iv) a cross-section model of *L. elegans* mitotic metaphase chromosomes; DNA blue, cenH3 red, and tubulin green. No structural boarder was found between sister chromatids (dotted line). **B**) Chromosome bending: i) CenH3- and tubulin-immunolabeling and ii) model of chromosome bending at metaphase/anaphase transition (cenH3 red, tubulin green, DNA blue). **C**) Terminal NOR position. i) FISH: 45S rDNA (green), telomeres (red), DNA (blue). ii) Model of two possible NOR locations (green) in holocentrics and resulting microtubule interaction. In case of an interstitial NOR, a mutual exclusion of NOR and centromere position would generate a quasi 'di-holocentric' chromosome. In case of a twist between sister chromatids within the less condensed interstitial NOR the same chromatid (left and right the NOR) could be pulled to opposite poles during divisions, causing anaphase bridges and subsequently chromosome breaks.

Light microscopic and ultra-structural investigations showed that mitotic *L. elegans* chromosomes display a longitudinal centromeric groove along each sister chromatid to which cenH3 and microtubules co-localize, except at chromosome ends (Fig 12a). At metaphase/anaphase transition, chromosomes are bent to a sickle-like shape likely mediated by microtubule forces and possibly facilitating movement of large holocentric chromatids (Fig 12b). A single 45S-rDNA locus, situated distal to *Arabidopsis*-telomere repeats, was localized at the end of one chromosome pair. A terminal centromere-free NOR may ensure mitotic chromosome stability (Fig 12c).

3.2.3 Meiosis in *L. elegans*

Early studies in *L. elegans* (formerly named *L. purpurea*) suggested an inverted order of meiotic sequences, i.e. an equational first and reductional second meiotic division (Kusanagi, 1962; Nordenskiold, 1962). However, no studies were conducted on the meiotic centromere distribution in *Luzula* or any other holocentric plant chromosomes to support holocentricity during meiosis and to confirm the occurrence of inverted meiosis. It was asked whether *L. elegans* shows holocentricity during meiosis, performs inverted meiosis, and to what degree meiosis in *L. elegans* differs from that in monocentric chromosome species (Heckmann et al., in preparation).

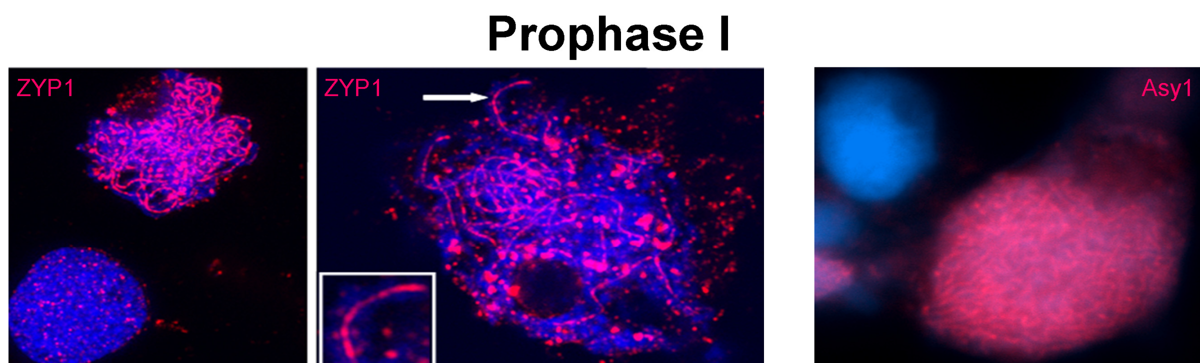


Figure 13 Synaptonemal complex (SC) and axis formation during prophase I in *L. elegans*. Immuno-detection of the SC-associated protein Zyp1 and of the axial element-associated protein Asy1 using anti-*Arabidopsis* Zyp1 and anti-*Arabidopsis* Asy1. Antibodies kindly provided by Chris Franklin.

Early prophase I events in *L. elegans* are cytologically similar to that in other species, as indicated by presence of synaptonemal complex (Zyp1 signals) and by axis formation (Asy1 signals) (Fig 13). *L. elegans* chromosomes are holocentric

throughout meiosis (Fig 14, 15). Besides *C. elegans*, that shows restricted kinetic activity (functional monocentric chromosomes) and does not require cenH3 and CENP-C during meiosis (Monen et al., 2005), in no other holocentric chromosome species the meiotic centromere organization has been traced.

At diakinesis only two predominant bivalent configurations are apparent, rod- or ring-like structures (Fig 14). Rod-like bivalents show a puffed end-to-end association. This connection between homologs is free of cenH3. FISH with *Arabidopsis*-type telomere sequences and with 45S rDNA showed that the puffed end displays in some cases a cross-shaped configuration reminiscent of a terminal chiasma involving the NOR or telomeres (Fig 14). In case of a twist between associated homologs, a cross-shape configuration would also be expected within this region. Alternatively, a twist within this region might explain why a cross-shape configuration is not always apparent.

