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MINI-REVIEW

Loading time of the centromeric histone H3 variant differs between plants and animals

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Kinetochores are protein complexes established at eukaryotic centromeres and responsible for the correct chromosome segregation during nuclear divisions. Kinetochore formation is initiated by substitution of histone H3 by CENH3 within some but not all centromeric nucleosomes. Correct timing and targeting of this process are essential for centromere function, but are not well understood. In this paper, we point out that CENH3 loading in plants occurs before mitotic sister centromere separation, while in animals, it was recently shown to occur after sister centromere separation. Additionally, monocentric chromosomes of higher plants display distinct sister kinetochores immediately after loading of CENH3 during late G2. Although the reason for the different timing of CENH3 deposition is not yet clear, it indicates different mechanisms of regulation for CENH3 loading between animals and plants.

Human and *Drosophila* CENH3 is loaded after mitosis

Initiation of kinetochore formation by incorporation of CENH3 (homologous to human CENP-A, Earnshaw and Rothfield 1985) into centromeric nucleosomes has been suggested by several investigations (e.g. Howman et al. 2000; Blower and Karpen 2001); however, previous knowledge on the timing of CENH3 deposition was vague. Contrary to the largely replication-dependent deposition of H3, and the other nucleosomal histones, for the few species investigated, CENH3 loading was assumed to occur mainly outside of the S-Phase (Table 1). Indirect evidence suggests that CENH3 of *Schizosaccharomyces pombe* can be deposited to the centromeres during the S and the G2 phases (Takahashi et al. 2005). Shelby et al. (2000) suggested that human CENH3 deposition occurs during G2 when maximum expression of RNA and protein levels occurs. CENH3 expression experimentally limited to S phase abolished centromere targeting (Shelby et al. 1997). However, Jansen et al. (2007) reported that for human cells, CENH3 loading occurred during telophase/early G1. While the exit from mitosis was found to be a requirement for CENH3 loading, tension across the centromeres (as hypothesised by Mellone and Allshire 2003) was not. Similar observations, i.e. CENH3 loading during anaphase, independent of DNA replication as well as of pulling forces of the mitotic spindle, but dependent on the progress of mitosis, were made for *Drosophila* by Schuh et al. (2007). These observations were interpreted as a likely conserved timing of CENH3 deposition in eukaryotes (Carroll and Straight 2007). This hypothesis may be true for animals, but it is at odds with the observations for *Arabidopsis* and other plants.

Plant CENH3 is loaded before mitosis and before separation of sister kinetochores

The most precise cell cycle window for CENH3 loading, up to 2006, had been determined for the dicot plant *Arabidopsis thaliana* by Lermontova et al. (2006). Their study was based on transgenic plants that expressed fluorescently labelled EYFP-AtCENH3 and on immunostaining of the endogenous protein using antibodies against AtCENH3. Endopolyploid *Arabidopsis* nuclei did not reveal an

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Table 1 Cell cycle phase of CENH3 loading in various eukaryotes

Species	Cell cycle phase of CENH3 loading	Reference
Schizosaccharomyces pombe	S/G2	Takahashi et al. 2005
Homo sapiens	Maximum expression at G2	Shelby et al. 2000
	Telophase/G1	Jansen et al. 2007
Drosophila melanogaster	Outside of S	Ahmad and Henikoff 2001
	Anaphase	Schuh et al. 2007
Arabidopsis thaliana	Late G2	Lermontova et al. 2006
Hordeum vulgare	Late G2	This paper
Luzula nivea	Prometaphase/metaphase	Nagaki et al. 2005
Cyanidioschyzon merolae	S to metaphase	Maruyama et al. 2007

increase in CENH3 in parallel with the increased ploidy level or with the amount of the 180-bp centromeric repeat (Fig. 1). Endocycles omit G2 and mitosis (D'Amato 1998), and it was therefore assumed that either of these phases might represent the window for CENH3 deposition. In fact, a fraction of G2 nuclei displayed double signal spots (as do prophase and metaphase chromosomes), indicating a split of sister kinetochores before mitosis, while the remaining sister centromere regions are still cohesive as shown by fluorescent in situ hybridisation signals for the centromeric 180-bp repeat. At the same time, a doubling of signal volumes and signal intensity for either EYFP-AtCENH3 or anti-AtCENH3 was observed, as compared with 2C nuclei (Fig. 1). This suggests loading of CENH3 during late G2

