Chromosoma (2011) 120:633–640
DOI 10.1007/s00412-011-0338-5

RESEARCH ARTICLE

Deposition, turnover, and release of CENH3 at *Arabidopsis* centromeres

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Received: 27 April 2011 / Revised: 14 July 2011 / Accepted: 29 July 2011 / Published online: 13 August 2011

Abstract The kinetochore is a complex multiprotein structure located at centromeres and required for the proper segregation of chromosomes during mitosis and meiosis. An important role in kinetochore assembly and function plays the centromeric histone H3 variant (CENH3). Cell cycle stage of CENH3 deposition to centromeres varies between different organisms. We confirmed by in vivo studies that deposition of *Arabidopsis* CENH3 takes place at centromeres during G2 and demonstrated that additionally a low turnover of CENH3 occurs along the cell cycle, apparently for replacement of damaged protein. Furthermore, enhanced yellow fluorescent protein (EYFP)-CENH3 of photobleached chromocenters is not replaced by EYFP-CENH3 molecules from unbleached centromeres of the same nucleus, indicating a stable incorporation of CENH3 into centromeric nucleosomes. In differentiated endopolyploid nuclei however, the amount of CENH3 at centromeres declines with age.

Introduction

Centromeres represent the chromosomal positions where kinetochore proteins assemble that are responsible for sister chromatid cohesion, chromosome movement, and cell cycle regulation (Allshire 1997; Choo 1997; Nicklas 1997; Amor et al. 2004; Farr 2004; Henikoff and Dalal 2005). The centromeric chromatin is composed of centromeric DNA repeats which however are neither required nor sufficient for centromeric identity (Karpen and Allshire 1997; Maggert and Karpen 2001; Amor and Choo 2002; Nasuda et al. 2005; Han et al. 2006). The position of centromeres on chromosomes of eukaryotic organisms is determined epigenetically rather than by a DNA sequence. A primary role in determination of centromere identity and kinetochore assembly is proposed for the centromeric histone H3 (CENH3) (Warburton et al. 1997; Karpen and Allshire 1997; Allshire and Karpen 2008).

In contrast to the situation described for other nucleosomal histones, CENH3 expression and deposition is not linked to S phase (Shelby et al. 2000). Surprisingly, different loading times during the cell cycle were reported for plant, fungal, and animal centromeres. Quantification of enhanced yellow fluorescent protein (EYFP)-CENH3 immunosignals during different stages of mitotic cell cycle and in endopolyploid nuclei as well as a quantification of immunosignals of endogenous CENH3 showed that in *Arabidopsis* CENH3 is depositing to centromeres mainly during G2 (Lermontova et al. 2006). In contrast to these data, measuring of fluorescence intensity of EGFP-CID/CENH3 in *Drosophila* embryos during mitotic cycle showed a steep increase in fluorescence intensity during anaphase. Also bleaching of EGFP-CID/CENH3 signals during metaphase has resulted in fluorescence recovery during anaphase (Schuh et al. 2007). For *Drosophila*, in addition to the regular deposition during anaphase, a low turnover of CENH3 at different cell cycle stages was reported (Schuh et al. 2007). Jansen et al. (2007) have demonstrated that in HeLa cells CENH3 is equally partitioned to sister centromeres after S phase and deposition of new CENH3 to centromeres occurs during G1. Using fluorescence recovery after photobleaching (FRAP),

Communicated by Erich Nigg

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Hemmerich et al. (2008) confirmed that human CENH3 is incorporating to centromeres during G1. No FRAP of GFP-CENH3 was observed in human S, G2, or metaphase cells, indicating that no second CENH3 loading pathway and no detectable turnover occur in these cells after G1. In budding yeast cells the main loading of CENH3 occurs during S (Pearson et al. 2004) and in addition a low level (~18%) of CENH3 turnover at telophase and G1 was observed (Pearson et al. 2004). In summary, metazoans incorporate CENH3 from anaphase/telophase to mid-G1 (Schuh et al. 2007; Jansen et al. 2007; Hemmerich et al. 2008), budding yeast during S phase (Pearson et al. 2004), fission yeast during S and G2 (Takayama et al. 2008), and plants and protozoans during G2 (Lermontova et al. 2006, 2007; Dubin et al. 2010). It is not yet clear why different phylogenetic groups load CENH3 at different cell cycle stages.

Here we applied FRAP analyses to study in vivo whether CENH3 of *Arabidopsis thaliana* is deposited at centromeres during G2, and to what degree incorporated CENH3 is a subject of protein turnover. Additionally, we tested whether CENH3 of individual chromocenters damaged by photobleaching can be replaced by CENH3 from undamaged centromeres of the same nucleus. Furthermore, we analyzed CENH3 immunosignals in sorted 2C–8C nuclei of differentiated cells from young and mature leaves of *A. thaliana* in order to test stability of CENH3 levels in aging tissues.

