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Unequal distribution of genes and chromosomes refers to nuclear diversification in the binucleated *Giardia intestinalis*

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ABSTRACT

The single-celled parasite *Giardia intestinalis* (Diplomonadida) has two equally sized nuclei in one cell. The nuclei have been considered identical. We have previously shown that they contain different chromosomal sets and proceed through the cell cycle with some asynchrony. Here, we demonstrate by fluorescence in situ hybridization that several genes from chromosome 5 are lost in one of the two nuclei of the WBc6 *Giardia* line. The missing segment stretches over at least 50 kb near the 5′ chromosome end. In both WB and WBc6 *Giardia* cell lines, chromosome 5 is trisomic in one nucleus and monosomic in the other nucleus. The described chromosomal deletion has always been observed at the monosomic chromosome in WBc6; however, the deletion was not detected in the parent line WB. The chromosomal segment was thus initially lost after biological cloning of WB, which gave rise to clone WBc6. We show that *Giardia* is capable of carrying out gene expression from only one nucleus. The two nuclei display a certain level of diversity, making each of them irreplaceable. The doubled karyomastigonts of diplomonads likely have separate functions both in the mastigont/flagellar organization and in chromosomal and gene content. To our knowledge, our results offer the first methodical approach to differentiating the two, so far indistinguishable nuclei.

1. Introduction

Giardia intestinalis (syn. *Giardia duodenalis*, *Giardia lamblia*) is a prevalent single-celled intestinal parasite colonizing the digestive tract and causing diarrhea in humans and domestic and farm animals. It alternates between stages of a flagellated trophozoite, which causes disease, and an infective cyst, which mediates the disease transmission. In a wider evolutionary context, *G. intestinalis* belongs to the diplomonad group (Excavata). The diplomonads are remarkable for the presence of two karyomastigonts in one cell. In general, a karyomastigont is a specialized organelle cluster composed of the nucleus and the flagellar apparatus, which usually consists of two basal bodies anchoring flagella and the associated roots (Yubuki et al., 2016). The diplomonad karyomastigonts were hypothesized to arise several times independently during evolution by duplication of a single enteromonad karyomastigont (Kolísko et al., 2008). However, little is known, after the putative duplication, about whether the karyomastigonts remained identical or diverged. If it was the latter, an interesting question is to what extent a eukaryotic cell is able to exert control over diversified motility apparatuses and, more importantly, to tolerate two non-identical nuclei. A suitable model for studying these questions among diplomonads is *G. intestinalis* due to its easy laboratory maintenance, medical and veterinary importance, and the generally good understanding of its cellular and molecular features including a fully sequenced genome.

For a long time, the *Giardia* cell has simply been considered to carry doubled structures. However, the mastigonts within a single cell of *Giardia* (two groups of four basal bodies/flagella with associated structures) have been shown to differ with regard to the age and origin of the flagella they contain and by their associated structures (Nohýnková et al., 2006). With regard to the two nuclei in a cell, these have been assumed to be identical, especially based on the works of Yu et al. (2002) and Kabnick and Peattie (1990). We have previously reported that the two nuclei of Giardia possess different numbers of chromosomes. Aneuploidy-near tetraploidy was described in *Giardia* cells and different clinical isolates, and laboratory lines have been reported as karyotypically divergent with chromosome number differences between the two nuclei (Tůmová et al., 2016).

The coexistence of two nuclei in one cell is an especially exciting fact, as DNA replication, gene expression and karyokinesis must be tightly coordinated in the cell cycle. Other than diplomonads, there are not many examples of permanent diplokaryosis among eukaryotes apart from microsporidia and some amoebae

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(Walochnick et al., 2010; Han and Weiss, 2017). Di- or multikaryotic cells may appear transcendent in many organismal groups such as ciliates, filamentous fungi or mammalian cells; however, these always originate from a cell with one nucleus, or the dikaryon represents a mating stage in sexual reproduction. In addition to diplokaryosis, which may originate by cell fusion or incomplete cytokinesis after karyokinesis, another process leading to doubling of the genetic content of a cell may happen. Whole genome duplication within one nucleus resulting from repeated replication has been shown to lead to organismal diversification in many eukaryotic groups during evolution, providing a new pool of genetic material for some paralogous genes to undergo subfunctionalization or neofunctionalization (Van de Peer et al., 2017). Although the mode of diplokaryotic origin is not convincingly known in diplomonads and *Giardia*, whole genome duplication and changing mono- and diplokaryotic states is not a relevant situation in these organisms.

We would like to gain an understanding of the coexistence of the two nuclei in diplomonads by characterizing their chromosome and gene content. As there are insufficient means by which the nuclei in diplo- and multikaryotic cells can be physically separated for individual analysis, methods of fluorescent in situ hybridization (FISH) and cytogenetics facilitate at least a basic understanding of their putative diversification. By using these methods, we present the first study showing the unequal distribution of individual chromosomes between the two nuclei and the presence of some genes in only one of the two diplomonad nuclei. Possible consequences of such nuclear diversification are discussed.

