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GTPase Ran strongly accumulates at the kinetochores of somatic chromosomes in the spermatogonial mitoses of *Acricotopus lucidus* (Diptera, Chironomidae)

Wolfgang Staiber

Institute of Genetics (240), University of Hohenheim,
Garbenstrasse 30, D-70599 Stuttgart, Germany
e-mail: wstaiber@uni-hohenheim.de

Abstract
Unequal chromosome segregation and spindle formation occurs in the last gonial mitosis in the germ line of the chironomid *Acricotopus lucidus*. During this differential mitosis all germ line limited chromosomes (=Ks) migrate undivided to only one pole of the cell, while the somatic chromosomes (=Ss) first remain in the metaphase plane, and with the arrival of the Ks at the pole they then separate equally. The evolutionarily conserved GTPase Ran plays a crucial role in many cellular processes. This includes the regulation of microtubule nucleation and stabilisation at kinetochores, and of spindle assembly during mitosis, which is promoted by a RanGTP concentration gradient that forms around the mitotic chromosomes (Kalab et al. in Science 295:2452–2456, 2002, Nature 440:697–701, 2006). In the present study a strong accumulation of Ran was detected by immunofluorescence at the kinetochores of the Ss in normal gonial and differential gonial mitoses of males of *A. lucidus.* In contrast, no Ran accumulation was observed at the kinetochores of the Ss in metaphases of brain ganglia mitoses or of aberrant spermatocytes or in metaphases I and II of spermatocyte meiotic divisions. Likewise there was no accumulation at the kinetochores of *Drosophila melanogaster* mitotic chromosomes from larval brains. The specific accumulation of Ran at the kinetochores of the Ss in differential gonial mitoses of *A. lucidus* strongly suggests that Ran is involved in a mechanism acting in this exceptional mitosis, which retains the Ss at the metaphase plane and prevents a premature separation and unequal segregation of the Ss during monopolar migration of the Ks.

Keywords: GTPase Ran · Accumulation · Kinetochore · Differential gonial mitosis · Unequal mitotic spindle · *Acricotopus lucidus*

Introduction

An unusual spindle formation and unequal chromosome segregation are special features of the last gonial mitosis in the chironomid *Acricotopus lucidus*. The result of this differential mitosis, in which initially all germ line-limited chromosomes (=Ks; K is derived from 'Keimbahn', Bauer and Beermann 1952) move as unseparated sister chromatids to one spindle pole, while the somatic chromosomes (=Ss) remain at the metaphase plane and then divide equally, is two daughter cells with quite different chromosome constitutions. The cell getting all the Ks and two sets of Ss passes through meiosis, while the cell receiving only two S sets develops into a nurse cell in the female and into an aberrant spermatocyte in the male (Staiber 2008). Both daughter cells remain connected by a cytoplasmic canal. The monopolar migration of the undivided Ks in the last gonial mitosis compensates for the elimination of about half of the Ks in the first division of the primordial germ cells (White 1973; Redi et al. 2001). The presence of the Ks in the germ line is essential for normal gametogenesis (Bantock 1970). During early syncytial embryonic divisions all Ks are eliminated from the future somatic nuclei (Staiber 2000).

Ran, a Ras-related small GTPase, is involved in the regulation of various processes during the cell cycle, including microtubule nucleation around chromosomes and spindle assembly, spindle checkpoint control, post-mitotic formation of the nuclear envelope and control of nucleocytoplasmic transport (reviewed in Clarke and Zhang 2008; Kalab and Heald 2008). During mitosis a chromosome-centered RanGTP concentration gradient is generated due to the opposed activities of the chromatin-associated guanine nucleotid exchange factor RCC1 (regulator of chromosome condensation 1) and the Ran GTPase-activating protein RanGAP1 (Carazo-Salas et al. 1999; Kalab et al. 2002). The existence of the RanGTP gradient was detected using fluorescence resonance energy transfer (FRET)-biosensors that visualised the Ran nucleotide state around metaphase chromosomes (Kalab et al. 2002, 2006). A high RanGTP concentrations results in the release of spindle assembly factors such as TPX2 and HURP from importins effecting microtubule nucleation, stabilisation at the kinetochores and spindle formation (Gruss et al. 2001, Sillje et al. 2006, Casanova et al. 2008).

