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ORIGINAL PAPER

Non-canonical Cajal bodies form in the nucleus of late stage avian oocytes lacking functional nucleolus

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Abstract In the somatic cell nucleus, there are several universal domains such as nucleolus, SC35-domains, Cajal bodies (CBs) and histone locus bodies (HLBs). Among them, CBs were described more than 100 years ago; however, we still do not have a final understanding of their nature and biological significance. The giant nucleus of avian and amphibian growing oocytes represents an advantageous model for analysis of functions and biogenesis of various nuclear domains. Nevertheless, in large-sized avian oocytes that contain transcriptionally active lampbrush chromosomes, CB-like organelles have not been identified yet. Here we demonstrate that in the pigeon (*Columba livia*) oocyte nucleus, characterized by absence of any functional nucleoli, extrachromosomal spherical bodies contain TMG-capped spliceosomal snRNAs, core proteins of Sm snRNPs and the protein coilin typical for CBs, but not splicing factor SC35 nor the histone pre-mRNA 3′-end processing factor symplekin. The results establish that coilin-rich nuclear organelles in pigeon late-stage oocyte are not the equivalents of HLBs but belong to a group of CBs. At the same time, they do not contain the snoRNP/scaRNP protein fibrillarin involved in 2′-O-methylation of snoRNAs and snRNAs. Thus, the nucleus of late-stage pigeon oocytes houses CB-like organelles that have an unusual molecular composition and are implicated in the snRNP biogenesis pathway. These data demonstrate that snRNP-rich non-canonical CBs can form in the absence of nucleolus. We argue that pigeon oocytes represent a new promising model to investigate CB modular organization, functions and formation mechanism.

Keywords Avian oogenesis · Cajal body · Coilin · Germinal vesicle · Histone locus body · Nuclear bodies · Nuclear compartments · Nucleolus · Nucleus · Oocyte · Splicing factors

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Introduction

Cajal bodies (CBs) are evolutionarily conserved nuclear organelles that form in both germ and somatic cell nuclei of many species including vertebrates, invertebrates, plants and yeast (Gall 2000; Gall et al. 2004; Cioce and Lamond 2005). These specialized nuclear organelles take part in RNA biogenesis and assembly of small nuclear ribonucleoprotein particles (snRNPs) (including spliceosomal snRNPs) and small nucleolar (sno) RNPs and, in addition, contribute to telomere maintenance and some other important cellular functions (Matera and Shpargel 2006; Pontes and Pikaard 2008). Although CBs were described for the first time at the beginning of the twentieth century (Gall 2000) and the precise investigation of the molecular composition and functions of the bodies started few decades ago (Andrade et al. 1991), we still do not have a final understanding of their nature and biological significance.

During the last decade the protein coilin was considered to be a universal molecular marker for CBs from different species and tissues, until it was identified as a component of histone locus bodies (HLBs) in Drosophila (Liu et al. 2009). Recent investigations of coilin mutants in flies and mice have shown that this component is essential for

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correct CB formation but is not required for survival of the organism (Tucker et al. 2001; Liu et al. 2009). Moreover in the absence of coilin, the snRNA modification machinery does work in Drosophila (Deryusheva and Gall 2009). Nowadays, to identify CBs in the nucleus, a number of its molecular components need to be analyzed including small nuclear RNAs (snRNAs), Sm-proteins, coilin, fibrillarin, small Cajal body-specific RNAs (scaRNAs) and SMN (survival of motor neurons) protein (Andrade et al. 1991; Liu and Dreyfuss 1996; Xie et al. 2007).

CBs appear to represent a group of organelles with a modular structure in which domains of different RNA processing pathways can combine together depending on organism and cell type. In experiments where factors required for U snRNP cytoplasmic maturation were depleted, the formation of residual nucleoplasmic foci containing coilin and snoRNP components was demonstrated (Lemm et al. 2006). Furthermore, in the absence of coilin, snoRNP components condense into nucleoplasmic foci (residual CBs) that do not recruit snRNP or SMN complex proteins (Bauer and Gall 1997; Tucker et al. 2001; Lemm et al. 2006). In addition to gene knockdown experiments, our knowledge about the role of CBs is significantly increased by studies of existing types of functional nuclear organization in vivo (Ferreira and Carmo-Fonseca 1995; Liu et al. 2006; Matera and Shpargel 2006; Pontes and Pikaard 2008; Nizami et al. 2010a).