2. Materials and methods

2.1. Cell lines

Five laboratory lines of *G. intestinalis* from assemblage A were examined. All originated from the same WB isolate (American Type Culture Collection (ATCC), UK, ATCC 30957) that was obtained in 1979 by duodenal aspirate from a symptomatic patient not responding to metronidazole treatment (Smith et al., 1982). The lines were collected for this study from the following different sources: two laboratory lines represented the WB isolate, namely, WB-M (a gift from Professor E.A. Meyer, Oregon Health Sciences University, Portland, USA; brought to Prague in 1989, after which it was frozen in liquid nitrogen, thawed in 2013 and then maintained in continuous culture until used) and WB-A (retrieved from the ATCC collection in 2013 and kept in a continuous culture), and three laboratory lines representing the WBc6 line (ATCC 50803) of the original QWB isolate, namely, WBc6-C (a gift from Professor Z. W. Cande, University of California, Berkeley, USA; received in 2012 and kept in continuous culture), WBc6-S (a gift from Dr. M. Lalle, Instituto Superiore di Sanita, Rome, Italy and originating from Professor S. Svärd, University of Uppsala, Sweden; received in 2016 and kept in continuous culture), and WBc6-H (a gift from Dr. M. Lalle and originating from Professor A. Hehl, University of Zürich, Switzerland; received in 2016 and kept in a continuous culture). Axenic cultures were routinely maintained in TYI-S-33 medium (pH 6.8) in screw-capped borosilicate glass tubes. The cultures were passaged twice per week by inoculating 500 μL of the chilled 4 day old culture into a new tube containing 7.5 mL of preheated TYI-S-33 medium. For all experiments, only the trophozoite life-cycle stage of G. intestinalis was used.

2.2. Fish

The FISH protocol and probe construction were carried out according to Tůmová et al. (2016). Briefly, the chromosome suspensions from the five *Giardia* cell lines were prepared as described by Tůmová et al. (2007). The chromosome spreads were partially air-dried, placed in 50% acetic acid solution for several seconds and then dried at 37 °C. The hybridization mixture contained 20 ng of labeled probe, 10 μg of salmon sperm, and 50% deionized formamide (Sigma-Aldrich, USA) in 2× SSC and was incubated at 82 °C for 5 min. Single colored FISH was developed using a TSA-Plus TMR System according to the manufacturer's instructions (PerkinElmer, Waltham, USA) using a digoxigenin-labeled probe and an anti-dig-horseradish peroxidase (HRP) antibody (Roche Applied Science, Indianapolis, USA). In two colored FISH, a sequential double hybridization signal development was processed according to the manufacturer's directions (PerkinElmer, Waltham, USA), as a combination of (i) a digoxigenin-labeled probe, anti-dig-HRP antibody, and TSA-Plus TMR and (ii) a biotin-labeled probe, streptavidin-HRP, and TSA-Plus Fluorescein. An Olympus BX51 fluorescence microscope equipped with a DP70-UCB camera was used for observation.

2.3. FISH probes

For FISH probe design, we selected 13 single copy or one low copy number gene (beta-tubulin), all located at chromosome 5 and annotated in the Giardia database reference genome (http://giardiadb.org, line WBc6) (summarized in Supplementary Table S1). The following open reading frames (ORFs) were used: GL50803_14615, GL50803_16312, GL50803_95653, GL50803_17023, GL50803_15112, GL50803_16374, GL50803_39904, GL50803_16264, GL50803_14702, GL50803_21232, GL50803_14846, GL50803_7439, and GL50803_101291. PCR products of approximately 2000 bp inside the ORFs of the genes were cloned into a pJET 1.2/blunt cloning vector (Fermentas, USA) and transformed into chemically competent TOP10 Escherichia coli cells (Invitrogen, USA). Purified PCR products amplified from plasmids isolated from a single bacterial colony (QIAprep Spin MiniprepKIT, Qiagen, Germany) were labeled by random priming with digoxigenin-11-dUTP (Roche) or biotin-11-dUTP (PerkinElmer, USA) using a DecaLabel DNA Labeling Kit (Fermentas, USA). The primers used, the product lengths and an exact chromosomal location are listed in Supplementary Table S1. The probe sequence was checked by sequencing the purified PCR products from minipreps. As controls, probes specific to other chromosomes were used for determination of probe binding to a chromosome of interest (Supplementary Table S2); the following probes were used: GL50803_40817 (chromosome 2), GL50803_96018 (chromosome 3), and GL50803_17495 (chromosome 4).

2.4. Controls for FISH accuracy

The probe binding efficiency was estimated at >100 cells as the percentage of the FISH-labeled to unlabeled nuclei. This value varied among probes from 80% to 98% of the positive nuclei. There are no clear morphological differences between *Giardia* chromosomes 1–5 that would allow determination of the probe binding to a particular chromosome. Thus, in two colored FISH, more than 20 combinations of two probes to chromosome 5, or a probe to chromosome 5 and to another chromosome, were used. The aim was to verify probe binding on individual condensed mitotic chromosomes to confirm (different probes to chromosome 5) or exclude (probes to different chromosomes) binding to the same chromosome in the same nucleus. The probe sizes, chromosome localization and primer sequences are listed in Supplementary Tables S1 and S2. The presence of a copy of the respective gene at the particular chromosome was verified through BLAST searches at http://giardiadb.org for the WBc6 (ATCC 50803) *Giardia* line. The selected probes were shown to not share sequence homology with

genome portions other than those stated. Randomly chosen slides were counted by two persons, and the probe binding patterns were evaluated from at least two independent FISH experiments for every probe/sample. Various probe combinations in a two colored FISH were tested in all five *Giardia* line samples. The efficacy of biotin- versus digoxigenin-labeled probes and signal developing was reciprocally tested and calibrated to the same binding efficacy.

2.5. Gene expression analysis

To determine whether the "missing genes" in one nucleus are expressed, we performed a semiquantitative PCR. Total RNA was isolated from 15 ml of *Giardia* monolayer cultures in TYI-S-33 medium by modified CTAB extraction (Healey et al., 2014) followed by LiCl precipitation (New England Biolabs, UK). Residues of genomic DNA were removed using a Turbo DNA-free kit (Life Technologies, USA). The concentration and purity of total RNA were determined by NanoDrop spectrophotometry and agarose gel electrophoresis. Purified RNA was reverse transcribed into cDNA using a ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, UK), and the resulting cDNA was diluted to a working concentration of 100 ng/μl.