Materials and methods

Time-lapse confocal microscopy analysis

Generation of the EYFP-CENH3 transgenic plants has been described previously (Lermontova et al. 2006). *A. thaliana* seeds transformed with AtEYFP-CENH3 were laid out on agar medium in cover slip chambers (Nalge Nunc Int.). Roots growing parallel to the cover slip bottom were analyzed in a LSM 510META confocal microscope (Carl Zeiss, Germany) using a ×63 oil immersion objective (n.A. 1.3). EYFP was excited with a 488-nm laser line and fluorescence recorded with a 505–550 bandpass filter. Images were collected at 4-min intervals for up to 60 min. At each time point single images were taken with open detector pinhole. Alternatively, image stacks with 2 μm *z*-steps were made with pinhole adjusted accordingly. Images of Z-stacks were projected in maximum intensity projection. Intensities of the signals were analyzed by the LSM software release 3.2.

FRAP analysis

FRAP experiments were performed with a 30-mW argon ion laser. Roots were observed with a ×63 oil immersion objective (n.A. 1.3). EYFP fluorescence (before and after bleaching) was visualized using 5% of the 488 nm laser line with the laser running at 50% power in combination with a 505–550-nm band pass filter and an open detector pinhole. A single prebleach image was recorded to measure prebleach intensity. For bleaching small well-defined circular areas the size of an individual cell, a cluster of centromeres or a single centromere were photobleached using 100% of the 488 laser line with three iterations (medium bleaching) to 10 iterations (complete bleaching). The bleaching scan lasted ~2.5 to 8.0 s and was immediately followed by an image scan. Images were taken at 4-min intervals for up to 60 min or at 14-s intervals for up to 3 min. Intensities of the signals were background normalized to produce relative fluorescence intensity data.

Fluorescence loss in photobleaching analysis

For fluorescence loss in photobleaching (FLIP), nuclei of interest were prescanned three times followed by additional 47 scans alternated by bleaching of a single chromocenter. Recordings were made with a ×63 oil emersion objective n. A. 1.3. For normal scanning, 4–5% laser power was used in combination with a 650-μm pinhole; scan speed was set at 6, image size was 512×100 pixels. Bleaching was performed with 100% laser power with four iterations over an area of 17×17 pixels. Total time of observation was 64 s. During this period the loss of fluorescence was measured in the bleached area as well as in surrounding unbleached chromocenters.

Isolation and flow sorting of nuclei

For the isolation of leaf nuclei, plants were grown in soil in a cultivation room. Leaves were fixed in 4% formaldehyde in Tris buffer (10 mM Tris, 10 mM Na_2EDTA, and 100 mM Triton X-100, pH 7.5) for 20 min. Nuclei were isolated, stained with 4',6-diamidino-2-phenylindole (DAPI; 1 mg/ml), and processed for flow sorting according to their fluorescence intensity reflecting the DNA content as described (Jasencakova et al. 2000). Nuclei of each fraction were sorted into separate Eppendorf tubes, subsequently dropped onto microscopy slides into a drop containing 100 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, 0.05% Tween20, and 5% sucrose, air-dried, and used for immunolabeling or stored at −20°C. Flow cytometry was performed on the flow sorter FACSAria (BD Biosciences) equipped with 375- and 488-nm lasers. DAPI emission was measured using a 450/40-nm filter.

Immunostaining and FISH

Immunostaining (Jasencakova et al. 2000) and fluorescence in situ hybridization (FISH) (Schubert et al. 2006) of nuclei/chromosomes was performed as described. Endogenous

CENH3 was detected using rabbit antibodies against a N-terminal peptide of *A. thaliana* CENH3 (1:500) and goat anti-rabbit rhodamine (1:200; Jackson Immuno Research Laboratories) or goat anti-rabbit Alexa 488 (1:200, Invitrogen). GUS protein expressed under the control of *CyclinB1*;1 promoter (*CycB1*; 1) was detected with anti-GUS antibodies (1:100; Molecular Probes) and goat anti-rabbit rhodamine (1:200; Jackson Immuno Research Laboratories).

As probe for FISH, pAL harboring the 178-bp centromere-specific repeat (Martinez-Zapater et al. 1986) was labeled with digoxigenin-dUTP by PCR with sequence-specific primers forward 5′-AGTCTTTGGCTTTGTGTCTT-3′ and reverse 5′-TGGACTTTGGCTACACC ATG-3′ (Kawabe and Nasuda 2005). The FISH signals were detected using mouse anti-Dig (1:250; Roche) and goat anti-mouse Alexa 488 (1:200; Molecular Probes).