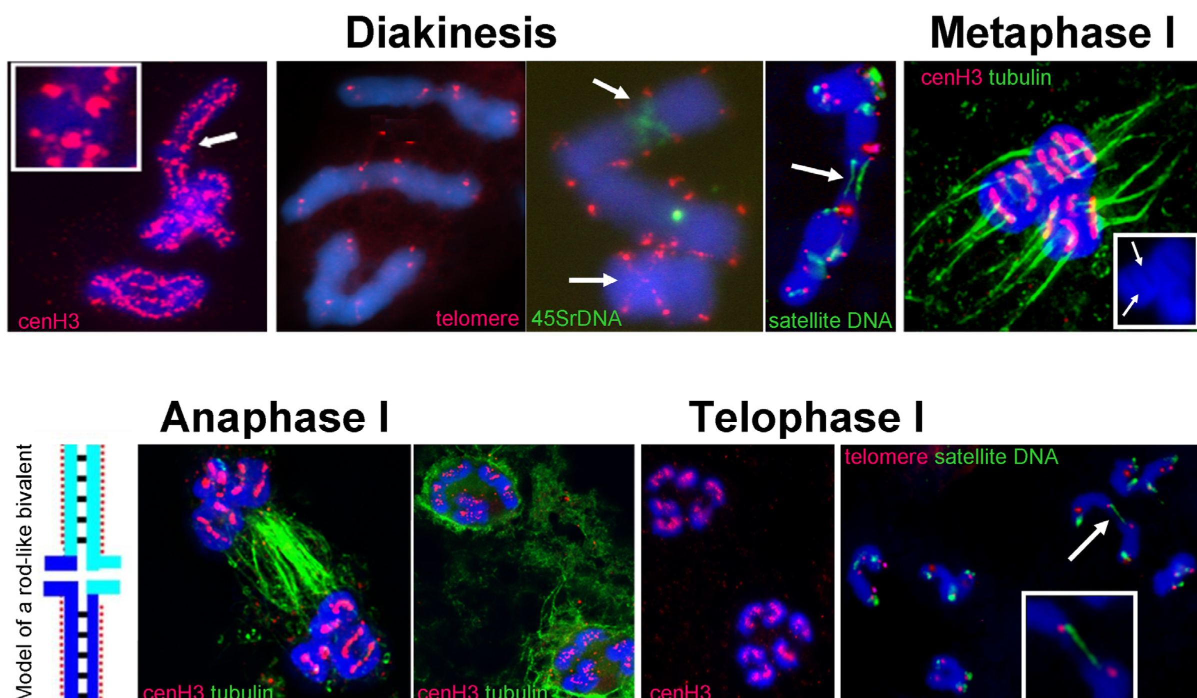


Figure 14 *L. elegans* chromosomes during the first meiotic division. Immunolabeling with cenH3 (red) reveals holocentric rod- and ring-like bivalents during diakinesis. Rod-bivalents show an end-to-end association free of cenH3 (arrow, inset). FISH with a *Arabidopsis*-type telomere (red) and a 45S rDNA (green) probe indicates cross-shaped (arrows) FISH signals and FISH with a satellite DNA (green) and a *Arabidopsis*-type telomere (red) probe shows chromatin stretches (arrow), in both cases within the region between telomeres of the end-to-end associated homologs. CenH3- (red) and tubulin-immunolabeling (green) from metaphase I until telophase I shows that meiotic chromosomes are holocentric and separation of sister chromatids occurs at meiosis I due to holokinetic activity along the length of individual chromatids; i.e. an equational first division during which sister chromatids rather

than homologous chromosomes are separated. Interestingly, the two sister kinetochores per homolog behave as two distinct functional units during metaphase I: four individual linear kinetochores per bivalent are visible. DAPI-stained metaphase I chromosomes show a pronounced centromeric groove structure along each sister chromatid (inset, arrows indicate 2 grooves along the 2 sister chromatids of one homologous chromosome). FISH with *Arabidopsis*-type telomere (red) and satellite DNA (green) probes shows that terminal satellite DNA is involved in the end-to-end association of homologs during meiosis I and that the association of non-sister chromatids is chromatin-based. Arrow indicates stretched satellite DNA (inset) between telomeres of two non-sister chromatids at telophase I (picture kindly provided by Maja Jankowska).

FISH showed that the 45S rDNA and terminal satellite DNA, cytologically distal the *Arabidopsis*-type telomeres, are involved in the end-to-end association of homologs during meiosis I (Fig 14). A satellite DNA-containing chromatin protrusion might mimic the morphological chromosome end while telomeres are at the physical DNA end as speculated for the terminal 45S rDNA (Heckmann et al., 2011b). However, terminal repetitive DNA is a common feature in holocentric autosomes (Heckmann and Houben, 2013). Thus, terminal heterochromatin facilitating the end-to-end association of homologs might be needed to deal with holocentricity during meiosis. Finally, if chiasmata are restricted to chromosome ends, most of the chromosome regions would not recombine. Taken into account that early prophase I events (when COs are induced) are cytologically similar to other species, it is likely that COs are established along chromosomes while finally possibly only terminal chiasmata persist. However, the question remains how to resolve interstitial chiasmata under maintenance of terminal ones? Alternatively, more likely 'sticky' terminal heterochromatin may enable an achiasmatic end-to-end association of homologs.

At metaphase/anaphase I transition, from the top view (with respect to metaphase I plate), bivalents appear rod-like, whereas from the side view they occur mainly U-shaped (Figs 14, 16). The predominant occurrence of rod bivalents suggests that one end-to-end connection within a ring bivalent gets preferentially resolved. The U-shape of rod bivalents is likely mediated by spindle forces comparably to the sickle-shape mitotic chromosomes at metaphase/anaphase transition (Fig 12b; Heckmann et al., 2011b). Microtubules attach to cenH3-containing chromatin along nearly the entire chromosome axes of the linear bivalents (Fig 14). From meta- until telophase I chromosomes are highly condensed and show a pronounced centromeric groove along each sister chromatid (Fig 14, metaphase I inset).