Because of its larger size, transcriptional activity and peculiarities of organization, the oocyte nucleus (also called the germinal vesicle, GV) of amphibians and some insects is an advantageous model for analysis of CB organization and functional relevance (Gall et al. 2004; Bogolyubov and Parfenov 2008). Even though growing avian oocytes are also characterized by large size of the nucleus (up to 400 μm) and a high transcriptional activity, they have not been used as a model for studying functional compartmentalization of the cell nucleus until recently (Maslova and Krasikova 2011). Importantly, the growing oocyte of adult birds is characterized by inactivation of nucleolar organizer region and by the absence of any functional nucleoli (Greenfield 1966; Gaginskaya et al. 2009). Inactivation of chromosomal nucleolar organizing regions in nucleus of growing oocytes of adult avian females was confirmed by DNA/RNA radioactive in situ hybridization on two species of birds, particularly on *Columba livia*, and it was also established that amplification of rRNA genes does not happen during avian oogenesis (Gaginskaya and Gruzova 1969, 1975). These peculiarities of avian oogenesis open up unique opportunities for studying the relationships between the nucleolus and other nuclear structures. Specifically, CBs often have an intimate relationship with nucleoli in other systems (reviewed in Lafarga et al. 2009; Gilder and Hebert 2011).

In avian GVs that contain transcriptionally active lampbrush chromosomes, CB-like organelles have not yet been identified. The most comprehensively described intranuclear bodies among all avian oocytes in terms of their morphogenesis and nature are the so-called centromere protein bodies (Gaginskaya and Gruzova 1969; Solovei et al. 1996; Saifitdinova et al. 2003; Krasikova et al. 2004). Some years ago these structures were thought to be the CBs, later came the convincing evidence that centromere protein bodies are not the equivalents of CBs. It was found that centromere protein bodies of avian GVs do not contain coilin, TMG-capped snRNAs, splicing factor SC35, RNA-polymerase II, or nucleolar proteins fibrillarin, B23 and Nopp140 (Krasikova et al. 2004). Moreover, DNA-topoisomerase II and the cohesin complex components that are not typical constituents of canonical CBs were identified in the centromere protein bodies of birds (Krasikova et al. 2004, 2005).

Remarkably, in the oocyte nucleus of one of the bird species, the rock pigeon (*C. livia*), two other types of extrachromosomal spherical bodies called 'hollow spheres' (HSs) and 'solid globes' (SGs) with unknown functions were described earlier (Khutinaeva et al. 1989). According to the histochemical staining experiments data, SGs and HSs were said to contain nucleic acids (NAs), but the nature of the bodies was obscure. In the present work, we investigate the molecular composition and possible functions of these remarkable extrachromosomal spherical bodies of pigeon GV, and for the first time describe CB-like organelles in these transcriptionally active growing oocytes characterized by the absence of any functional nucleoli. Our data suggest that the extrachromosomal NA-containing nuclear bodies (SGs and HSs) in avian oocytes are CB-like structures. We argue that pigeon oocytes with their enormous transcriptionally active nucleus lacking a functional nucleolus represent a promising new model for studying CB modular organization, as well as their function and formation mechanism.

Materials and methods

Immunofluorescent staining of oocytes

Oocytes of 1 mm and less in diameter and follicular epithelium cells dissected from adult females of the rock pigeon (*Columba livia*) were manually separated under a Leica MZ16 stereomicroscope. All manipulations with animals were carried out in accordance with relevant federal guidelines and institutional policies. To improve penetration of antibodies, separated oocytes and pieces of ovary were treated with collagenase (1 mg/ml, Sigma) in 1× phosphate buffered solution (PBS) (1.47 mM KH_2PO_4, 4.29 mM $Na_2HPO_4 \times 7H_2O$, 137 mM NaCl, 2.68 mM KCl) for