Results

Cells of *A. thaliana* load CENH3 on centromeres during G2 and display a low turnover

From previous experiments, quantifying the fluorescence intensity of EYFP-CENH3 and of endogenous CENH3 immunosignals, we concluded that loading of CENH3 to plant centromeres occurs mainly during G2 (Lermontova et al. 2006; 2007). To confirm this result by an independent in vivo approach, we applied long-term FRAP analysis and quantification of fluorescence intensity. Measurement of EYFP-CENH3 fluorescence intensity starting from metaphase revealed that no additional loading of EYFP-CENH3 took place during anaphase/early G1 (Fig. 1a, d). Instead, fluorescence intensity dropped in the course of bleaching and persisted at a low level during the next 30 min (Fig. 1b, e). The same observation was made when one daughter nucleus of a cell in anaphase/telophase was bleached (Fig. 1c). Fluorescence intensities of cells that were ready to enter mitosis (late G2 cells, identified by the presence of double signals for EYFP-CENH3) were compared with those of surrounding cells in other cycle stages (G1 to early G2; Fig. 2). This measurement has confirmed that in late G2 cells the amount of CENH3 is twice as high as in the surrounding cells (Fig. 2b). We followed selected cells within 60 min in order to demonstrate that they enter mitosis (Fig. 2a, 25, 30, and 60 min). To confirm additionally that nuclei with double signals for CENH3 are in G2, we performed a double immunostaining experiment with anti-CENH3 and anti-GUS antibodies on transgenic plants transformed with *CycB1*; 1-GUS construct (Colon-Carmona et al. 1999). *CycB1*;1 is expressed during G2; therefore, it can be used as a marker for this stage. Indeed, all cells showing double signals for CENH3 (green) showed immunostaining of GUS (red) in cytoplasm or cytoplasm and nucleus (Fig. 2c). Bleaching of a single fluorescent locus, a group of loci or an entire interphase nucleus (the latter shown in Fig. 3a) resulted in slow and incomplete fluorescence recovery. Short-term FRAP analysis after bleaching of single fluorescent loci of interphase nuclei yielded fluorescence recovery of up to ~6% within 116 s (Fig. 3b). To ensure that recovery is due to replacement of damaged protein by newly synthesized one and not due to fluorophore recovery, seedlings of EYFP-CENH3 transformants were fixed in 4% formaldehyde and used for the FRAP. In this case, no recovery was observed after photobleaching, indicating that EYFP-CENH3 bleaching is irreversible. Therefore, it was concluded that the main loading of CENH3 takes place during late G2, because a double fluorescence intensity was only found in nuclei that within ~1 h had past mitosis (Fig. 2a). A slow turnover of CENH3 occurs during G1 to early G2 phase of the cell cycle in *A. thaliana* cells.

To examine the potential mobility of CENH3 incorporated into centromeres, we performed FLIP experiments, in which individual chromocenters of 10 nuclei were repeatedly bleached and fluorescence of other chromocenters was continuously measured (Fig. 4). We observed only a very low reduction of fluorescence intensity at unbleached chromocenters (Fig. 4) which was within the range of reduction resulting from image scanning. These data indicate that CENH3 incorporates stably into centromeric nucleosomes and does not exchange between centromeres inside a nucleus.

The level of CENH3 at chromocenters is decreasing with the leaf age

After we had shown that CENH3 incorporation into centromeric nucleosomes is stable, we addressed the question whether the level of centromeric CENH3 is maintained during plant growth and development. We compared CENH3 immunostaining patterns in 2C and 4C nuclei isolated from young and mature leaves of *A. thaliana* wild-type plants, accession Columbia (Fig. 5a). Three types of immunolabeling were observed: (1) strong labeling at centromeric chromocenters and weak labeling of nucleoplasm, (2) weak labeling of chromocenters and nucleoplasm, and (3) weak nucleoplasm labeling only (Fig. 5b). About 60% of 4C nuclei isolated from young leaves showed strong labeling of centromeric chromocenters, while only 16% of 4C nuclei isolated from mature leaves showed such labeling pattern (Fig. 5c). In contrast, 2C nuclei isolated from young and from mature leaves showed similar immunostaining patterns as 4C nuclei from young leaves, indicating that the found differences between 4C nuclei are not due to poor immunostaining of nuclei from older tissues. Nevertheless, the percentage of 2C nuclei with strong labeling of chromocenters was lower in differentiated (60%) than in meristematic tissues (93%)