Contrary to a monopolar centromere orientation in monocentrics, sister kinetochores of *L. elegans* behave as two distinct functional units during meiosis I mediating bipolar attachment to microtubules. Hence, four individual linear kinetochores are formed instead of two fused focal kinetochores (Fig 14). Whereas in monocentrics sister chromatid cohesion is released in a two-step way (first along chromosome arms during meiosis I and second at centromeres during meiosis II), in *L. elegans* sister chromatid cohesion is released along holocentric chromosomes with the possible exception at the end-to-end association between non-sister chromatids. Thus, sister chromatid separation occurs at meiosis I due to holokinetic activity along the length of individual chromatids. Therefore, sister chromatids are separated rather than homologous chromosomes. It is unknown whether the end-to-end association of non-sister chromatids during anaphase and telophase I is based on persisting chiasmata, terminal cohesion or other mechanisms. The association seems to be chromatin-based mediated by satellite DNA in *L. elegans* (Fig 14). Thus, observed chromatids are most likely connected at telophase I (Fig 14) *via* heterochromatin threads (satellite DNA) similar to achiasmatic heterochromatin threads found during *Drosophila* oocyte meiosis (Hughes et al., 2009).

During a rather short interkinesis, association of non-sister chromatids along their length occurs; i.e. connection between non-sister chromatids is established most likely by an achiasmatic cohesion mechanism. This would guarantee that until meiosis II non-sister chromatids become associated. FISH experiments (Fig 15) indicated alignment of homologous non-sister chromatids (pair wise distribution of 45S rDNA signals). The association along the entire length of non-sister chromatids persists until anaphase II (Fig 15). Whether known cohesion proteins such as Rec8 are involved in the re-establishment of cohesion after meiosis I is unknown.

At anaphase II associated non-sister chromatids are separated in a mitosis-like second meiotic division. At metaphase II holokinetic (cenH3 signals along the entire chromatid length) non-sister chromatids align perpendicular to the spindle axis at the equational plate and microtubules are attached all along their length (Fig 15). Notable metaphase II chromosomes are smaller (highly condensed) than mitotic ones. At anaphase II the two non-sister chromatids segregate holokinetically to opposite poles (Fig 15). Four daughter cells, each with a haploid set of chromosomes, are finally generated after formation of nuclear envelope and of cell wall during telophase II.

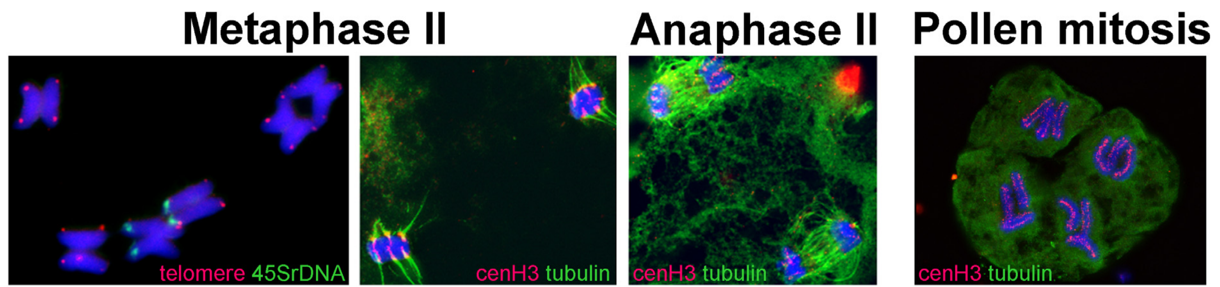


Figure 15 *L. elegans* chromosomes during the second meiotic division and the first pollen mitosis. Metaphase II: FISH with 45S rDNA (green) and *Arabidopsis*-type telomere (red) probes demonstrates association of homologous non-sister chromatids along their length. Immunolabeling with cenH3 (red) and tubulin (green) shows that holocentric non-sister chromatids are separated at metaphase II/anaphase II *via* holokinetic spindle attachment. Tetrads at first pollen mitosis show holocentric chromosomes after immunolabeling with anti-cenH3 (red) and anti-tubulin (green).

From the present data, the first meiotic division in *L. elegans* seems to be equational and the second meiotic division reductional. Thus, our observation supports the assumption that *Luzula* chromosomes perform an inverted sequence of meiotic events (inverted meiosis) in order to deal with holocentricity during meiosis (Fig 16).