In the present study, the distribution of Ran was analysed by indirect immuno-fluorescence in the exceptional differential gonial mitosis of *A. lucidus*, which displays unseparated monopolar moving chromosomes, and arranged chromosomes still in the metaphase plane, in a single cell. Interestingly, a strong accumulation of Ran was detected at the kinetochores of the remaining Ss. Except for one S-like K, which shares highly repeated centromere-specific DNA sequences with the Ss, and sometimes stays in the metaphase plane together with the Ss in differential mitosis (Staiber 1988; Staiber et al. 1997), all other Ks showed no Ran accumulation in gonial metaphases.

The substantial accumulation of Ran at the kinetochores of the Ss in differential gonial mitosis strongly supports the idea that Ran is involved in a mechanism that regulates the successive monopolar and bipolar migrations of Ks and Ss and controls the equal segregation of

the Ss, thereby ensuring that the germ line cell that will enter into meiosis receives two whole sets of Ss and of Ks.

Material and methods

Chromosome preparation and immunofluorescence

Chromosome preparations of spermatogonial mitoses, male meiotic stages and brain ganglia mitoses of young fourth instar larvae and prepupae from a laboratory stock of *Acricotopus lucidus* (Diptera, Chironomidae), and of brain mitoses of third instar larvae from a *Drosophila melanogaster* Oregon wild-type strain, were made as described in Staiber (2012). Preparations were then treated with 0.4% Triton® X-100 in phosphate buffered saline (PBS), pH 7.4, under gentle agitation for 4 h, blocked with 4% non-fat dry milk in PBS, pH 7.4, for at least 4 h, over-layed with a polyclonal rabbit anti-Ran antibody (Cell Signaling; #4462, lot 1; New England BioLabs, Braunschweig, Germany) diluted 1:50 in PBS with 3% bovine serum albumin (BSA). They were then incubated in a moist chamber under plastic coverslips at 5°C for about 40 h. After removal of the coverslip the preparations were washed four times for 10 min each in 0.2% Tween®20 in PBS, pH 7.4, at room temperature and covered with fluorescein (FITC)-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, Dianova, Hamburg, Germany) diluted 1:50 in PBS with 3% BSA at 5°C overnight. After three final washes, the chromosomes were stained with 0.2 µg/mL 4′,6-diamidino-2-phenylindole (DAPI; Serva, Heidelberg, Germany) in PBS, pH 7.4, for 5 min, rinsed in PBS and mounted in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA). Images were taken with a Canon 450D digital camera on a Zeiss Axioplan epifluorescence microscope (Carl Zeiss, Germany) using a Plan-Neofluar 100/1.3 objective, and were further processed with Corel Photo Paint and CorelDraw software (Ottawa, ON, Canada).

Microtubule staining in living cells

Living spermatogonial cells were prepared and stained with 1 µM Oregon Green 488 paclitaxel (Molecular Probes, Invitrogen, Eugene, OR, USA) and 1 µg/ml 4',6'-diamidino-2-phenylindole (DAPI; Serva, Heidelberg, Germany) as described in Staiber (2007).

Immunoblot analysis

For the preparation of protein extracts, early embryos of *A. lucidus* (0 - 4 ½ h hours after egg deposition), *Drosophila* Schneider 2 (S2) cells and human K562 cells were washed in PBS and homogenised on ice in RIPA I buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% Triton® X-100, 0.1% SDS) and protease inhibitor cocktail (Roche, Basel, Switzerland). The homogenates were placed on ice for 20 min and centrifuged for 15 min at 4°C in a Biofuge 22R (Heraeus Sepatech, Osterode, Germany). A loading buffer was added to the supernatant and the probes were run on 12.5% SDS-PAGE followed by immunoblot blot analysis using the rabbit anti-Ran

antibody (1:250, Cell Signaling; #4462, lot 1; New England BioLabs, Braunschweig, Germany) and then a goat anti-rabbit antibody (IgG) conjugated with alkaline phosphatase (1:500, Jackson ImmunoResearch, Dianova, Hamburg, Germany).