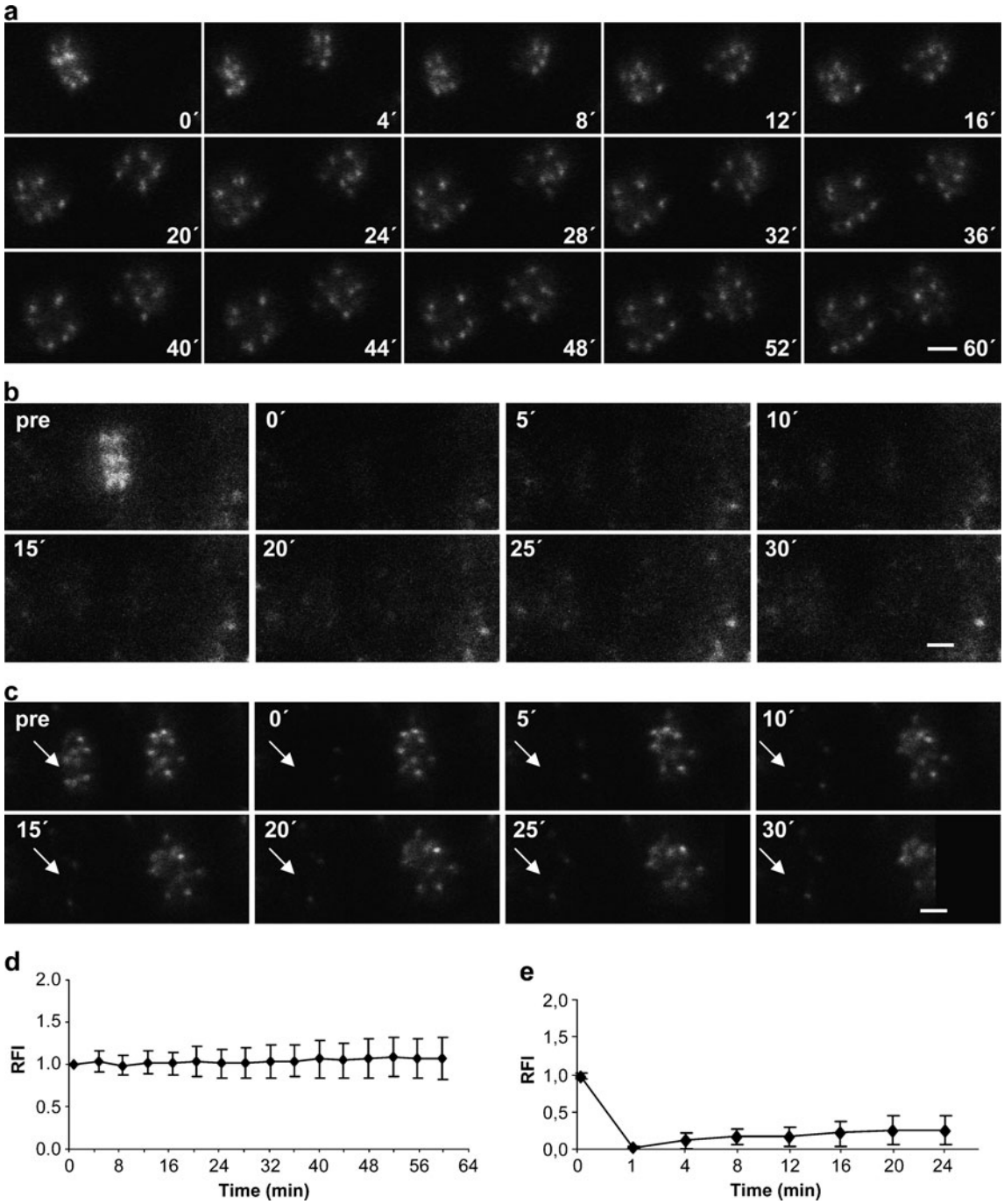

Fig. 1 FRAP of mitotic nuclei. **a** EYFP-CENH3 fluorescence signals from metaphase to G1 within 60 min. **b**, **c** EYFP-CENH3 fluorescence recovery after bleaching at metaphase and anaphase, respectively. *Arrows* in (**c**) indicate the position of the bleached anaphase daughter nucleus. *Bars*=2 μm. (Some nuclei were traced up to 60 min after bleaching with similar result.) **d** The relative fluorescence intensity (RFI±SD) of EYFP-CENH3 for five selected nuclei over 60 min. **e** EYFP-CENH3 RFI±SD after bleaching at metaphase

(Lermontova et al. 2006). Since we observed differences in distribution of CENH3 immunostaining patterns between 2C and 4C nuclei of mature leaves, we continued analysis with nuclei of higher ploidy levels (8C). Due to the low level of endopolyploidization in young tissues, a sufficient amount of nuclei with a ploidy level higher than 4C can be isolated only from mature leaves. We found in 8C nuclei of mature leaves similar proportions of the three CENH3 immunostaining patterns (14% type 1, 58% type 2, and 28% of type 3) as in 4C nuclei (Fig. 5c).