Expression of selected genes was validated via semiquantitative reverse transcription (RT)-PCR and electrophoretic separation of amplified fragments. PCR primers were designed for each analyzed gene from the individual reference coding sequences of *G. intestinalis* ATCC 50803 assemblage A isolate WBc6 obtained from GiardiaDB. All designed primers are summarized in Supplementary Table S3.

All PCRs were carried out in a volume of 25 μl with 1× Taq buffer containing $(NH_4)_2SO_4$, 2 mM $MgCl_2$ (Thermo Scientific, Lithuania), 0.2 mM each 2′-deoxynucleotide 5′-triphosphates (dNTP Mix, PCR Grade) (Qiagen, USA), 10 pmol of each primer, 0.5 units of Taq DNA polymerase (recombinant) (Thermo Scientific, Lithuania), and 100 ng of genomic DNA template. Cycling conditions were set as follows: a preliminary denaturation step at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and an elongation step at 72 °C for 5 min. PCR-amplified fragments were separated by electrophoresis on a 1% agarose gel stained with Gel Red™ Nucleic Acid Gel Stain 10,000× in water (Biotium, USA).

3. Results

3.1. Several genes from chromosome 5 are missing from the same nucleus of the two nuclei of the WBc6 Giardia lines

Hybridization of probes to various portions of chromosome 5 showed that the *Giardia* lines WBc6-C, WBc6-H and WBc6-S lack five genes in one of the two nuclei (probe IDs: DUAL GL50803_15112, MEM GL50803_16374, HCM GL50803_39904, WD40 GL50803_16264 and RRP5 GL50803_14702) (Fig. 1, Table 1). The missing area, estimated from the comparison with the reference genome from the *Giardia* database, is at least 50 kb. It is near the 5́-end of chromosome 5, namely, at positions 1,280,596 – 1,331,328 bp in the GiardiaDB isolate WB sequence. Out of 2000 WBc6 cells analyzed with these five probes, only 48 (2.4%) revealed labeling in both nuclei. To confirm that the missing genes are always absent from the same nucleus, colocalization experiments with two different probes were done. The probe MEM (GL50803_16374) was selected as a marker, and all probes, reported in single experiments as missing in one nucleus, cohybridized with the MEM probe to chromosomes in the same nucleus (Figs. 1, 2). The other eight probes designed to hybridize to the area surrounding the deletion and to other chromosome 5 regions, hybridized to both nuclei in all WBc6 lines (Fig. 2, Supplementary Fig. S1, Table 1). As controls, cohybridization experiments with probes against chromosome 5 and chromosomes 2–4 revealed FISH signals on different chromosomes (Supplementary Fig. S2).

As already mentioned in the section 2, all three WBc6 lines (WBc6-C, WBc6-H, WBc6-S) represent laboratory lines of the biological clone WBc6 of the original clinical isolate WB. Interestingly, in both *Giardia* WB lines (WB-M, WB-A) representing the original isolate, the five genes shown to be absent in one nucleus of the *Giardia* WBc6 lines (documented above) were present in both nuclei (Fig. 3). Concomitantly, all other probes hybridized to both nuclei of the WB lines.

3.2. Modified chromosome 5 copy number between the two nuclei in WB and WBc6 Giardia lines

The FISH experiments with probes to chromosome 5 binding to both nuclei (eight probes in WBc6 lines and 13 in WB lines; see Table 1) revealed that both WBc6 and WB *Giardia* lines always have one signal in one nucleus (assigned as nucleus 1) and most often 2–4 signals in the other nucleus (assigned as nucleus 2) (Figs. 2, 3).

The number of signals in nucleus 2 varied slightly among the probes, e.g., the "SER probe" labeling nuclei 1 and 2 (GL50803_7439, $n = 100$): three signals (61%), two signals (22%), one signal (17.4%) in nucleus 2; the "170 probe" labeling nuclei 1 and 2 (GL50803_17023, $n = 112$): three signals (73.2%), two signals (17.8%), four signals (7.1%) in nucleus 2. The "MEM probe" labeling only nucleus 2 (GL50803_16374): three signals (56%), two signals (33%), four signals (6.6%) and others 4.4% ($n = 291$) in nucleus 2.

In contrast to this finding, all probes binding to nucleus 1 gave only a surprisingly uniform single signal in nucleus 1 (99%, $n = 650$). Based on this result, we assume that chromosome 5 in nucleus 1 is monosomic and chromosome 5 in nucleus 2 is mainly trisomic. In two colored FISH, cohybridization of probes to nucleus 2 revealed that their binding patterns match the same chromosomes, despite the fact that they did not give a uniform binding pattern with regard to the signal number in nucleus 2 (Fig. 2). Sometimes, the genes detected by the probes were missing at some chromosomes 5 in nucleus 2. In contrast to this, probes designed to different chromosomes revealed binding to chromosomes other than chromosome 5 (Supplementary Fig. S2). The suggested process leading to the observed situation caused by numerical and structural aberrations is shown in Fig. 4.

3.3. The single chromosome 5 is located in the faster progressing nucleus in WB and WBc6 Giardia lines

A certain level of cell cycle asynchrony was previously described for mitotic *Giardia* nuclei, pronounced in the delayed prophase chromosome condensation and a later anaphase onset in one of the two nuclei (Tůmová et al., 2007, 2015). By binding the FISH probes, we were able to identify in WB and WBc6 lines that the rapidly proceeding nucleus is always nucleus 1 with the monosomic chromosome 5 (98%, $n = 250$), which bears the above described subterminal deletion in WBc6 lines (Fig. 3, Supplementary Fig. S3). We thus found a tool to differentiate the two nuclei in WBc6 and WB lines for various experimental set-ups, relying on either a FISH probe hybridization or a more simple observation of the delay in the prophase chromosome condensation.