Results

Gonial cells of *A. lucidus* possess, in addition to the two chromosome sets of Ss, 2n = 6, a variable number of 6 to 16 Ks (Staiber 1988). In the last 'differential' gonial mitosis an asymmetric spindle is formed which is clearly visible when all Ks move undivided to one pole, while the Ss remain arranged in the metaphase plane (Fig. 1a). The distances from the metaphase plane to the two spindle poles are significantly different. Extensive arrays of microtubules extend poleward, moving the Ks towards one pole. In contrast, opposite-directed microtubule arrays of about equal density, but of different length, extend from the unseparated Ss to both poles (Fig. 1a). During anaphasic movement of the equally segregating Ss the polar microtubules push the spindle poles apart (Fig. 1b).

Immunostaining of differential mitoses with an anti-Ran antibody reveal intense fluorescent signals at the kinetochores of the remaining Ss, which show conspicuous close pairing of the homologues, while the Ks are devoid of signals (Fig. 1c). The separated poleward moving anaphase Ss display no Ran staining at the kinetochores (Fig. 1d). An accumulation of Ran at the S kinetochores becomes apparent in prometaphase of differential gonial mitosis (Fig. 2e) and is clearly visible in metaphase (Fig. 2f). Clear Ran signals at the kinetochores of the Ss were also detected in metaphases of normal gonial mitoses preceding the differential mitosis (Fig. 1g), but not in the following anaphases with equally segregating Ss and Ks (Fig. 1h).

According to the product information of the manufacturer, the polyclonal anti-Ran antibody used in this study was produced by immunising rabbits with two synthetic peptides corresponding to amino acids surrounding Arginin 29 and Asparagin 142 of human Ran. A species cross-reactivity of the antibody was predicted for *Drosophila* based on 100% sequence homology. To check this, protein extracts from *D. melanogaster* Schneider 2 (S2) cells and human K562 cells, and from early embryos of *A. lucidus,* were separated by SDS-PAGE and analysed by subsequent immunoblotting using the anti-Ran antibody (Fig. 1t). Clear bands at about 24 kDa appeared in all protein extracts (Fig. 1t) corresponding to the predicted molecular weight of 24.4 kDa deduced from the amino acid sequence of human Ran (P62826, UniProtKB/Swiss-Prot).

In contrast to differential gonial mitoses, no accumulation of Ran was detected at the kinetochores of the paired Ss in metaphases of larval brain ganglia mitoses of *A. lucidus* (Fig. 1i), and at the kinetochores of ganglion metaphase chromosomes of *D. melanogaster* (Fig. 1j).

In each Ran immunostaining series of ganglion mitoses of *Acricotopus* and *Drosophila*, preparations of differential gonial mitoses of *Acricotopus* were used as positive controls. Likewise, no Ran accumulation was observed at the kinetochores of the Ss and Ks in

spermatocyte metaphases I (Fig.1k) and metaphases II (Fig. 1l) of *A. lucidus,* and at the kinetochores of the metaphase-like condensed Ss in the connected aberrant spermatocytes (Fig. 1k, l).