20 min at room temperature (RT) and washed three times for 5 min in PBS. After fixation in 2% paraformaldehyde (PFA) (Serva) solution in PBS during 2 h at RT and washing in PBS, oocytes and pieces of ovary were permeabilized in 0.2% Triton X-100 (ICN Biomedicals Inc) in PBS at +4°C overnight (ON) and washed in PBS. To prevent non-specific binding of antibodies (Abs), oocytes and follicular epithelium cells were blocked with 1% blocking reagent (Roche) in PBS for 40 min. Afterwards, the preparations were incubated with certain primary Abs diluted in 1% blocking reagent (Roche) for 1 h and with corresponding secondary Abs diluted in 1% blocking reagent (Roche) for 1 h. After each incubation step, the preparations were washed three times for 5 min in PBS to remove unbound Abs. Finally, oocytes and pieces of ovary were counterstained with DAPI (4,6-diamidino-2-phenylindole, 1 mg/ml, Sigma) and mounted in DABCO antifade solution (Merck).

Immunofluorescent staining of intact oocyte nuclei

The nuclei from 0.5 to 5.0 mm diameter oocytes from egg-laying females of the rock pigeon (*C. livia*) were isolated manually with thin tungsten needles in GV isolation medium [83 mM KCl, 17 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 1 mM $MgCl_2$ 1 mM dithiothreitol (DTT)] according to the standard technique (Solovei et al., 1993). All manipulations with GVs utilized a stereomicroscope Leica MZ16. Staining of undamaged GVs was performed as described by Krasikova (2007). Briefly, isolated GVs were either immediately fixed in 2% PFA in PBS for 30 min at RT and washed in PBS for 10 min or directly analyzed by confocal laser scanning microscopy. 3D-preserved unfixed nuclei were stained with the nucleic acid (NA) specific fluorochrome Sytox green (0.07 μM, Molecular Probes) in PBS for 5–10 min at RT and were immediately analyzed with confocal laser scanning microscope. Fixed nuclei that were isolated from oocytes were incubated for 1 h with certain primary Abs diluted in PBS and washed for 10 min in PBS, then incubated with the appropriate secondary Abs diluted in PBS for 1 h and washed for 10 min in PBS. Immunostained GVs were immediately transferred into glass chambers and analyzed with confocal laser scanning microscope.

Immunofluorescent staining of germinal vesicle spreads

Spreads of pigeon GV content from oocytes of 0.5–2.0 mm in diameter were prepared from ovaries of adult females according to the standard technique of avian lampbrush chromosomes (LBCs) isolation (Solovei et al. 1993). After 30 min of fixation in 2% PFA in PBS, spreads were incubated in 50, 70% ethanol (+4°C) for 5 min and kept in 70% ethanol ON +4°C for best fixation.

The preparations of GV spreads for immunostaining were not dried before use and were stained as described previously (Krasikova et al. 2004). Preparations were incubated for 5 min in 70, 50, 30% ethanol, in PBS with 0.01% Tween-20 and were blocked with 1% blocking reagent (Roche) in PBS for 40 min. After that they were incubated with certain primary Abs diluted in 1% blocking reagent (Roche) for 1 h and with corresponding secondary Abs, dehydrated and mounted in DABCO antifade solution (Merck) containing DAPI (1 mg/ml, Sigma).

Antibodies used

The following primary Abs were used: rabbit polyclonal Ab (pAb) R288 against coilin (dilution 1:100) (Andrade et al. 1991); rabbit pAb H-300 against coilin (dilution 1:100) (Santa Cruz Biotechnology); mouse monoclonal antibody (mAb) against SR-protein SC35 (dilution 1:50) (Abcam); mouse mAb Y12 against symmetrical dimethyl-arginine (dilution 1:50) (Lerner et al. 1981); mouse mAb K121 against 2,2,7-trimethyl guanosine (dilution 1:150) (Santa Cruz Biotechnology); mAb No-185 against No38 protein (dilution 1:5) (Schmidt-Zachmann et al. 1987); mouse mAb No114 against Nopp140 protein (dilution 1:5) (Schmidt-Zachmann et al. 1984); mouse mAb 17c12 against fibrillarin (dilution 1:5) (Pollard et al. 1997); mouse mAb 38F3 against fibrillarin (dilution 1:100) (Santa Cruz Biotechnology); mouse mAb HYB331-01 against double-stranded DNA (dilution 1:100) (Abcam); mouse mAb against symplekin (dilution 1:250) (BD Transduction Laboratories). The following secondary Abs were used: Cy3-conjugated goat anti-rabbit IgG (dilution 1:500) and Alexa-488-conjugated goat anti-mouse IgG (dilution 1:300) (Jackson ImmunoResearch Laboratories).