3.4. Expression of the genes missing from one nucleus in WBc6 Giardia lines is carried out from the other nucleus

We wanted to determine whether the genes present in only one nucleus are expressed or exist as silent pseudogenes. Expression of the selected genes was validated via semiquantitative RT-PCR and

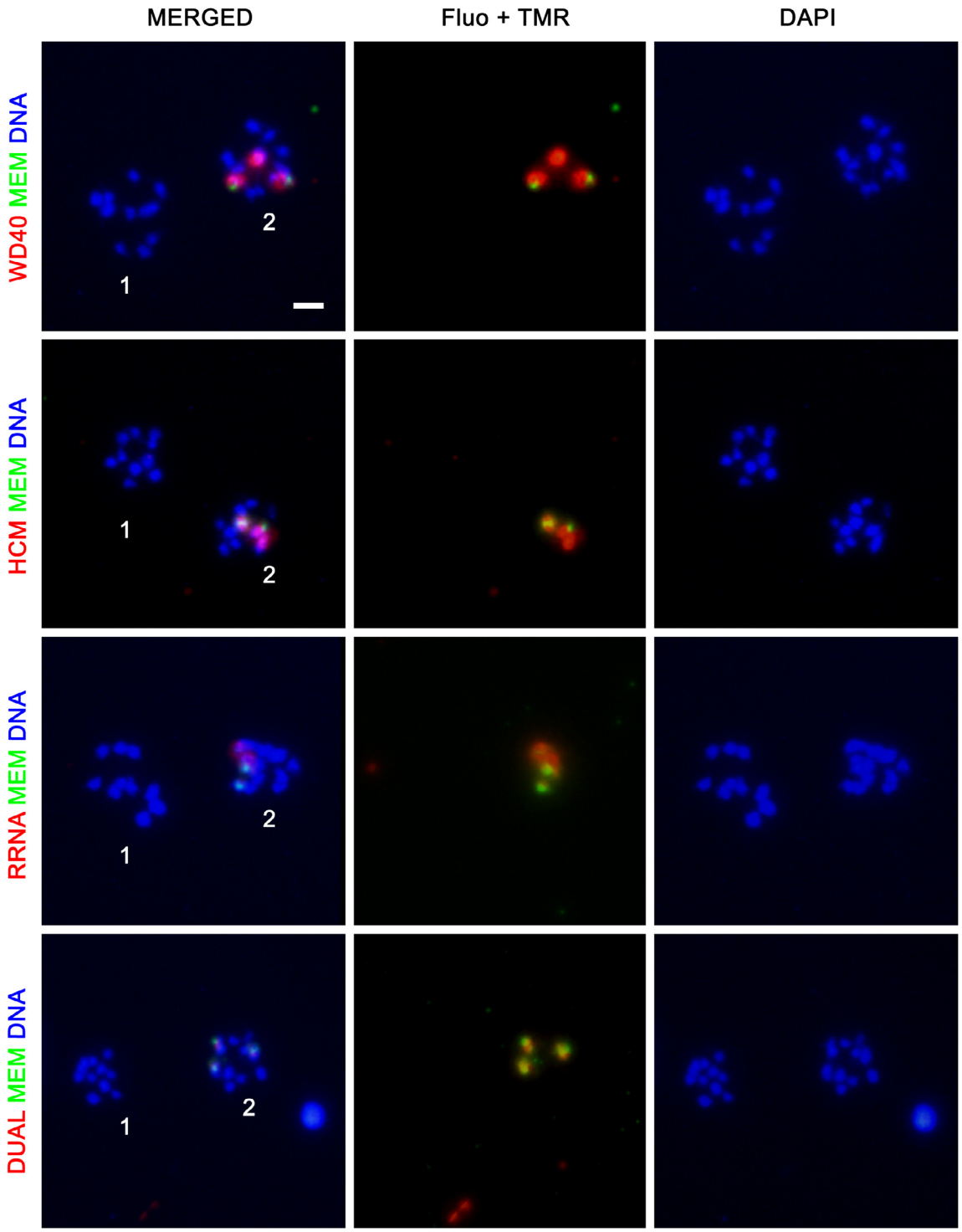

Fig. 1. Deletion in the same nucleus of the two nuclei of WBc6 *Giardia intestinalis* lines. Fluorescence in situ hybridization of probes against genes WD40, HCM, RRP5, DUAL (red signal from tetramethylrhodamine) with a probe against the MEM gene (green signal from fluorescein). There was no binding of these probes to nucleus 1, indicating the deletion. In contrast to this, a positive reaction was always obtained using probe combinations in the same nucleus, designated nucleus 2. The probes mainly colocalized to the same chromosomes in nucleus 2, which belongs to the longest chromosomes in the karyotype spreads (chromosome 5). The condensed chromosomes are stained with DAPI (blue). Bar represents 1 μm.

electrophoretic separation of amplified fragments. By testing 10 different genes including the five missing from one nucleus (Supplementary Table S3), we were able to show that the gene expression can run from one giardial nucleus only. All five genes missing in nucleus 1 in WBc6-C (probe IDs: DUAL GL50803_15112, MEM GL50803_16374, HCM GL50803_39904, WD40 GL50803_16264 and RRP5 GL50803_14702) were detected in *Giardia* trophozoite cDNA (Fig. 5). Both *Giardia* lines with the deletion (WBc6-S, WBc6-H) showed the same expression profile as WBc6-C (data not shown). As a negative control, we selected the CWP1 gene (GL50803_5638), whose transcription is regulated in the encystation process, and its expression first starts after transfer of *Giardia*

Table 1
Probes for fluorescence in situ hybridization to chromosome 5 of *Giardia intestinalis* nuclei. The probes were binding to either one (nucleus 2) or both nuclei (nucleus 1 and nucleus 2), as indicated for all *Giardia* cell lines tested. WBc6 cell lines (WBc6-C, WBc6-H, WBc6-S) are laboratory lines of the biological clone WBc6. This clone originated from the WB clinical isolate, here represented by its two laboratory lines WB-M and WB-A.