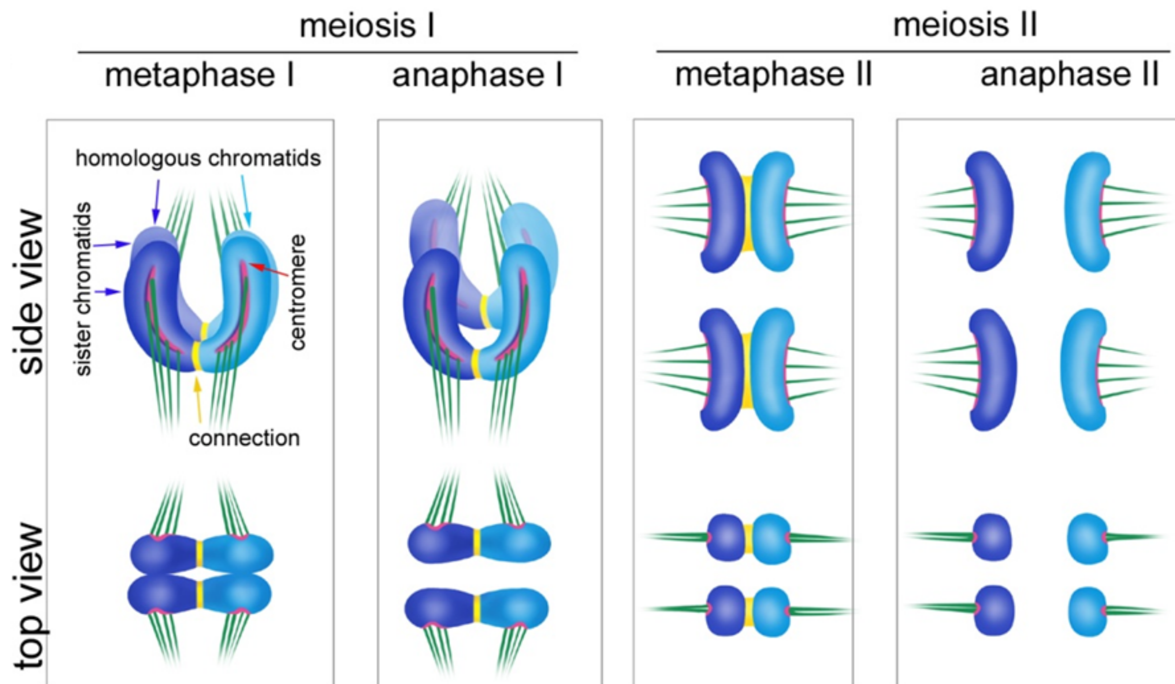


Figure 16 Structural model of meiotic chromosomes in *L. elegans*. Chromosomes are shown from the side and the top view (with respect to the metaphase plate) for better understanding. Holocentric U-shaped bivalents (homologs are end-to-end associated) align at metaphase I in such a manner that sister chromatids rather than homologous chromosomes are separated during meiosis I. During

meiosis II, associated non-sister chromatids are holokinetically separated. Therefore, the first meiotic division is equational and the second one is reductional. Hence, an inverted sequence of meiotic events from a cytological point of view compared to the typical reductional-equational sequence observed in monocentric organisms takes place.

However, in future the following questions need to be addressed: How is the meiotic recombination process regulated in *L. elegans* and what keeps the homologs together at their ends during meiosis? Are cross-overs randomly distributed or restricted to chromosome termini? Does a delayed resolution of terminal chiasmata ensure the correct separation of sister chromatids at meiosis I? Are cohesins involved in the end-to-end association of non-sister chromatids after metaphase I or other mechanisms? How is the alignment of non-sister chromatids realized at meiosis II, are cohesins involved?

To address these questions, a variety of available plant meiotic antibodies (kindly provided by various researchers; *Arabidopsis* Asy1, Zyp1C, MLH1, MSH4, RPa1a, Scc3, and Sgo1 and *Zea maize* Asy1, Am1, Zip1, and Sgo1) known to recognize proteins involved in meiotic kinetochore formation, recombination and cohesion was tested for cross-reactivity in *L. elegans*. Unfortunately, most antibodies did not cross-react with *L. elegans*, suggesting either a restricted conservation of protein similarity or, less-likely, that the corresponding homologs are not expressed in *L. elegans*. Only in case of Asy1 and Zyp1 (Fig 13) distinct immunolabeling patterns were observed similar to those described for species with monocentric chromosomes. Therefore, identification of corresponding genes in *L. elegans* based on deep sequencing of the meiotic transcriptome is currently in progress. After gene identification corresponding antibodies will be raised and used for immunostaining.

4. Summary

I) The transcriptional and post-translational regulation of the centromere-specific histone H3 (cenH3) was studied in *Arabidopsis thaliana*.

The *A. thaliana cenH3* promoter (*cenH3*pro) proved to be active in dividing tissues (Heckmann et al., 2011a). Just 512 bp upstream of the transcriptional start site are sufficient for reporter gene expression. Regulatory elements within the second intron confer regular expression in root meristems. That E2F transcription factors regulate *cenH3* was shown by (i) ChIP with E2F-specific antibodies, ii) plants stably overexpressing E2Fs, iii) transient co-expression of E2Fs and a *cenH3*pro reporter construct and iv) mutagenesis of the two E2F binding sites within *cenH3*pro. E2F-mediated transcriptional regulation of *cenH3* seems to be conserved between organisms.

AtAurora1 and 3 phosphorylate AtcenH3 *in vitro*; AtAurora3 phosphorylates Atcenh3 at serine65 (Heckmann et al., unpublished). Experiments to elucidate the biological role of AtcenH3S65ph are initiated. An AtcenH3S65ph-specific antibody was generated and various *A. thaliana* transformants mimicking hypo- or hyperphosphorylation of S65 have been generated.

II) The genome organization with special emphasis on the repetitive DNA composition and the chromatin properties as well as the behaviour of holocentric chromosomes during mitosis and meiosis were studied in the plant *Luzula elegans*.

The *L. elegans* genome (3.81 Gbp/1C) consists of 61% highly repetitive DNA with the Angela clade of Ty1/copia LTR-retrotransposons being the dominant repeat fraction (Heckmann et al., 2013). 37 distinct satellite DNA sequence families were identified. Twenty of them were localized *in situ* and a karyogram based on these probes was generated discriminating the 3 equally sized chromosome pairs. Satellite sequences are enriched at centromere-free chromosome ends, while LTR retrotransposons are uniformly dispersed along chromosomes. Terminal enriched repetitive DNA commonly found in holocentric autosomes (Heckmann and Houben, 2013) may ensure mitotic chromosome stability and/or correct meiosis. Centromere-specific repetitive sequences were not found in *L. elegans*. Post-translational histone H3

modifications and DNA methylation were uniformly distributed and no obviously late replicating regions were found. Super-high resolution microscopy revealed distinct intermingled units of different chromatin types. The data suggest a different genome organization for species with holo- *versus* such ones with monocentric chromosomes.