Fluorescence in situ hybridization with U7 snRNA antisense oligomer

The preparations of GV spreads and small pieces of pigeon oviduct were used for fluorescence in situ hybridization. The U7 snRNA antisense biotinylated oligomer 5′-CTA AAAGAGCTGAAATCACTG-3′ was used as a hybridization probe according to the method described by Frey and Matera (1995). Pieces of oviduct were fixed in 2% PFA in PBS for 30 min, washed in three changes of PBS for 15 min and permeabilized in PBS containing 0.2% Triton X-100 for 5 min with subsequent washes in PBS. Dehydrated pieces of tissue and preparations of GV spreads were hybridized with hybridization mix (4× SSC, 10% dextran sulfate, 1 μg/μl of competitor tRNA and 1–2 ng/μl of biotinylated U7 snRNA antisense oligomer) at RT ON. They were then washed in 4× SSC three times at 37°C. For detection of hybridization signal, slides were incubated

with avidin conjugated to Alexa 488 (Molecular Probes Inc.). Finally, the preparations of GV spreads were counterstained with DAPI (1 mg/ml, Sigma) and mounted in DABCO antifade solution (Merck). Pieces of oviduct were stained with DAPI at +4°C ON and mounted in DABCO antifade solution.

Fluorescence microscopy

GV spreads were examined with Leica fluorescence microscope DM 4000B (Leica Wetzlar GmbH, Germany). Images were taken with 40×/1 and 100×/1.30 oil immersion objectives, appropriate filter cubes for such fluorescence dyes as Alexa 488 (green), Cy3 (red) and DAPI (blue) and recorded using a monochrome digital camera DFC 350FX at RT. For image analysis and optimization of contrast and relative signal intensities following programs were used: CW 4000 FISH (Leica Cambridge Ltd.) and Photoshop 8.0 (Adobe Systems).

Confocal laser scanning microscopy and image analysis

To investigate the three-dimensional structure of oocyte nuclei after staining, a series of optical sections through 3D-preserved nuclei, oocytes and follicular cells were collected at RT. The confocal laser-scanning microscope Leica TCS SP5 equipped with a HCX Plapo lambda blue 20 × 0.7 and HCX Plapo lambda blue 40 × 1.25 oil immersion objectives and a diode laser (405 nm) for DAPI, an argon laser (488 nm) for Alexa 488 and Sytox Green, and helium–neon laser (543 nm) for Cy3 (Leica Microsystems CMS GmbH, Germany) was used. For each optical section, signals in different channels were acquired sequentially and then images were merged using LAS AF software (Leica-Microsystems CMS GmbH, Germany). In the process of scanning in z direction, scanning pitch corresponded to maximum resolution of the objective. Brightness and signal to noise ratio were improved using appropriate gain and offset values. To reduce noises on acquired images the three frames image averaging was displayed. For each confocal image, there was corresponding transmitted light image. Computational analysis of 3D-image stacks and 3D-reconstructions were performed using instruments of "Process" ("Colors", "Crop", "3D Projection") and "Quantify" ("Line Profile") tabs in Las AF software.

Electron microscopy

For electron microscopy, whole oocytes of 0.5–3.0 mm in diameter and GVs isolated from oocytes of adult pigeons were used. Oocytes and GVs were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer with 2.5 M sucrose, pH 7.4, and post-fixed in 1% OsO4. With the help of an LKB-III ultramicrotome 70 nm sections were cut. Thin sections were stained with uranyl acetate and plumbum citrate as described by Reynolds (1963). Images were acquired at 60 kV using the BS-500 (Tesla, Czech Republic) electron microscope.