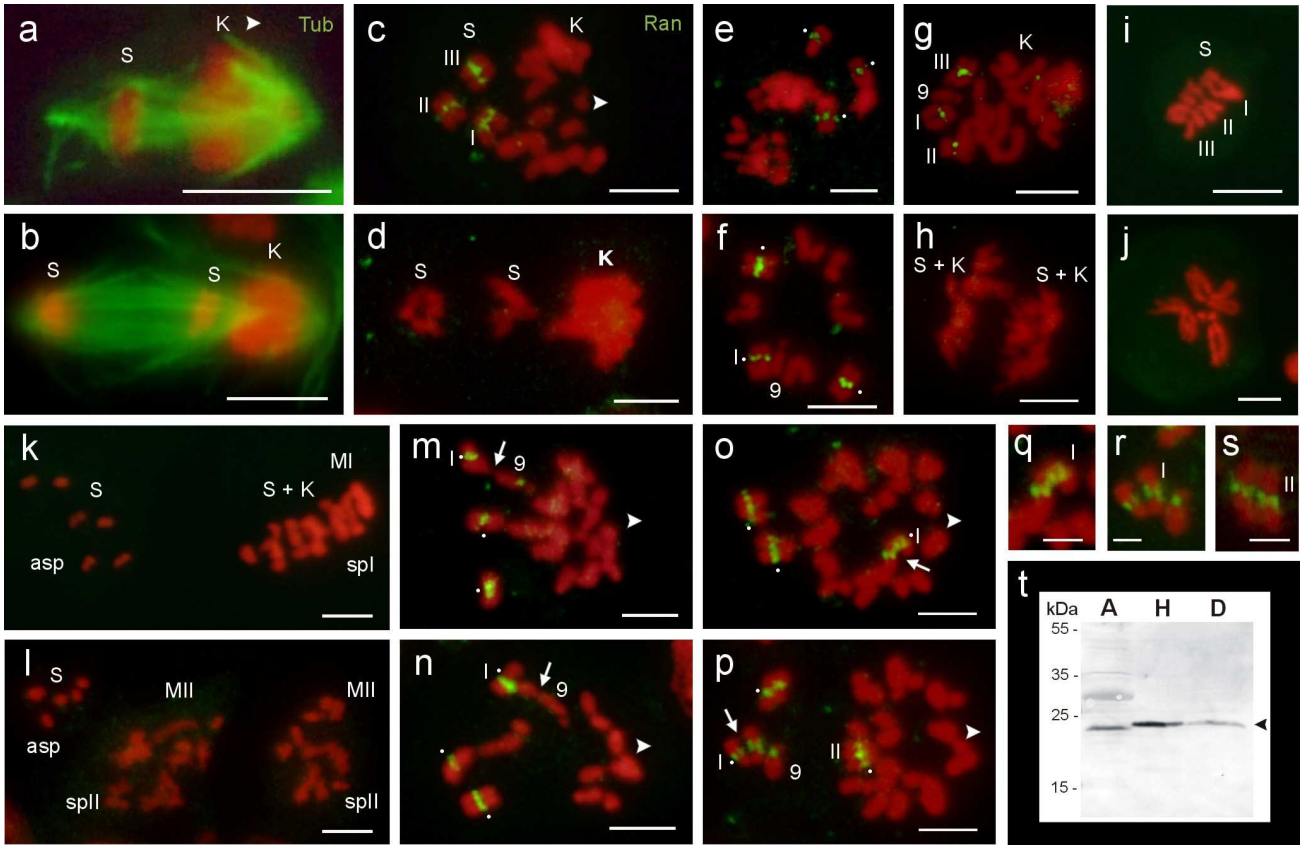

Fig. 1 a-f Differential spermatogonial mitoses of *A. lucidus*. **a** Distribution of spindle microtubules visualised by Oregon Green labelled paclitaxel during monopolar migration of the unseparated Ks (*K*) and remaining Ss (*S*), and **b** during bipolar movement of the equally segregating Ss. Chromosomes are stained with DAPI and pseudocolored red. **c-s** Ran immunofluorescence staining. **c** Strong Ran accumulation is present at the kinetochores of the six Ss, which stay back in the metaphase plane and are closely paired (*I-III*), while the poleward (*arrowhead*) moving Ks exhibit no signals. **d** The equally segregating anaphase Ss are devoid of Ran signals. The weak fluorescent dots on the compact group of Ks arranged around the spindle pole and on the chromosome arm of one S are unspecific signals. **e** The Ss (*dots*) display clear Ran signals in prometaphase and **f** show strong accumulation of Ran at the kinetochores in metaphase of differential mitoses, in contrast to the Ks. Germ line-limited chromosome K9 (*9*) is associated with SI. **g, h** Normal spermatogonial mitoses preceding differential mitosis, in which Ss and Ks segregate equally. **g** Metaphase with Ran accumulation at the kinetochores of the Ss and **h** anaphase with unlabeled bipolar moving Ss and Ks. **i, j** The metaphase chromosomes in brain ganglia mitoses of *A. lucidus* (**i**) and *D. melanogaster* (**j**) exhibit no Ran accumulation at the kinetochores. **k** Kinetochores of Ss and Ks in metaphase I of a primary spermatocyte (*spI*) and **l** in metaphases II of secondary spermatocytes (*spII*) and of the Ss in the connected aberrant spermatocytes (*asp*) are devoid of Ran signals. **m**, **n** Differential mitoses showing the remaining strongly labelled Ss, and K9 with (**m**) and without (**n**) Ran signals at the kinetochore. K9 is partial paired with SI (*arrows*) and is drawn in direction to the other poleward (*arrowhead*) migrating Ks. **m** Some of the Ks exhibit some faint but unspecific fluorescent signals. **o**, **p** Differential mitoses with separated homologous SIs (*arrows*) clearly showing Ran labelling at the kinetochores of the two sister chromatids of each S. Chromosomes SI in **o** and SII in **p** were displaced by the preparation from the metaphase plane into the poleward moving group of Ks. **q-s** Sections with separated homologous Ss from differential mitoses clearly localising the Ran accumulation to the kinetochores. **q, r** Sections of **o** and **p**. **t** Immunoblot analysis of protein extracts of *A. lucidus* early embryos (*A*), human K562 cells (*H*), and *D. melanogaster* Schneider S2 cells (*D*) using the anti-Ran