To test whether the reduction of centromeric CENH3 immunosignals in mature leaves correlates with changes in compaction of centromeric chromocentres, we compared the FISH signals for the centromeric 178 bp (Martinez-Zapater

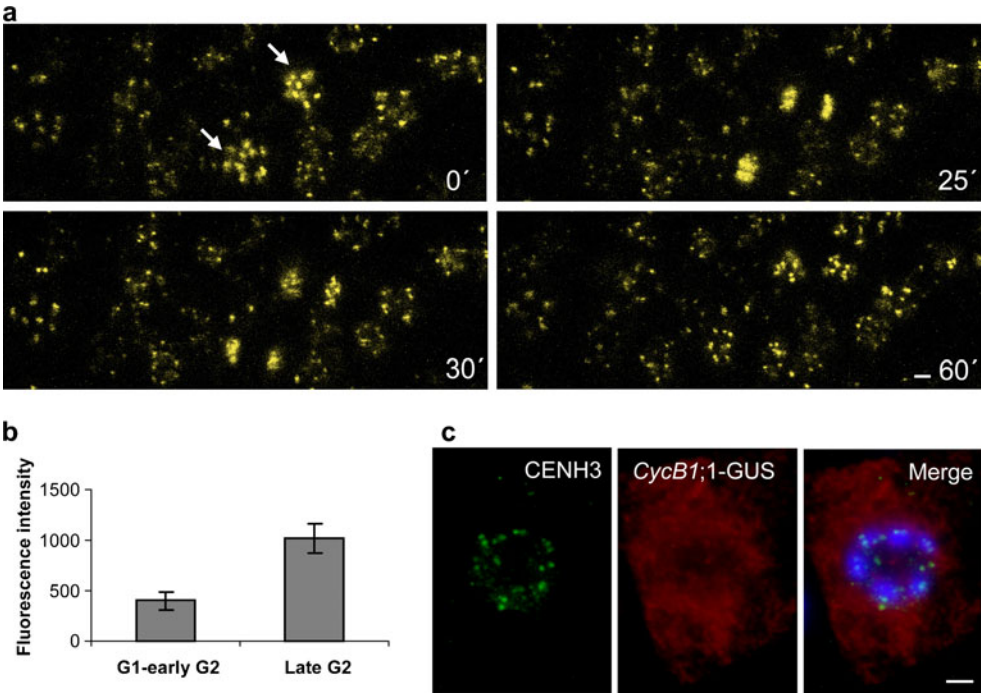

Fig. 2 Comparison of fluorescence intensity of root tip G2 nuclei with that of surrounding nuclei. **a** Live cell imaging of root tip meristem with two G2 nuclei (*arrows*) followed for 60 min. Late G2 nuclei were identified by the presence of double signals for EYFP-CENH3 and were followed until they entered mitosis (within at least 1 h). **b** Fluorescence intensity of late G2 nuclei compared with that of all other surrounding nuclei, which are either in G1, S, or early G2. **c** Localization of CENH3 (*green*) and GUS (*red*) in G2 cells from root tip of *CycB1*;1-GUS transformants. *Bars*=2 μm

et al. 1986) repeat in 2C and 4C nuclei isolated from young and mature leaves. No differences in distribution and intensity of signals were observed between nuclei of young and mature leaves, indicating that decreased centromeric CENH3 immunosignals are not due to dispersion or decay of centromeric DNA.

Discussion

The mechanism of cell-cycle-dependent CENH3 deposition to centromeres remains to be elucidated in detail. Several possibilities are shown in Fig. 6: (1) opposite a CENH3-containing nucleosome a gap appears in one of the daughter chromatids, filled during G2 by a new CENH3-containing nucleosome. This would require availability of all nucleosomal histones during G2. (2) One daughter chromatid gets a "normal" nucleosome opposite the CENH3-containing one. During G2 the entire normal nucleosome is substituted or only H3 is exchanged against CENH3. (3) CENH3-containing nucleosomes are split during replication into "hemisomes" (Dalal et al. 2007) and complemented during G2 with histone tetramers containing CENH3; or (4) during replication "heterosomes" (having one CENH3 and one H3 molecule) are formed in both chromatids, and H3 is substituted by CENH3 during G2. At present no unambiguous decision in favor of one of these possibilities is possible. It is also unclear why different groups of organisms load CENH3 during different cell cycle stages. Previous quantification of immunosignals suggested loading of EYFP-CENH3 and of endogenous CENH3 of *Arabidopsis* during G2 (Lermontova et al. 2006). Application of FRAP and in vivo quantification of EYFP-CENH3 fluorescence intensity confirmed and extended the previous observation by the following results: (1) late G2 nuclei showed a two-fold EYFP-CENH3 intensity compared to the surrounding nuclei; (2) EYFP-CENH3 fluorescence intensity did not increase during anaphase and early G1; and (3) after bleaching of metaphase or one daughter nucleus during anaphase only a weak recovery of EYFP-CENH3 fluorescence occurs, indicating a low level of turnover.