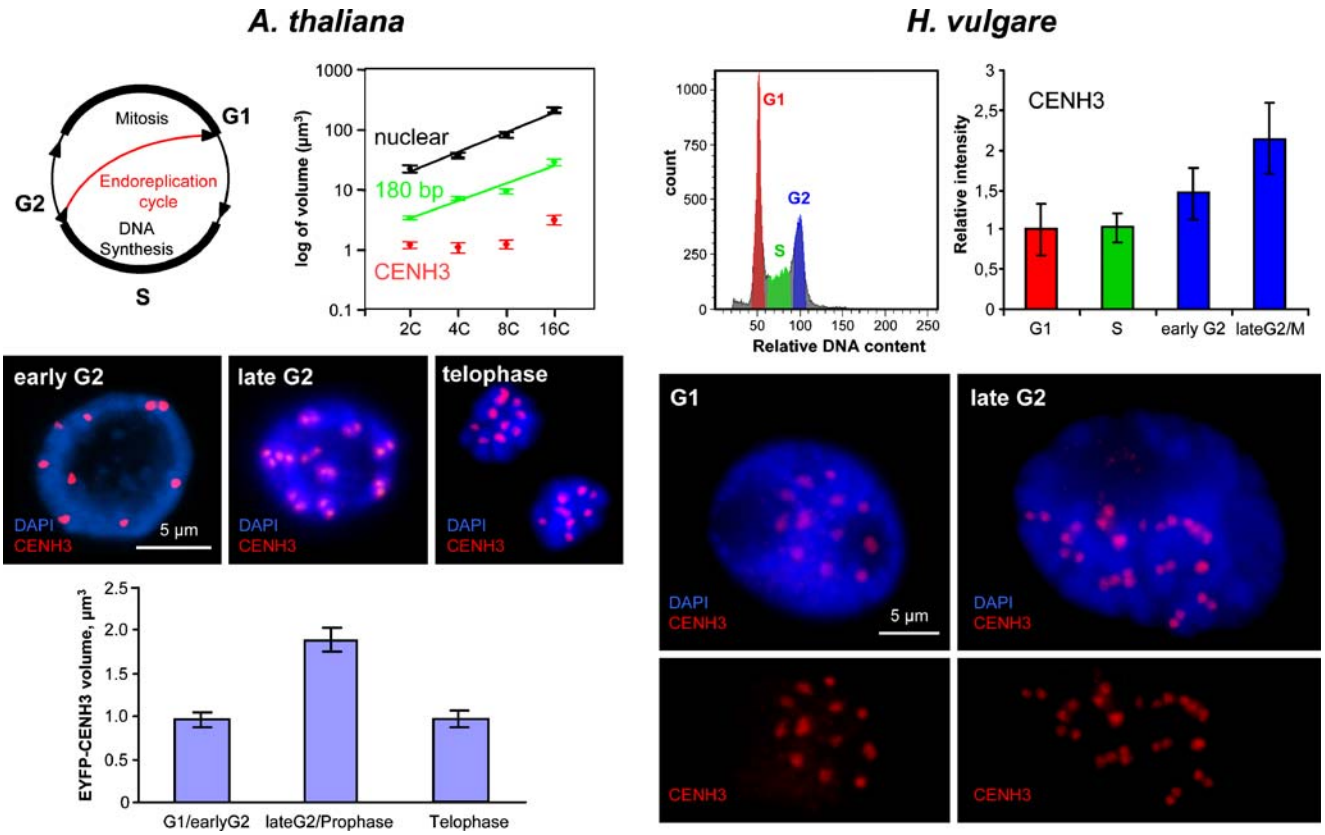

Fig. 1 CENH3 loading in *A. thaliana* and *Hordeum vulgare* (barley). *Left* Scheme of the mitotic cycle vs the endocycle in *A. thaliana*. CENH3 amounts do not increase in parallel with the amount of 180-bp centromeric repeats and the nuclear volume in endopolyploid nuclei; accumulation of CENH3 followed by sister kinetochore splitting occurs in late G2. *Right* Histogram of nuclei suspension from barley root tip meristems and relative amounts of CENH3 as measured by fluorescence intensity after indirect immunostaining with anti-rice CENH3 antibodies; CENH3 loading occurs before sister kinetochore splitting in late G2. For flow-sorting of nuclei, immunostaining and measurement of signal volume and intensity, see Lermontova et al. (2006)

immediately before appearance of distinct sister kinetochores. On holocentric chromosomes of the monocot *Luzula nivea*, the most intense accumulation of CENH3 apparently occurs from prophase to metaphase (Nagaki et al. 2005) and within nuclei of the red alga *Cyanidioschyzon merolae* from S to metaphase (Maruyama et al. 2007). Nuclei of barley root tip meristems with a 4C DNA content (indicative of a replicated diploid genome), immunostained with anti-CENH3 antibodies (Nagaki et al. 2004), already revealed double signal dots at each centromere in late G2 nuclei. The fluorescence intensity in late G2 to metaphase nuclei was twice as high as in G1 (2C) and in S phase nuclei, while early G2 nuclei showed single signals and an intermediate fluorescence intensity (Fig. 1). This finding indicates that premitotic CENH3 deposition, likely before the spatial separation of sister kinetochores, is conserved among plants.