Western blotting

Each sample for western blots contained extract from germ cell nuclei. Samples were mixed with 5 µl SDS-loading buffer (350 mM Tris–HCl, 30% glycerol, 10% sodium dodecyl sulfate (SDS), 600 mM DTT, 0.012% bromphenol blue, pH 6.8) and heated at 97°C for 3 min before separation on a 10% SDS resolving gel with a 4% stacking gel as described by Laemmli (1970). The gel was calibrated with molecular weight marker Dual Color Standard (Bio-Rad Laboratories, Inc.). For immunoblotting, proteins were electrophoretically transferred to PVDF membranes (MP Biomedicals, LLC) using 90 V for 1.5 h at 4°C. The transfer efficiency was confirmed by Ponceau S staining. Before incubation with antibody, the PVDF membranes were cut into strips and blocked with 3% nonfat dry milk in PBS for 2 h at RT. PVDF strips were incubated for 2 h RT primary antibody diluted 1:100 in 1% BSA in PBS-0.05% Tween 20. Strips were then washed three times for 15 min with PBS-0.2% Tween 20 to remove unbound antibody and incubated with goat anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase (AP) (Sigma) diluted 1:2000 in 1% BSA in PBS-0.05% Tween 20 for 2 h. After three 10 min rinses with PBS-0.2% Tween 20, immunoreactive bands were detected by adding 1.5 ml of detection mixture per strip containing AP-buffer (50 mM Tris HCl Ph 9.5, 120 mM NaCl, 5 mM $MgCl_2$), 17 µg/µl BCIP (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt, Fermentas) and 0.33 µg/µl NBT (nitro blue tetrazolium, Fermentas) 0.33 µg/µl. The immunoreactive bands were detected by Image Station GDS-800 (UVP, USA).

Results

Nuclei of growing pigeon oocytes contain extrachromosomal spherical bodies

In giant transcriptionally active nuclei of large-sized oocytes of *C. livia* (the rock pigeon), extrachromosomal spherical bodies can be subdivided into two major types according to their ultrastructure revealed using electron microscopy (Fig. 1). Bodies of the first type have dense homogeneous matrix and therefore were called 'solid globes' (SGs); SGs are, on average, about 6 µm in diameter (Fig. 1a). Bodies of the second type have a thin layer of dense matrix on their periphery and a big transparent