Probe No.	Probe ID	Gene ID	Gene Location at Chromosome 5	Present in Nucleus 1 and/or 2	
				WBc6-C/WBc6-H/WBc6-S	WB-M/WB-A
1.	146	GL50803_14615	824,815–827,232[1]	1 and 2	1 and 2
2.	163	GL50803_16312	829,622–833,573	1 and 2	1 and 2
3.	956	GL50803_95653	897,958–900,360	1 and 2	1 and 2
4.	170	GL50803_17023	900,334–902,697	1 and 2	1 and 2
5.	DUAL	GL50803_15112	1,280,596–1,282,719	2	1 and 2
6.	MEM	GL50803_16374	1,287,564–1,289,774	2	1 and 2
7.	HCM	GL50803_39904	1,297,673–1,300,078	2	1 and 2
8.	WD40	GL50803_16264	1,316,561–1,319,686	2	1 and 2
9.	RRP5	GL50803_14702	1,325,800–1,331,328	2	1 and 2
10.	212	GL50803_21232	1,401,149–1,403,875	1 and 2	1 and 2
11.	148	GL50803_14846	1,422,292–1,425,426	1 and 2	1 and 2
12.	SER	GL50803_7439	1,456,301–1,458,259	1 and 2	1 and 2
13.	BT	GL50803_101291	3,604,400–3,605,743	1 and 2	1 and 2

[a]Genes at chromosome 5 start at location 813,665.

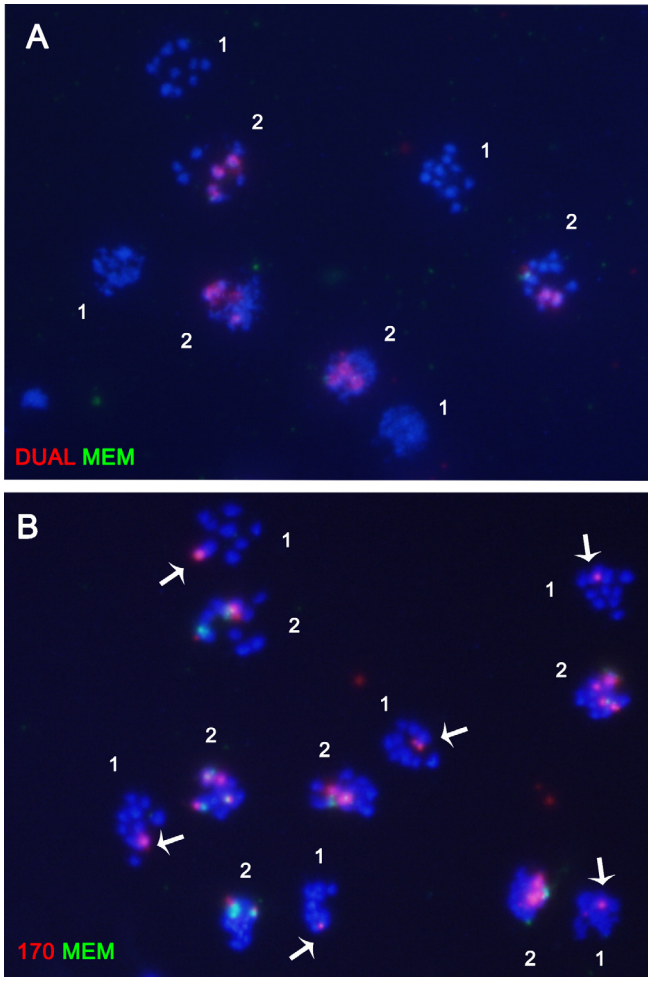

Fig. 2. Modified chromosome 5 copy number between the two nuclei of a WBc6 *Giardia intestinalis* lines. Fluorescence in situ hybridization of two probes against genes from chromosome 5. (A) Probes DUAL (red) and MEM (green); nuclei stained with DAPI. Both probes labeled chromosomes only in nucleus 2 (two or three signals). (B) Probes 170 (red) and MEM (green); nuclei stained with DAPI. The 170 probe labeled both nuclei, in nucleus 1 just one signal (arrow), in nucleus 2 two to four, mainly three signals. Note the colocalization of the two probes to the same chromosomes 5.

trophozoites into the encystation medium (Davis-Hayman et al., 2003). Thus, its expression was not detected in trophozoites. We additionally followed the tagged expression of the MEM gene (missing from nucleus 1 in WBc6 lines) and estimated its cellular localization as predicted by its sequence as a cellular surface protein (Supplementary Fig. S4).