Instead of a 'diffuse' centromere organization along holocentric chromosomes, mitotic *L. elegans* chromosomes display a centromeric groove along each sister chromatid except at chromosome ends (Heckmann et al., 2011b). Chromosomes bend during metaphase/anaphase transition likely mediated by microtubule forces. A single 45S-rDNA locus is localized at the end of one chromosome pair distal to *Arabidopsis*-type telomere repeats and might ensure mitotic stability of the NOR-bearing chromosome.

Meiotic *L. elegans* chromosomes are holocentric throughout meiosis (Heckmann et al., in preparation). During early prophase I, meiosis proceeds as in other species. However, only end-to-end associated bivalents are observed at metaphase I. This connection is likely mediated by terminal satellite DNA and/or cross-over. Sister kinetochores behave as two distinct functional units during meiosis I mediating bipolar microtubules attachment contrasting the sister centromere fusion of monocentric chromosomes. Sister chromatids rather than homologous chromosomes are separated during anaphase I. Subsequently, association of non-sister chromatids occurs leading to half-bivalents at metaphase II, followed by a mitosis-like second meiotic division. The data suggests an equational first and a reductional second meiotic division. Thus, *L. elegans* chromosomes likely perform an inverted meiosis.

5. Zusammenfassung

I) Die transkriptionelle and post-translative Regulierung des zentromer-spezifischen Histons H3 (cenH3) wurde in *Arabidopsis thaliana* studiert.

Der *A. thaliana cenH3* Promoter (*cenh3*pro) wirkt in teilungsaktiven Geweben (Heckmann et al., 2011a). Bereits 512 bp strangaufwärts des Transkriptionsstarts reichen aus für eine Reportergenexpression. Regulatorische Elemente innerhalb des zweiten Introns vermitteln eine reguläre Expression in Wurzelmeristemen. Dass E2F Transkriptionsfaktoren *cenH3* regulieren wurde gezeigt durch (i) ChIP mit E2F-spezifischen Antikörpern, ii) stabil E2F-überexprimierende Pflanzen, iii) transiente Co-Expression von einem *cenh3*pro Reporterkonstrukt und E2Fs und iv) Mutagenese beider E2F-Bindestellen im *cenh3*pro. E2F-vermittelte transkriptionelle *cenH3* Regulierung scheint zwischen Organismen konserviert zu sein.

AtAurora1 und 3 phosphorylieren AtcenH3 *in vitro*; AtAurora3 phosphoryliert AtcenH3 an Position Serin 65 (Heckmann et al., nicht publiziert). Experimente um die biologische Rolle von AtcenH3S65ph aufzuklären sind initiiert. Dazu wurden ein AtcenH3S65ph-spezifischer Antikörper und mehrere *A. thaliana* Transformanten die eine Hypo- oder Hyperphosphorylierung von S65 nachahmen generiert.

II) Die Genomorganisation, vor allem die Komposition der repetitiven DNA und die Chromatineigenschaften, sowie das Verhalten von holozentrischen Chromosomen während der Mitose und Meiose wurden in der Pflanze *Luzula elegans* untersucht.

Das *L. elegans* Genom (3,81 Gbp/1C) besteht zu 61% aus hoch-repetitiver DNA mit der Angela Klade von Ty1/copia LTR-Retrotransposons als dominierendes repetitives Element (Heckmann et al., 2013). 37 unterschiedliche Familien von Satelliten-DNA wurden identifiziert. 20 von diesen wurden *in situ* lokalisiert und basierend darauf wurde ein Karyogram erzeugt, welches die mikroskopische Unterscheidung der 3 gleichgroßen Chromosomenpaare erlaubt. Satellitensequenzen sind angereichert an zentromer-freien Chromosomenden, wohingegen LTR-Retrotransposons gleichmäßig entlang der Chromosomen verstreut sind. Terminal angereicherte repetitive DNA, gewöhnlich in holozentrischen Autosomen zu finden (Heckmann and

Houben, 2013), könnte mitotische Chromosomenstabilität und/oder eine korrekte Meiose ermöglichen. Es wurden keine zentromer-spezifischen repetitiven Sequenzen gefunden. Post-translative Histon H3 Modifikationen und DNA-Methylierung waren gleichmäßig im Genom verteilt und es wurden keine auffällig spät-replizierenden Regionen gefunden. Super-hoch auflösende Mikroskopie zeigte eine distinkte Vermischung unterschiedlicher Chromatinformen. Die Daten deuten eine unterschiedliche Genomorganisation in Spezies mit holozentrischen gegenüber denen mit monozentrischen Chromosomen an.

Anstelle einer 'diffusen' Zentromerorganisation sind mitotische Chromosomen von *L. elegans* durch eine zentromerische Furche entlang der Schwesterchromatiden außer an den Chromosomenden gekennzeichnet (Heckmann et al., 2011b). Während des Überganges von der Metaphase zur Anaphase zeigen Chromosomen eine U-Form wahrscheinlich bedingt durch Mikrotubulikräfte. Der 45S-rDNA Lokus ist bevorzugt am Chromosomenende und könnte mitotische Stabilität von NOR-Chromosomen ermöglichen.