antibody detected Ran at about 24 kDa (*arrowhead*) in all extracts. *Scale bars* represent 5 µm (**a**-**p**) and 2 µm (**q**-**s**)

In metaphases of differential mitoses (Fig 1f), and during monopolar migration of Ks (Fig. 1c, m-p), all Ss displayed clear Ran accumulation at the kinetochores but not the Ks, with exception of one special K, K9 (classification of the Ks in Staiber 1988). K9 is sometimes closely associated with SI (Fig. 1f, g) or stays back in the metaphase plane as the Ss (Fig. 1m, n, p), and shows partial pairing with SI (arrowheads in Figs. 1 m,n). In these cases some of the remaining K9s show clear Ran signals at the kinetochore (Fig. 1m, p). For example, in the immunostained preparation of Figure 1m in 25 of 31 well spread differential mitoses, the remaining Ss displayed strong Ran accumulation at the kinetochores; in 9 of the Ran-stained differential mitoses K9 was partially paired with SI and showed in two of these mitoses clear Ran signals at the kinetochores. An exceptional accumulation of Ran was detected at no other chromosomal sites than at the kinetochores. The weak fluorescent dots on some of the Ks in the gonial mitosis in Figure 1g, on some of the poleward moving Ks in the differential mitoses in Figures 1d and 1m, and on the chromosome arm of one anaphase S in Figure 1d are unspecific signals.

Occasionally, the closely paired homologous Ss of differential mitoses could be separated from each other by strong pressure during squash preparation (arrows in Figs. 1o, p). Such immunostained Ss clearly demonstrate, that Ran is accumulated at the kinetochores of both sister chromatids of each of the Ss (Fig. 1q-s).

Discussion

In mitosis a RanGTP concentration gradient surrounding the chromosomes in a cloud-like manner is formed by the catalytic activities of the chromatin-associated guanine nucleotide exchange factor RCC1, and Ran's GTPase activating factor RanGAP1, as visualised in mitotic *Xenopus* egg extracts and human cells (Kalab et al. 2002, 2006). High concentrations of RanGTP near the chromosomes cause the release of spindle assembly factors from inhibiting importin-complexes, inducing the nucleation and assembly of kinetochore and spindle microtubules (Dasso 2006; Tulu et al. 2006; Kalab and Heald 2008).