Since loading of CENH3 to centromeres before separation of sister chromatids is a common feature of *Arabidopsis*, barley, *Luzula*, red algae, yeast (surveyed in Lermontova et al. 2007), and protozoans (Dubin et al. 2010), premitotic CENH3 loading is the phylogenetic older mechanism and seems more reasonable than loading during anaphase to G1, because it remains elusive why metazoan kinetochores can perform mitosis with half of the "normal" CENH3 amount (replication-mediated dilution is compensated only after sister chromatid separation). Data obtained on loading of CENH3 (CID) to centromeres in *Drosophila* are contradictory. Ahmad and Henikoff (2001) claimed that in *Drosophila* Kc167 cells GFP-CENH3 was deposited throughout the cell cycle. Later, Schuh et al. (2007) showed that in early embryonic cells lacking G1 and G2 phases deposition of CENH3 is occurring during anaphase. Recently, Mellone et al. (2011) have demonstrated that in *Drosophila* S2 cell lines newly synthesized CENH3 (CID) can be recruited to centromeres during metaphase. Although the mechanism of CENH3 assembly is not yet clear, a number of chaperones guiding CENH3 assembly have been identified and charac-

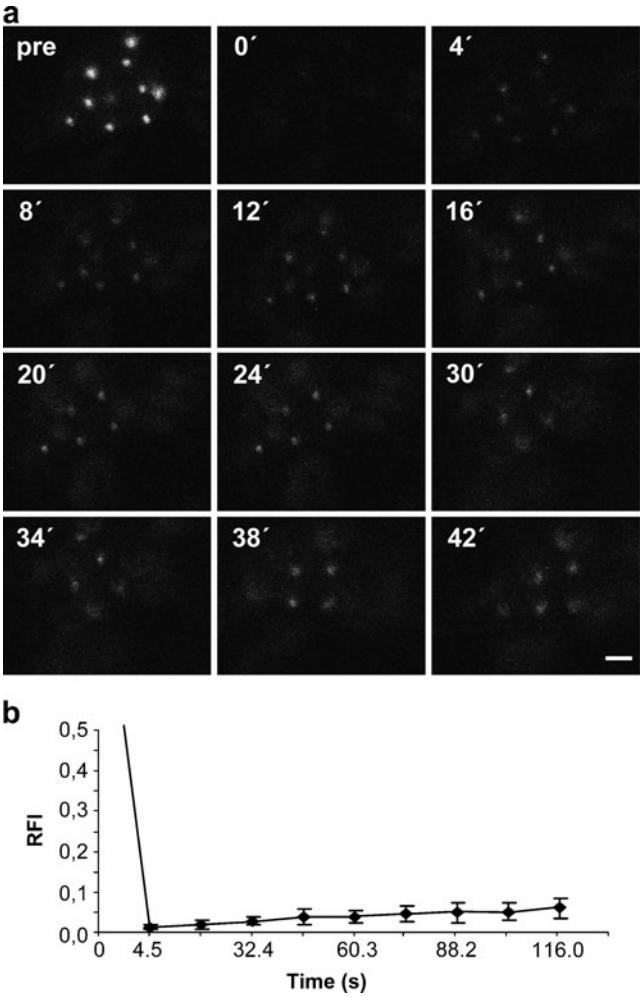

Fig. 3 FRAP of interphase nuclei. **a** EYFP-CENH3 FRAP of an interphase nucleus. After bleaching, fluorescence recovery was monitored over 42 min. *Bar*=2 μm. **b** Fluorescence recovery after bleaching of individual chromocenters of interphase nuclei over 116 s (mean of 10 individual chromocenters from 10 interphase nuclei±SD)

terized in yeast, *Drosophila*, mammals, and *Caenorhabditis elegans* (Silva and Jansen 2009; Torras-Llort et al. 2009; Dalal and Bui 2010). In human, the Holliday Junction Recognition Protein (HJURP) was proposed to play a key role as CENH3 chaperone responsible for the targeting to and deposition of newly synthesized CENH3 at centromeres (Foltz et al. 2009; Dunleavy et al. 2009; Shuaib et al. 2010). HJURP is conserved among mammals, and homologues can be identified for chicken, frog, and zebra fish, but not for plants. A low similarity of HJURP to the Scm3 protein of yeast was reported (Sanchez-Pulido et al. 2009). In plants, no homologue for Smc3 could be identified. Whether or not the reason for post-mitotic deposition of CENH3 in animals is linked with the appearance of a new CENH3 chaperone is not yet clear. Likely, plants and metazoans do not only differ as to the time of CENH3 deposition at centromeres but also as to the CENH3 assembly factors involved.