Meiotische *L. elegans* Chromosomen sind holozentrisch während der gesamten Meiose (Heckmann et al., in Vorbereitung). Während der frühen Prophase I läuft die Meiose ab wie in anderen Spezies. Am Ende der Metaphase I sind jedoch nur noch End-zu-End assoziierte Bivalente zu beobachten. Diese Verbindung wird wahrscheinlich durch terminale Satelliten-DNA und/oder Cross-over vermittelt. Schwesterkinetochore verhalten sich wie zwei eigenständige funktionelle Einheiten und vermitteln einen bipolaren Mikrotubuliansatz während der Meiose I. Im Gegensatz zu der Situation in monozentrischen Arten werden in *L. elegans* Schwesterchromatiden anstelle von homologen Chromosomen während der Anaphase I getrennt. In der Metaphase II kommt es zur Assoziation von nicht-Schwesterchromatiden, gefolgt von einer mitosegleichen zweiten meiotischen Teilung. Die Daten deuten eine ausgleichende erste und eine reduzierende zweite meiotische Teilung an. Daher durchlaufen *L. elegans* Chromosomen wahrscheinlich eine invertierte Meiose.

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7. *Curriculum vitae*

Personal details

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Education

08/1989 – 06/1993	Basic primary school in Bad Holzhausen
08/1993 – 07/2002	Secondary school in Luebbecke: 'Allgemeine Hochschulreife'
09/2002 – 06/2003	Compulsory community service in Luebbecke at the 'PARITÄTISCHE Verein für freie Sozialarbeit e.V.'
10/2003 – 03/2009	Degree in Biology at Kassel University: 'Diplom Biologe'
05/2008 – 03/2009	Diploma thesis at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben in the research group Karyotype Evolution headed by Prof. Ingo Schubert. Title: 'Charakterisierung des Expressionsmusters des zentromerischen Histons CENH3 in *Arabidopsis thaliana*' (Characterization of the expression pattern of the centromeric histone CENH3 in *Arabidopsis thaliana*)
04/2009 – 06/2009	Research assistant at the IPK in Gatersleben in the research group Karyotype Evolution headed by Prof. Ingo Schubert.
since 07/2009	PhD student at the IPK in Gatersleben in the research group Chromosome Structure and Function headed by Dr. habil. Andreas Houben. Title: Structure and regulation of centromeres in mono- and holocentric chromosomes

Gatersleben, 15.04.2013

(S. Heckmann)

List of Publications

Marques, A., Fuchs, J., Ma, L., **Heckmann, S.**, Guerra, M., Houben, A. (2011) Characterization of eu- and heterochromatin of Citrus with a focus on the condensation behaviour of 45S rDNA chromatin. Cytogenetics and Genome Research 134: 72-82.

Heckmann, S., Schroeder-Reiter, E., Kumke, K., Ma, L., Nagaki, K., Murata, M.M., Wanner, G., Houben, A. (2011) Holocentric chromosomes of *Luzula elegans* are characterized by a longitudinal centromere groove, chromosome bending and a terminal nucleolus organizer region. Cytogenetics and Genome Research 134: 220-228.

Heckmann, S., Lermontova, I., Berckmans, B., De Veylder, L., Bäumlein, H., Schubert, I. (2011) The E2F transcription factor family regulates *CENH3* expression in *Arabidopsis thaliana*. The Plant Journal 68: 646-656.

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Poster presentations

Heckmann, S., Nagaki, K, Houben, A. Title: Inverted meiosis – in the holocentric chromosome species *Luzula elegans*, 19.09 – 23.09.2009 , EMBO Conference Series - MEIOSIS, Isle sur la Sorgue, France

Lermontova, I., **Heckmann, S.,** Moraes, I., Rutten, T., Fuchs, J., Schubert, I. Title: Functional characterisation of the plant centromeric histone variant CENH3, 09.10 – 10.10.2009, Institutes day, IPK, Gatersleben, Germany

Heckmann, S., Nagaki, K, Houben, A. Title: Inverted meiosis – in the holocentric chromosome species *Luzula elegans*, 22.03 – 24.03.2010, DFG Priority Programme SPP1384 – Mechanisms of Genome Haploidization/ Annual Meeting, EMBL, Heidelberg, Germany

Heckmann, S., Nagaki, K, Houben, A. Title: Inverted meiosis – in the holocentric chromosome species *Luzula elegans*, 15.06 – 18.06.2010, Plant Science Students Conference 2010, IPK Gatersleben, Gatersleben, Germany

Lermontova, I., **Heckmann, S.,** Moraes, I., Rutten, T., Fuchs, J., Schubert, I. Title: Functional characterisation of the plant centromeric histone variant CENH3, 19.06 – 23.06.2010, EMBO Workshop on: Chromosome Segregation and Aneuploidy, Edinburgh, UK

Heckmann, S., Lermontova, I., Houben, A., Bäumlein, H., Schubert, I. Title: Regulation of CENH3 expression in *Arabidopsis thaliana*. 07.07 – 10.07.2010, Chromatin2010 – Transcription, chromatin structure and DNA repair in development and differentiation, Zeche Zollverein, Essen, Germany

Heckmann, S., Nagaki, K., Schroeder-Reiter, E., Macas, J., Houben, A. Title: Unusual holocentric chromosomes of the grass *Luzula elegans*. 28.03 – 30.03.2011, DFG Priority Programme SPP1384 – Mechanisms of Genome Haploidization - Annual Meeting, University Halle-Wittenberg, Halle, Germany