4. Discussion

The *Giardia* cell is a subject of scientific interest from many reasons; one of them is the coexistence of two equally sized nuclei within a single cell of a *Giardia* trophozoite. These have been considered identical, based on the work of Kabnick and Peattie (1990), who detected rRNA genes in both nuclei, and Yu et al. (2002), who used probes against selected genes to indicate that each nucleus of Giardia contains a complete set of genetic information. We previously reported that the two nuclei of Giardia are not identical in that they possess different chromosomal numbers (Tůmová et al., 2007, 2016). Apart from the abovementioned works, there are no other studies that address the distribution of the chromosomes and/or genes between the two nuclei in diplomonads. Thus, we took advantage of previously developed methods such as preparation of chromosomal spreads (Tůmová et al., 2007) and sensitive two colored FISH (Sterkers et al., 2011; Tůmová et al., 2016) to localize individual genes on chromosomes 5 in mitotic and interphase nuclei of *Giardia*. This chromosome is the largest among the otherwise morphologically indistinguishable chromosomes (Adam et al., 1988), having a size of 4.4 Mb and carrying 2906 genes (GiardiaDB, http://giardiadb.org). We found a gene that was present in only one nucleus, and by mapping the surrounding region by FISH, we estimated the missing portion to be at least 50 kb. There is a possibility that the missing portion even stretches over a larger area of up to 500 kb from where we detected the genes of interest. Based on this, we were able to call the nuclei with the deletion nucleus 1 and the nucleus without the deletion nucleus 2.

Interestingly, there were differences between individual *Giardia* cell lines which we collected for the purpose of this analysis. All biological clones of WBc6 had the deletion at chromosome 5 in nucleus 1, in contrast to the two lines of their parent WB isolate. This indicates a structural aberration of chromosome 5 in nucleus 1, which appeared after the biological cloning of WBc6 in 1983 and was maintained in the WBc6 cell lines collected from all over the world (the USA, Sweden and Switzerland). The genome of the *Giardia* WB clone C6 (GL_50803) was sequenced in 2007 (Morrison et al., 2007); however, in the *Giardia* community, the commonly used terminology frequently interchanges WB and WBc6, even in genomic comparisons (Ankarklev et al., 2015). On the other hand,