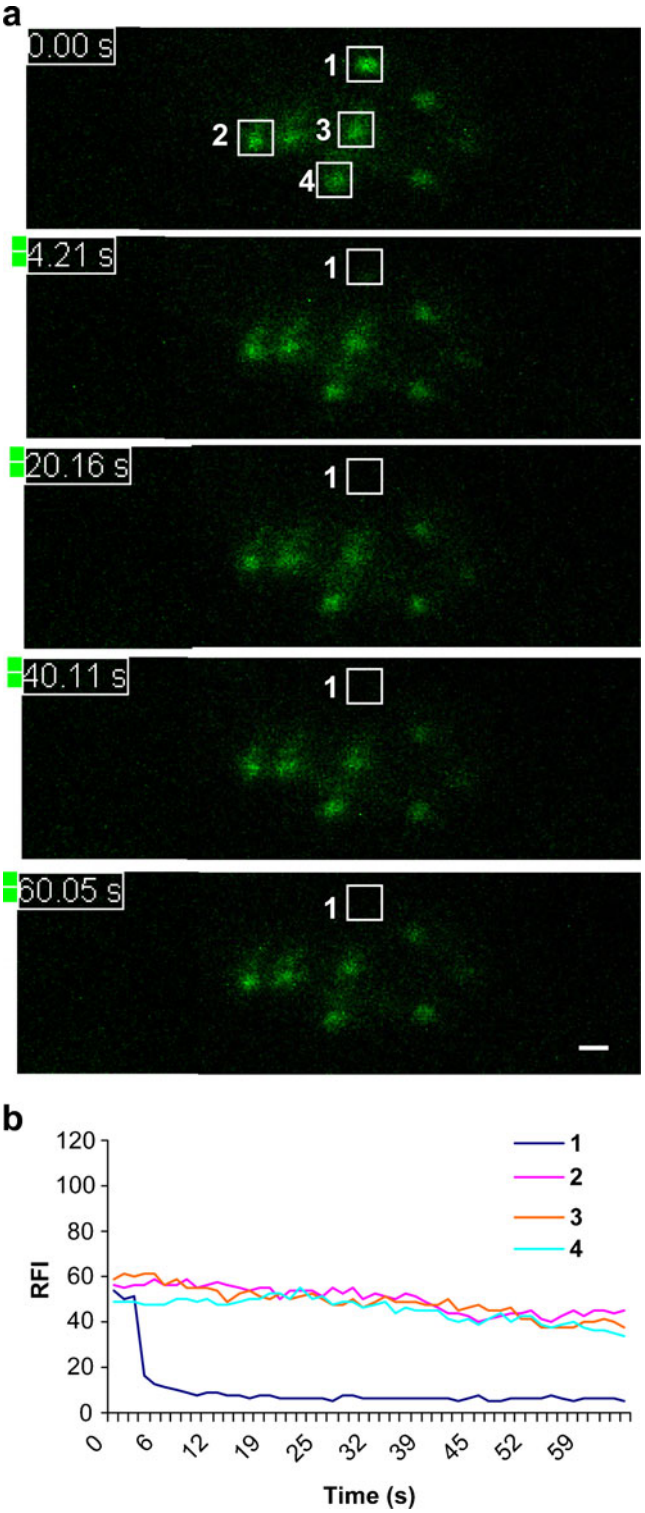

Fig. 4 FLIP on 14-day-old *A. thaliana* seedlings expressing an EYFP-CENH3 fusion construct. **a** Nuclei of interest were prescanned three times and then individual chromocenters were repeatedly bleached at 100% laser power over 47 time points. *Bar*=2 μm. **b** Fluorescence intensity was measured within the bleached (*box 1*) and unbleached chromocenters (*boxes 2 to 4*). Bleaching caused by scanning was measured in control cells

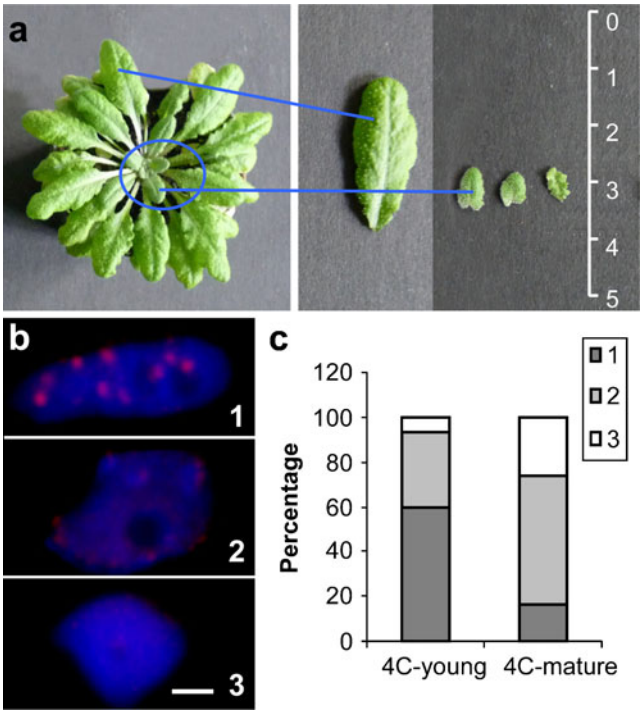

Fig. 5 Distribution of CENH3 immunolabeling patterns in nuclei isolated from young and mature leaves of *A. thaliana* wild-type. **a** Position of leaves used for analysis. **b** The three different patterns of CENH3 immunosignal (*1* strong labeling at centromeric chromocenters and weak labeling of nucleoplasm, *2* weak labeling of chromocenters and nucleoplasm, and *3* weak nucleoplasm labeling only). *Bar*=2 μm. **c** Frequency of nuclei displaying these three immunostaining patterns in 500 sorted 4C nuclei of young and mature leaves each