Heckmann, S., Lermontova, I., Demidov, D., Berckmans, B., De Veylder, L., Bäumlein, H., Schubert, I., Houben, A. Title: Transcriptional and posttranslational regulation of CENH3 in *Arabidopsis thaliana*. 12.10 – 14.10.2011, EMBO workshop on: Histone Variants and Genome Regulation, IGBMC, Strasbourg, France

Heckmann, S., Kumke, K., Nagaki, K., Schroeder-Reiter, E., Macas, J., Houben, A. Title: Chromosome behaviour and genome organization in the holocentric chromosome plant *Luzula elegans* (Juncaceae). 23.05 – 25.05.2012, Gatersleben Research Conference – Chromosome Biology, Genome Evolution, And Speciation, IPK Gatersleben, Gatersleben, Germany

Jankowska, M., **Heckmann, S.**, Houben, A. Title: Inverted meiosis in the holocentric plant *Luzula elegans*. 04.06 – 07.06.2012, Plant Science Student Conference 2012, IPK Gatersleben, Gatersleben, Germany

Jankowska, M., **Heckmann, S.**, Houben, A. Title: Inverted meiosis in the holocentric plant *Luzula elegans*. 03.09 – 05.09.2012, The International PhD Student Conference on Experimental Plant Biology, Mendel Museum, Brno, Czech Republic

Lermontova, I., **Heckmann, S.**, Koroleva, O., Rutten, T., Fuchs, J., Schubert, I. Title: Regulation of CENH3 expression and consequences of CENH3 depletion or truncation in *Arabidopsis*. 05.09 – 07.09.2012, Chromatin and Epigenetics – 43rd Annual Conference of the German Genetics Society - Annual Conference of GRK1431, Haus der Technik, Essen, Germany

Karimi-Ashtiyani, R., Demidov, D., Scholl, P., **Heckmann, S.**, Banaei-Moghaddam, AM., Houben, A. Title: Posttranslational modification of CENH3 and its incorporation into parental centromeres in allopolyploid hybrids. 24.09 – 26.09.2012, Institutes day, IPK Gatersleben, Gatersleben, Germany

Heckmann, S., Jankowska, M., Houben, A. Title: How does holocentricity influence meiosis and the genome organization of *Luzula elegans*? 24.09 – 26.09.2012, Institutes day, IPK Gatersleben, Gatersleben, Germany

Lermontova, I., **Heckmann, S.**, Koroleva, O., Rutten, T., Fuchs, J., Schubert, I. Title: Regulation of CENH3 expression and consequences of CENH3 depletion or truncation in *Arabidopsis*. 01.10 – 05.10.2012, EMBO workshop on: Structure, Function and Regulation of Centromeres and Kinetochores, Institut d'Estudis Catalans (IEC), Barcelona, Spain

Heckmann, S., Kumke, K., Nagaki, K., Schroeder-Reiter, E., Macas, J., Houben, A. Title: Holocentric chromosomes of *Luzula elegans* are characterized by a longitudinal centromere groove and an inverted order of meiotic events. 01.10 – 05.10.2012, EMBO workshop on: Structure, Function and Regulation of Centromeres and Kinetochores, Institut d'Estudis Catalans (IEC), Barcelona, Spain

Oral presentations (only talks listed presented by me)

Heckmann, S., Lermontova, I., Bäumlein, H., Schubert, I. Title: Characterization of the expression pattern of the centromeric histone CENH3 in *Arabidopsis thaliana*, 10.06.2009, University of Kassel, Kassel, Germany

Heckmann, S., Lermontova, I., Demidov, D., Berckmans, B., De Veylder, L., Bäumlein, H., Schubert, I., Houben, A. Title: Transcriptional and posttranslational regulation of the Aurora-substrate CENH3 in Arabidopsis, SFB 648 meeting, 04.02.2011, Halle/Saale, Germany

Heckmann, S., Lermontova, I., Demidov, D., Berckmans, B., De Veylder, L., Bäumlein, H., Schubert, I., Houben, A. Title: Transcriptional and posttranslational regulation of CENH3 in Arabidopsis, DST-DAAD Project Based Personal Exchange Programme, 16.03.2011, Jammu University, Jammu, India

Heckmann, S., Schroeder-Reiter, E., Macas, J., Kumke, K., Ma, L., Nagaki, K., Murata, M.M., Wanner, G., Houben, A. Title: Holocentric chromosomes during mitosis and meiosis in the wood-rush *Luzula elegans*, 05.10.2011, University of Birmingham, Birmingham, UK

Heckmann, S., Schroeder-Reiter, E., Macas, J., Kumke, K., Ma, L., Nagaki, K., Murata, M.M., Wanner, G., Houben, A. Title: Holocentric chromosome organization and behaviour during mitosis and meiosis in *Luzula elegans*. Deutsch - japanisches Seminars zum Thema: Frontiers of Plant Chromosome Research: Centromeres and Artificial Chromosomes, 31.10.2011, IPK Gatersleben, Gatersleben, Germany

Conferences/meetings attended

19.09 – 23.09.2009, EMBO Conference Series - MEIOSIS, Isle sur la Sorgue, France

22.03 – 24.03.2010, DFG Priority Programme SPP1384 – Mechanisms of Genome Haploidization/ Annual Meeting, EMBL, Heidelberg, Germany

15.06 – 18.06.2010, Plant Science Students Conference 2010, IPK Gatersleben, Gatersleben, Germany

25.06.2010, SFB 648 workshop, University Halle-Wittenberg, Halle/Saale, Germany

07.07 – 10.07.2010, Chromatin2010 – Transcription, chromatin structure and DNA repair in development and differentiation, Zeche Zollverein, Essen, Germany