Human Ran is an evolutionary well conserved 216 amino acid protein that shares, for example, 86% identity with the *D. melanogaster* Ran protein (isoform A, GenBank: AAF48008; Koizumi et al. 2001). The anti-Ran antibody used in this study clearly detected Ran in Western blots of protein extracts from human and *Drosophila* cells, and from *Acricotopus* early embryos (Fig. 1t). For *Drosophila* embryos Silverman-Gavrila and Wilde (2006) estimated the amount of Ran at 0.06% of total embryonic protein, by comparing bands of known concentrations of recombinant Ran with the Ran bands of embryo protein extracts in Western blots.

Deviating from the established concentration gradient of RanGTP centered around the chromosomes in the present study a specific and strong accumulation of Ran was immuno-localised at the kinetochores of the Ss in prometaphases and metaphases of normal gonial and differential gonial mitoses of *A. lucidus*. Most likely, the guanine nucleotide-bound state of the

immunodetected Ran is RanGTP. Kinetochores of mitotic chromosomes from spermatogonial divisions of *A. lucidus* exhibit a typical trilaminar structure with microtubules attached to the outer layer, as determined on ultrathin sections in the transmission electron microscope (Staiber 2008).

In experiments with nocodazole-treated human cells Torosantucci et al. (2008) observed a local accumulation of RanGTP at kinetochores in prometaphases before and immediately after release from a nocodazole block, that had induced microtubule disassembly. For the detection, a conformational antibody that specifically recognises the GTP-bound form of Ran was used (Richards et al.1995). Five minutes after drug removal during early microtubule regrowth distinct RanGTP signals, but of much lower intensity, were still detectable at the kinetochores. Torosantucci et al (2008) concluded that a certain accumulation of RanGTP at the kinetochores is necessary before the onset of microtubule nucleation, but that in nocodazole-treated cells the absence of microtubules inhibits the aggregation of the GTPase activating protein RanGAP1 near the kinetochores (Joseph et al. 2002), leading to the observed abundant accumulation of RanGTP at the kinetochores.

Another case of a specific accumulation of Ran at kinetochores, but occurring in mitoses of untreated cells, was reported for *Caenorhabditis elegans*, a species possessing holocentric chromosomes (Bamba et al 2002). There, in late metaphases of early embryonic divisions, a clear Ran immunostaining was detected at 'the outer edges of each chromosome, which correspond to kinetochore regions' (Bamba et al. 2002).

In differential mitosis of *A. lucidus* one special K, K9, sometimes displayed the same specific Ran accumulation at the kinetochores as the Ss, when remaining in the equatorial plane together with the Ss, and showed close association or even partial pairing with SI (Fig. 1m). K9 has developed from SI during evolution as demonstrated by fluorescence in situ hybridisation using chromosome painting probes of the three Ss, and shares, as the only K, highly repeated centromere specific DNA sequences with the Ss (Staiber et al. 1997, Staiber and Schiffkowsky 2000; Staiber 2012). This S-like composition of the K9 centromere is possibly responsible for the occasionally observed kinetochore accumulation of Ran, and the S-like behaviour of K9 in differential mitosis.

One characteristic feature of differential mitosis is that the Ks congregate in the metaphase plane as the Ss do, but do not separate and migrate as the first to only one spindle pole. The remaining unseparated Ss are attached with dense bundles of kinetochore microtubules oriented to both spindle poles, as shown in Figure 1a. This indicates that during monopolar migration of the Ks the spindle assembly checkpoint is still active for the Ss, preventing the activation of the APC/C (anaphase promoting complex/cyclosome) and the release of separase. The first event after arrival of the Ks at the pole is spindle checkpoint inactivation, and the S sister chromatids are then separated and segregate equally. How this feedback is regulated is unknown.

Most likely there is a connection between the strong Ran accumulation at the kinetochores of the Ss, as detected in the normal and differential gonial mitoses, and events specifically occurring during the unequal differential mitosis, since such a kinetochore enrichment of Ran was not observed on the Ss in brain ganglion mitoses or in spermatocyte metaphases I and II of *A. lucidus*, and likewise not on ganglion metaphase chromosomes of *D. melanogaster* (Fig. 1i-l).