Open questions as to targeting and timing of CENH3 deposition

It is still an open question as to what triggers centromere-specific deposition of CENH3 when a centromere becomes established for the first time, and later on, how is a centromere recognised as the site for CENH3 deposition for its maintenance. According to Black et al. (2007), the CENH3 targeting domain itself mediates conformational rigidity to CENH3-containing nucleosomes, thus, providing the mark to specify the location of new CENH3 deposition. In spite of this, the local preexistence of CENH3 containing nucleosomes is apparently not necessary, as rare evolutionary de novo centromere formation is possible (Karpen and Allshire 1997; Amor and Choo 2002; Nasuda et al. 2005). Furthermore, overexpression of CENH3 results in ectopic CENH3 deposition (Van Hooser et al. 2001) and, at a high level of overexpression (greater than tenfold), in the formation of ectopic centromeres (Heun et al. 2006). However, orphan CENH3-containing nucleosomes that serve as a seed for de novo deposition in their vicinity during overexpression cannot be excluded. Indeed, naked DNA of an active *Candida albicans* centromere (85 kb) does not recruit CENH3 when reintroduced into cells (Baum et al. 2006). Even more questions arise as to the determination of the time in the cell cycle for CENH3 deposition. Does the time of loading depend mainly on the expression pattern of CENH3 and/or of proteins (e.g. KNL-2; Maddox et al. 2007) required for CENH3 loading? Is CENH3 deposition during the S-phase unsuitable because of competition with H3, and therefore avoided by many or most eukaryotes? Is ectopic centromere formation after overexpression due to preferential incorporation at the expenses of H3 during S-phase or rather due to the substitution of H3 during the 'normal' loading time? Is a diminishing of nucleosomes with CENH3 at centromeres required for a proper chromatin structure at centromeres during nuclear division (Jansen et al. 2007)? This could be speculated when loading occurs after nuclear division (post-reduction loading) and CENH3 becomes 'diluted' during the subsequent replication phase, as in humans and *Drosophila*. Alternatively, in plants, is the 'filling up' of centromeres with CENH3-containing nucleosomes by CENH3 deposition after replication (pre-reduction loading) required for proper segregation during nuclear division? If, as it seems, both variants of loading occur (Fig. 2), then different mechanisms to regulate CENH3 loading have to be assumed. If pre-reduction loading can be confirmed for fungi, then post-reduction loading apparently evolved after the divergence of metazoans and plants >1 billion years ago.

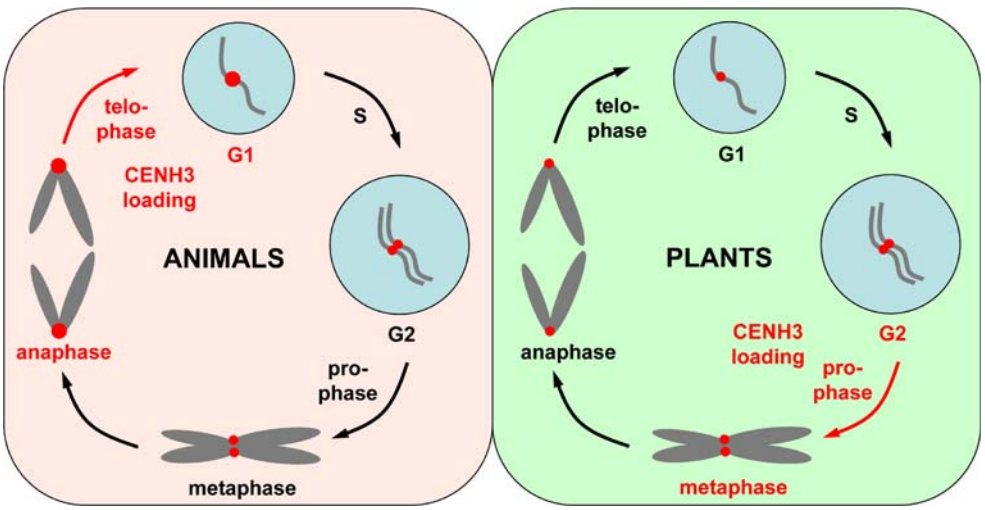

Fig. 2 Cell cycle timing of CENH3 deposition in plants (late G2 to prophase) and animals (anaphase to G1). Indirect evidence suggests CENH3 loading during S to G2 in fission yeast (Takahashi et al. 2005). If confirmed, this would indicate that post-reduction loading mechanisms for CENH3 evolved after divergence of fungi from Metazoa

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