09.09.2010, Minisymposium on Centromeres, Leibniz-Institut für Altersforschung, Jena, Germany

20.09 – 24.09.2010, Plant Epigenetics 2010 – European Networking Summer School (ENSS), IPK, Gatersleben, Germany

26.11 – 27.11.2010, SFB 648 workshop, Leucorea, Wittenberg, Germany

04.02.2011, SFB 648 workshop, University Halle-Wittenberg, Halle/Saale, Germany

28.03 – 30.03.2011, DFG Priority Programme SPP1384 – Mechanisms of Genome Haploidization - Annual Meeting, University Halle-Wittenberg, Halle/Saale, Germany

19.05 – 22.05.2011, Communication in Plants and their Responses to the Environment, Auditorium Maximum, Martin-Luther-University Halle-Wittenberg, Halle/Saale, Germany

12.10. – 14.10.2011, EMBO workshop on: Histone Variants and Genome Regulation, IGBMC, Strasbourg, France

31.10 – 03.11.2011, Deutsch - japanisches Seminars zum Thema: Frontiers of Plant Chromosome Research: Centromeres and Artificial Chromosomes, IPK Gatersleben, Gatersleben, Germany

23.05 – 25.05.2012, Gatersleben Research Conference – Chromosome Biology, Genome Evolution, And Speciation, IPK Gatersleben, Gatersleben, Germany

01.10 – 05.10.2012, EMBO workshop on: Structure, Function and Regulation of Centromeres and Kinetochores, Institut d'Estudis Catalans (IEC), Barcelona, Spain

Stays in other Research Centres

11.03 – 20.03.2011, DST-DAAD Project Based Personal Exchange Programme, Jammu University, Jammu, India: Supervisor Prof. M. Dhar - Immunolocalization of centromere-associated proteins and (peri-)centromere-associated histone modifications in *Plantago lagopus* B chromosomes

22.09 – 11.10.2011, University of Birmingham, Birmingham, UK: Supervisor Prof. F.C.H. Franklin - Study of meiosis in *Luzula elegans*

8. Eidesstattliche Erklärung

Hiermit erkläre ich, Stefan Heckmann, dass diese Arbeit von mir bisher weder der Naturwissenschaftlichen Fakultät I – Biowissenschaften – der Martin-Luther-Universität Halle-Wittenberg, noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde und ich noch keine vergeblichen Promotionsversuche unternommen habe.

Ich erkläre ferner, dass ich diese Arbeit selbstständig, ohne Fremde Hilfe und nur unter zur Zuhilfenahme der angegebenen Hilfsmittel und Literatur angefertigt habe und weiterhin keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe, sowie die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Gatersleben, 15.04.2013

(S. Heckmann)

9. Publications on which this thesis is based on and declaration on the contribution to these publications

9.1 Heckmann, S., Schroeder-Reiter, E., Kumke, K., Ma, L., Nagaki, K., Murata, M.M., Wanner, G., Houben, A. (2011) Holocentric chromosomes of *Luzula elegans* are characterized by a longitudinal centromere groove, chromosome bending and a terminal nucleolus organizer region. Cytogenetics and Genome Research 134: 220-228.
Available at: http://www.karger.com/Article/FullText/327713

Experimental work except scanning electron microscopy (performed by E. Schroeder-Reiter) was done by me. My contribution to the work corresponds approximately 80%.

Gatersleben, 15.04.2013 Gatersleben, 15.04.2013

(S. Heckmann) (K. Kumke)

9.2 Heckmann, S., Lermontova, I., Berckmans, B., De Veylder, L., Bäumlein, H., Schubert, I. (2011) The E2F transcription factor family regulates *CENH3* expression in *Arabidopsis thaliana*. The Plant Journal 68: 646-656.
Available at: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2011.04715.x/full

Most experimental work was done by me. Transgenic *Arabidopsis* plants overexpressing E2F transcription factors were provided by B. Berckmans. ChIP was performed by B. Berckmans. My contribution to the work corresponds approximately 80%.

Gatersleben, 15.04.2013 Gatersleben, 15.04.2013

(S. Heckmann) (I. Lermontova)

9.3 Heckmann, S., Macas, J., Kumke, K., Fuchs, J., Schubert, V., Ma, L., Novák, P., Neumann, P., Taudien, S., Platzer, M., Houben, A. (2013) The holocentric species *Luzula elegans* shows interplay between centromere and large-scale genome organization. The Plant Journal 73: 555-565.
Available at: http://onlinelibrary.wiley.com/doi/10.1111/tpj.12054/full

Experimental work except Illumina sequencing (S. Taudien and M. Platzer), bioinformatic analysis (P. Neuman, P. Novák and J. Macas), flow-cytometry (J. Fuchs) and high resolution microscopy (V. Schubert) was done by me. My contribution to the work corresponds approximately 60%.

Gatersleben, 15.04.2013 Gatersleben, 15.04.2013

(S. Heckmann) (J. Fuchs)

9.4 Heckmann, S., Houben, A. (2013) Holokinetic Centromeres (pp. 83-94). In: Jiang, J and Birchler, J (Eds.): Plant Centromere Biology, Vol 1. Wiley-Blackwell: in press.
Available at: http://www.wiley-vch.de/publish/dt/books/bySubjectLS00/bySubSubjectLS90/1-119-94921-1/?sID=2vl3vmfirkjgnj7fnfd918jjg1

Equal contribution to manuscript preparation. My contribution to the work corresponds approximately 50%.

Gatersleben, 15.04.2013 Gatersleben, 15.04.2013

(S. Heckmann) (A. Houben)