One obvious explanation is that in differential mitosis the high Ran concentration at the kinetochores of the remaining Ss stabilises the kinetochore microtubules: and although the Ss are arranged in the metaphase plane and have reached a bipolar orientation, with kinetochores being under tension (Fig. 1a), this prevents the inactivation of the spindle assembly checkpoint during monopolar migration of the undivided Ks and so blocks an unequal segregation of the Ss. Such high levels of RanGTP at the kinetochores of the Ss may result from a locally increased concentration or activity of Ran's guanine exchange factor RCC1 (Halpin et al. 2011), or by a lower kinetochore recruitment of RanGAP, which mediates, as mentioned before, the GTP-hydrolysis of Ran (Roscioli et al. 2012). Recently, Lee et al. (2012) demonstrated that in mitosis RanGTP also regulates, via Aurora B kinase, the stability of kinetochore microtubule attachments after chromosome congression in the metaphase plane, and that high RanGTP concentrations, are necessary to maintain stable attachments. On the other hand, Arnaoutov and Dasso (2003) could inactivate the spindle checkpoint in *Xenopus* egg extracts with experimentally elevated levels of RCC1, and as a probable result of this by raised RanGTP levels, and concluded 'that mitotic checkpoint arrest can be eliminated by an increased GTP binding status of Ran'. The differential effects of Ran on mitotic progression in these cases might be explained by concentration-dependent different modes of action of Ran.

In the regular gonial mitoses prior to the differential mitosis the kinetochores of the sister chromatids of Ss and Ks are bi-oriented and under tension at metaphase (amphitelic microtubule attachments), separate from each other and move to opposite spindle poles. This indicates that the spindle assembly checkpoint supervises here the Ss and Ks. For an unseparated monopolar moving K in differential mitosis it is not known if microtubules emanating from the one spindle pole are attached to only one of the sister kinetochores (monotelic) or to both (syntelic). In both cases no opposite tension forces act on the K sister kinetochores and they do not separate. One can only speculate whether the spindle assembly checkpoint also interacts with the Ks in differential mitosis.

Certainly, it can be assumed that elevated RanGTP levels are also necessary around the kinetochores of the Ks in normal and differential gonial mitoses as well as of the Ss in ganglion mitoses to induce microtubuli nucleation, but possibly the Ran concentration is much lower in comparison with the Ran accumulation at the S kinetochores so that in these cases Ran is not detectable with the immunofluorescence sensitivity achieved in this study.

The migration of all Ks as unseparated sister chromatids to only one pole in the last gonial mitosis compensates for the elimination of about half of the Ks in the first gonial mitosis

of the primordial germ cell (Bauer and Beermann 1952; Redi et al. 2001). This upregulation of the number of Ks is therefore indispensable for the continuance of the Ks in the germ line of *A. lucidus*. In addition, this kind of doubling of the Ks ensures that in the following meiosis each of the Ks has a homologue as a pairing partner.

It is not clear why an accumulation of Ran at the S kinetochores was found not only in the unequal differential gonial mitosis, but already in the previous normal gonial mitoses, in which Ss and Ks segregate equally (Fig. 1g, h). One can suppose, that the Ran-dependent mechanism ensuring an equal segregation of the Ss is generally active in all gonial mitoses of *A. lucidus.*

In summary, the unusual accumulation of Ran detected at the kinetochores of the remaining Ss in differential gonial mitosis strongly supports the idea that Ran is involved in a mechanism that prevents the inactivation of the spindle assembly checkpoint of the Ss until the unseparated Ks have arrived at the spindle pole, thereby ensuring the equal segregation of the Ss. This guarantees that the germ line cells entering into meiosis contain two complete sets of Ss and of Ks, which in turn is essential for the correct process of meiosis.

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Conflict of interest The author declares that he has no conflict of interest.

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