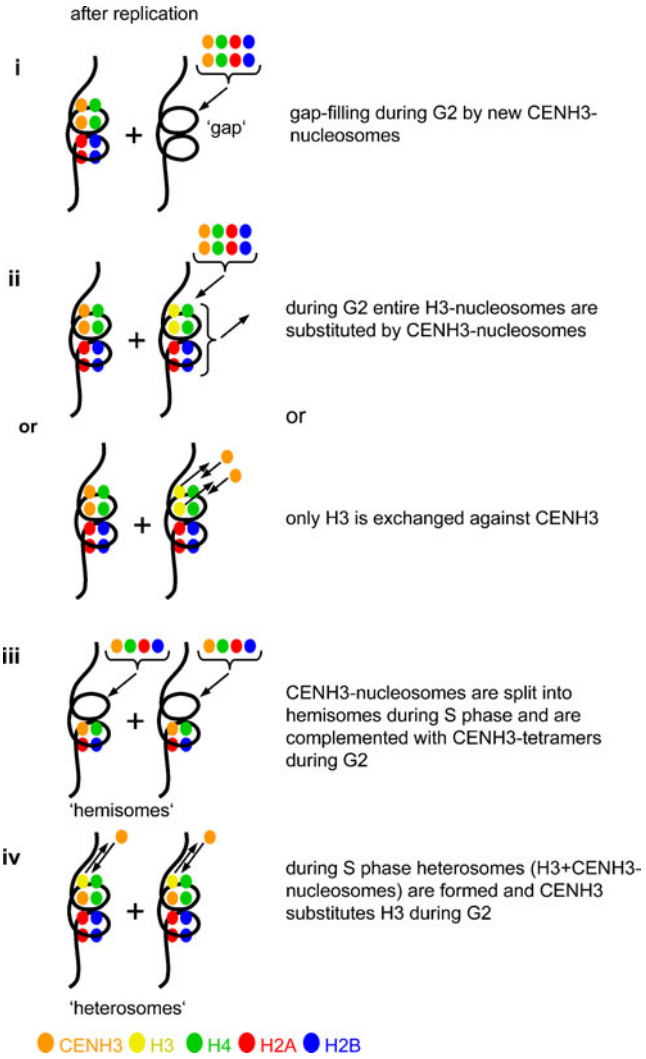

Fig. 6 Models for maintenance of CENH3-containing centromeric nucleosomes after replication in plants. (The same assumptions could be made for other kingdoms considering the cell cycle time during which they deposit CENH3)

The low fluorescence recovery of EYFP-CENH3 after bleaching of metaphase, anaphase, and interphase cells indicates existence of a slow turnover of CENH3 at centromeres. Thus, there are two pathways of CENH3 deposition in *Arabidopsis*: one for main loading during G2 and the other for replacement of damaged protein during any other cell cycle stage. The extent of turnover might depend on the degree of damage. CENH3 damage by photobleaching represents an artificial situation leading to a substantially increased turnover which normally may not occur. A low protein turnover in addition to the main loading was also observed in yeast (Pearson et al. 2004) and *Drosophila* (Schuh et al. 2007) but not in human cells (Hemmerich et al. 2008). The CENH3 turnover in *A. thaliana* is not caused by replacement of damaged CENH3 by CENH3 molecules from centromeres of the same nucleus which were not exposed to photobleaching.

We showed that CENH3 stably incorporates into centromeric nucleosomes. The fact that CENH3 remains at centromeric positions in nuclei of differentiated tissues, although CENH3 expression in non-meristematic tissues (Heckmann et al. 2011) is very low, additionally indicates its stability.

Analysis of 2C–8C nuclei from young and mature leaves of *A. thaliana* wild-type revealed that 2C nuclei isolated from leaves of both stages have similar immunostaining patterns, while 4C nuclei from mature leaves have less CENH3 than 4C nuclei from young leaves. 8C nuclei of mature leaves showed immunostaining pattern similar to that of 4C nuclei. This may indicate that CENH3 turnover in endopolyploid nuclei slows down during tissue maturation, while the amount of CENH3 in 2C nuclei of mature leaves is stably maintained and enables these nuclei to continue mitotic cycling. Reduced turnover of CENH3 in mature tissues is most likely the result of low CENH3 expression. An age-dependent reduction of CENH3 level within nuclei was not reported previously except for human pancreatic islet-cells in which the level of CENH3 is declined with the age (Lee et al. 2010).

Acknowledgments We thank Andrea Kunze and Rita Schubert for technical assistance; Karin Lipfert and Ursula Tiemann for help with preparation of figures; and Jörg Fuchs for help with flow sorting. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to I.L. (LE2299/1-1).

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