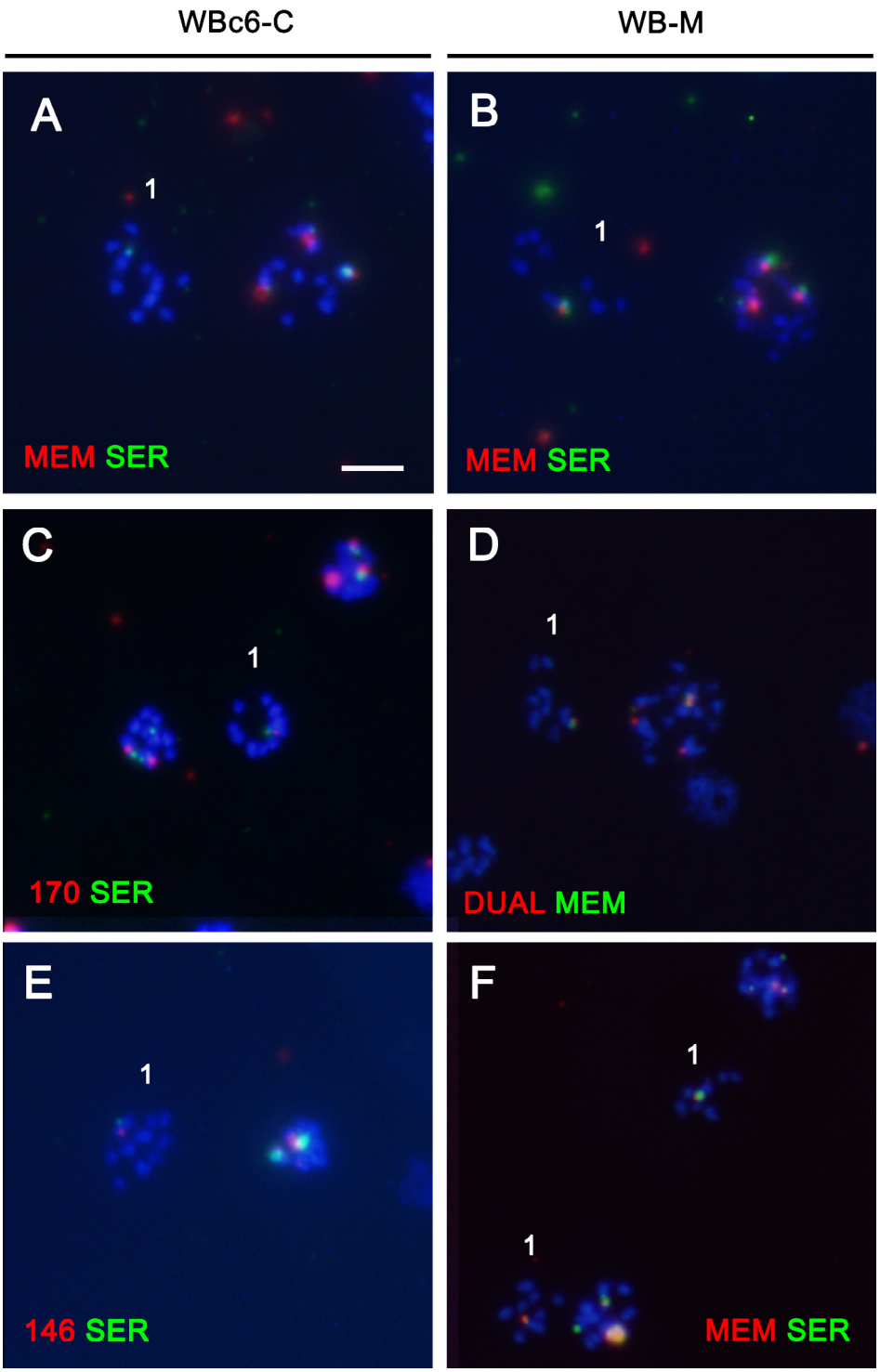

Fig. 3. Comparison of the parent WB *Giardia* line and its biological clone WBc6 by cohybridization of probes to chromosome 5. Chromosome 5 is present in nucleus 1 in one copy only and in nucleus 2 in three copies in both WB and WBc6. (A) In the WBc6-C line with karyotype 10 + 10 bearing the deletion in nucleus 1, the MEM probe binds to nucleus 2 only; however, the SER probe binds to chromosomes 5 in both nuclei. The MEM and SER probes colocalize to the same chromosomes in nucleus 2. (B) In WB-M with karyotype 8 + 13, both the MEM and SER probes colocalize to chromosome 5 (the longest chromosome) in nucleus 1 and to 3–4 chromosomes 5 in nucleus 2. (C, E) The probes 170 and SER, and 146 and SER (all outside the deletion) cohybridize to one chromosome 5 in nucleus 1 and to two out of three chromosomes in nucleus 2 in WBc6-C. (D) In WB-M, a positive signal from the DUAL and MEM probes on one chromosome 5 in nucleus 1 and on three to four chromosomes in nucleus 2. (E, F) The monosomic chromosome 5 is always contained in the faster progressing nucleus during mitosis in WB and WBc6 – i.e., with already condensed chromosomes. Nucleus 2 with the chromosome 5 trisomy has a delay in the prophase chromosome condensation. Bar represents 2 μm.

it should be mentioned that by common means of sequencing and deep-sequencing, it is difficult or almost impossible to detect a deletion on a *Giardia* chromosome due to the high ploidy and no possibility of analyzing the two nuclei separately. We cannot exclude the existence of more chromosomal structural aberrations on chromosome 5 or other *Giardia* chromosomes. Indeed, frequent small insertion-deletion events have been identified as alignment gaps in genomes of WB (WBc6, respectively) and other *Giardia* iso-

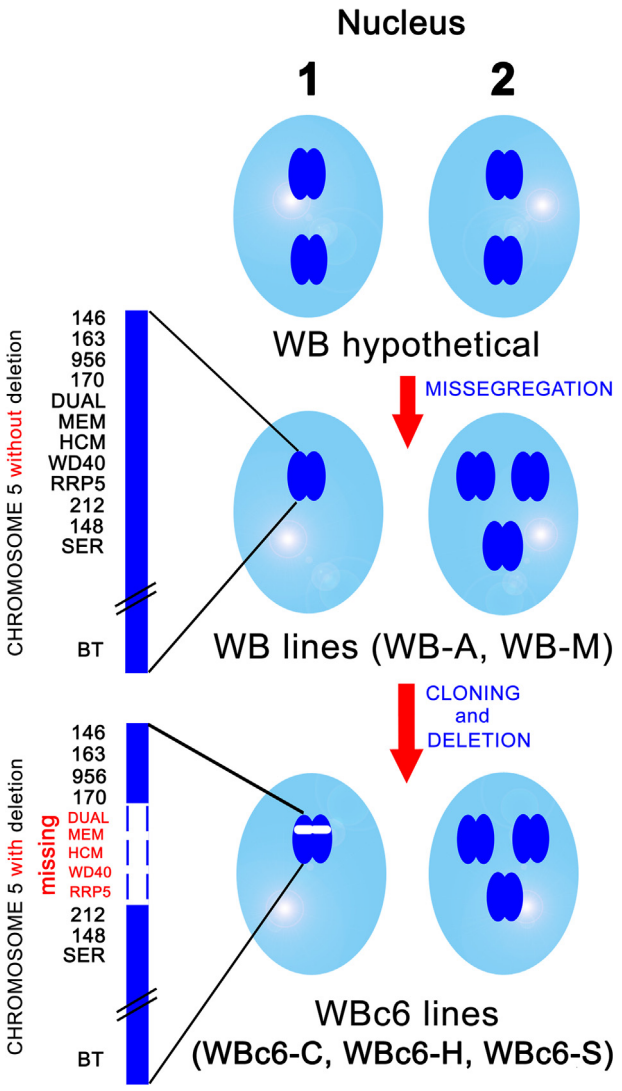

Fig. 4. Scheme representing the origin of monosomy and deletion on chromosome 5 in *Giardia* WBc6 lines. The putative way starts with chromosomal numerical aberrations in an original *Giardia* population (WB hypothetical). By missegregation or another process, the chromosome number of chromosome 5 was modified to monosomy in nucleus 1 and trisomy in nucleus 2 present in *Giardia* WB-M and WB-A lines. Biological cloning gave rise to WBc6 from WB. In the newly established WBc6 clone, putative structural aberrations occurred, which led to deletion at chromosome 5. This deletion was observed in all studied lines of WBc6 (WBc6-C, WBc6-H, WBc6-S). The deletion is schematically shown at chromosome 5 with the missing area (white). The genes shown as missing in nucleus 1 chromosome 5 (DUAL, MEM, HCM, WD40, RRP5) in WBc6 lines were, however, present in the nucleus 2 chromosome 5.

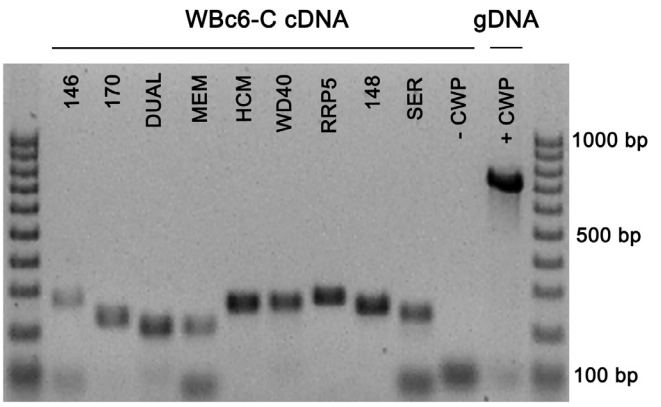

Fig. 5. Genes missing in one nucleus are transcribed from the other nucleus in a *Giardia* WBc6-C line. Semiquantitative reverse transcriptase PCR using WBc6-C cDNA revealed the gene expression from both nuclei (probes 146, 170, 148, SER) and from nucleus 2 only (probes DUAL, MEM, HCM, WD40, RRP5). The CWP1 probe was used as a negative control, as it is expressed in the *Giardia* cyst stage only, not in trophozoites. The presence of the CWP1 gene was shown by its amplification from *Giardia* genomic DNA (gDNA).

lates by deep sequencing (Jerlström-Hultquist et al., 2010). Our differentiation of the *Giardia* nuclei to nucleus 1 and nucleus 2 opens for the first time the possibility to track them individually during the cell and life cycle (encystation and excystation), and to differentiate them for the purposes of genetic manipulation. Indeed, plasmids have been reported to enter only one nucleus (Poxleitner et al., 2008), and homologous recombination was also reported to occur only in one nucleus (Carpenter et al., 2012). It will be of interest to specify whether the limited genetic manipulation of only one nucleus always correlates with the same nucleus.

Another new finding is the monosomy of chromosome 5 in nucleus 1 and the predominant trisomy in nucleus 2 in WB and WBc6 *Giardia* lines. In the WB-M line, the karyotype pattern was 8 + 13 (i.e., eight chromosomes in nucleus 1 and 13 in nucleus 2) (Tůmová et al., 2016; this study). It is thus likely that other chromosomes are also not present in exactly two copies per nucleus in this cell line. Modified chromosome size and copy number variation have been described from the pulse field gel electrophoresis experiments (Le Blancq et al., 1992; Upcroft et al., 1996) and from cytogenetic karyotyping (Tůmová et al., 2007, 2016). The modified chromosome number of chromosome 5 is especially interesting with regard to the total chromosome number in the WBc6 lines and the WB-A line with the karyotype 10 + 10 (i.e., 10 chromosomes in each of the two nuclei). This refers to the fact that, despite the chromosome number corresponding to exactly two copies of each of its five chromosomes per nucleus, a classic diploidy of each nucleus cannot be assumed. Consequently, other chromosomes are likely supernumerary in nucleus 1 and missing in nucleus 2 to make the chromosome number of 10. Detailed studies using multiple probes to a particular chromosome must be done to confirm the copy number of each chromosome per *Giardia* nucleus. Chromosome 4, in contrast to chromosome 5, is present most often in two copies in both nuclei as shown by binding of four different FISH probes (Tůmová et al., 2016). One of the probes against chromosome 4, however, indicated a loss of the particular gene copy at one of the two chromosomes in one nucleus and indicated for the first time an unequal gene distribution between the two nuclei (Tůmová et al., 2016). Mapping of a larger area around the missing gene – the approach applied in this study – may help to understand the extent of the deletion at chromosome 4 as well. It is difficult to interpret the signal numbers generated by individual probes because their binding to chromosomes 5 in nucleus 2, in contrast to binding in nucleus 1, was not uniform. We suggest that the process of gene rearrangements by gene losses or duplication to other chromosome portions, or to other chromosomes, must be taken into consideration in *Giardia*.

To exclude that deletion events leading to a total loss of genes from one nucleus are restricted to silent pseudogenes only, we wanted to know whether the particular genes, which were found missing in nucleus 1, are transcribed at least from nucleus 2. We performed semiquantitative RT-PCR and showed that the genes are constitutively transcribed in *Giardia* WBc6 trophozoites and thus only from one nucleus. This finding shows that these genes can be functional and that *Giardia* is able to assure transcription from only one nucleus. Additionally, it is clear from our data that the two nuclei do not progress synchronously during mitosis (Tůmová et al., 2007, 2015; this study). The monosomic chromosome is embraced in the faster progressing nucleus both in WB

and WBc6 *Giardia* lines. If there is a simple mechanistic reason why the nucleus with one chromosome 5 condenses and proceeds faster than a nucleus bearing three copies of the largest giardial chromosome, it remains speculative.

Here, we describe a unique situation of a eukaryotic cell with two slightly different nuclei. To our knowledge, this is the first case reporting such a situation. *Giardia* is a polyploid (aneuploidy-near tetraploidy) organism, and the increased ploidy may facilitate genome diversification, as four gene copies are provided for each locus under normal circumstances. Some of the gene copies may be lost without any physiological effect. A similar situation was described in eukaryotic organisms, mostly vertebrates and polyploid plants, which underwent whole genome duplication (WGD) events. Although most of the duplicated genes resulting from WGD events are subsequently lost, it is thought that these events provide new genetic material for some paralogous genes to undergo subfunctionalization or neofunctionalization (Sémon and Wolfe, 2007; Van de Peer et al., 2017). It has been proposed that polyploidization events may increase the genomic rearrangement rate, possibly directly accelerating evolution itself (Otto, 2007; Rensing, 2014). Most duplicated genes (paralogs) are quickly erased during evolution, and only some are retained. Whereas WGD events and tandem duplicates tend to show low asymmetric expression, genes transposed into different genomic regions show higher gene expression divergence (Wang et al., 2012). It is an open question as to what extent the expression of gene paralogs on the four chromosomal copies in *Giardia* are influenced by their particular nuclear microenvironment, being located in different nuclei with differing chromosome numbers and uncoordinated cell cycle progression. Despite this non-canonical situation, the *Giardia* cells of the investigated lines are fully viable and highly proliferative. The nuclear differentiation opens new possibilities in generating novel genetic variability, important for virulence and survival of this single-celled parasite. The complexity of regulatory networks operating between the two different nuclei of this eukaryotic cell must be astonishing.

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Appendix A. Supplementary data

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