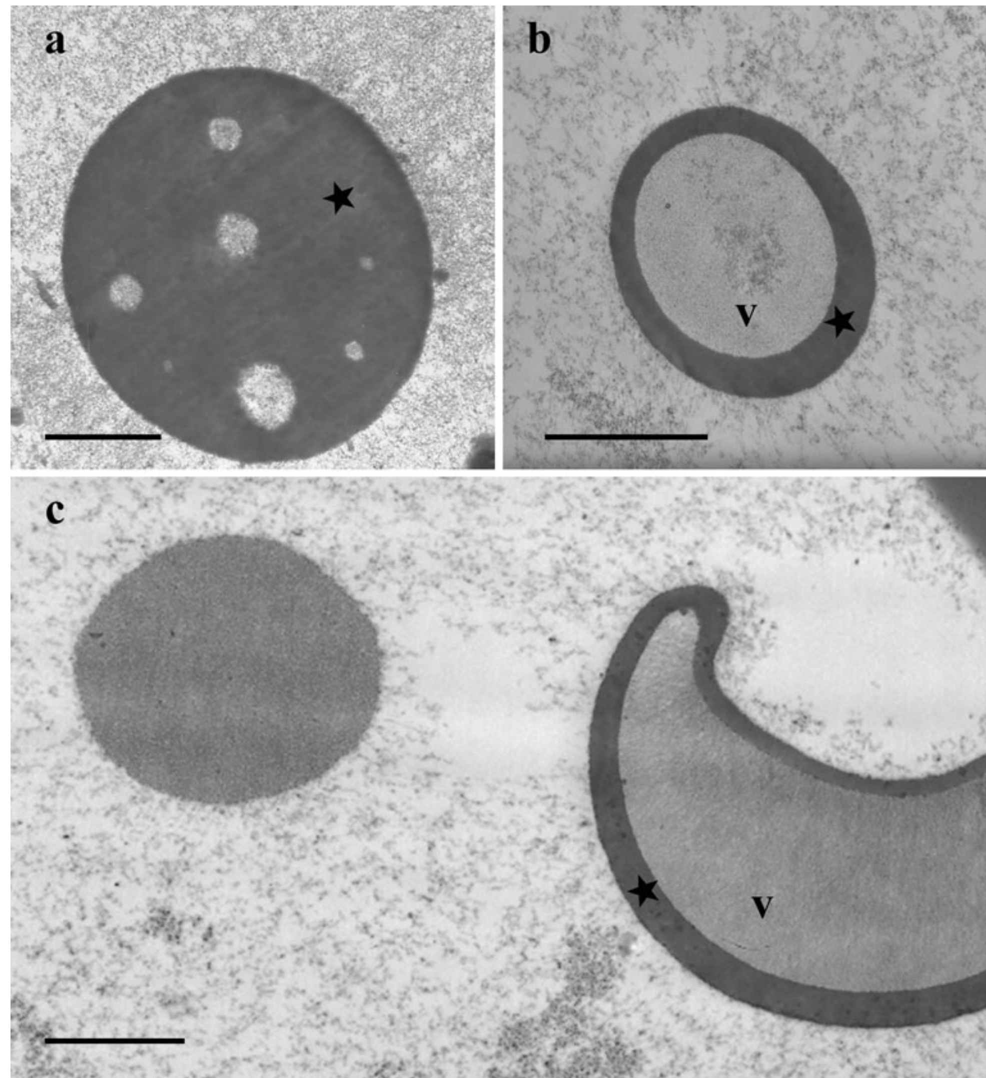

Fig. 1 Electron microscopy microphotographs of pigeon large-sized oocytes ultrathin sections through germinal vesicles showing a solid globe (SG) (**a**), a hollow sphere (HS) (**b**) and SG and HS together (**c**). *V* transparent vacuole, *asterisk* dense fibrillar matrix. *Scale bars* 1 µm (**a**), 2 µm (**b**) and 2.5 µm (**c**)

vacuole inside (Fig. 1b). These bodies were called 'hollow spheres' (HSs) and are larger than SGs, usually reaching around 12 µm in diameter. Based upon electron microscopy microphotographs, the matrix of both types of the bodies does not contain any obvious inclusion of granules (Fig. 1c). Note that the content of vacuole of HS can differ from the surrounding nucleoplasm.

Using confocal laser scanning microscopy, we described in detail the 3D-architecture of intact GVs from large oocytes of adult female pigeons. We first analyzed nuclear structures in 3D-preserved nuclei, microsurgically isolated from oocytes of 0.5–5.0 mm in diameter, by staining with the nucleic acid (NA)-specific fluorescent dye Sytox green (Fig. 2a, b). At the stage of transcriptional activity, lampbrush chromosomes (LBCs) in the nuclear space of pigeon GVs are distributed uniformly and, as expected, are not associated with nuclear envelope. Pigeon LBCs have both simple lateral loops and special loops (particularly terminal giant loops) the latter ones being often fused together (Khutinaeva et al. 1989). As opposed to GVs of chicken (*Gallus gallus domesticus*) and Japanese quail (*Coturnix coturnix japonica*), in pigeon GVs we can see the grouping of LBCs due to the fusion of centromere associated protein bodies that do not stain with Sytox green (A.K., unpublished observations). On preparations of freshly isolated GVs of the rock pigeon, there is a great number (up to several dozens) of extrachromosomal distinct spheres in the nucleoplasm. Usually the location of the spheres in GVs of pigeon is irregular: in growing oocytes they can be situated either near the nuclear envelope or in the center of the nucleus. These spheres exhibit high levels of fluorescence upon staining with Sytox green (Fig. 2). In contrast to SGs, there is an unlabeled vacuole inside each HS. Notably, HS staining with Sytox green was not as bright as that of SGs, probably because of the occurrence of non-fluorescent vacuole inside (Fig. 2e, f). These results can be explained either by

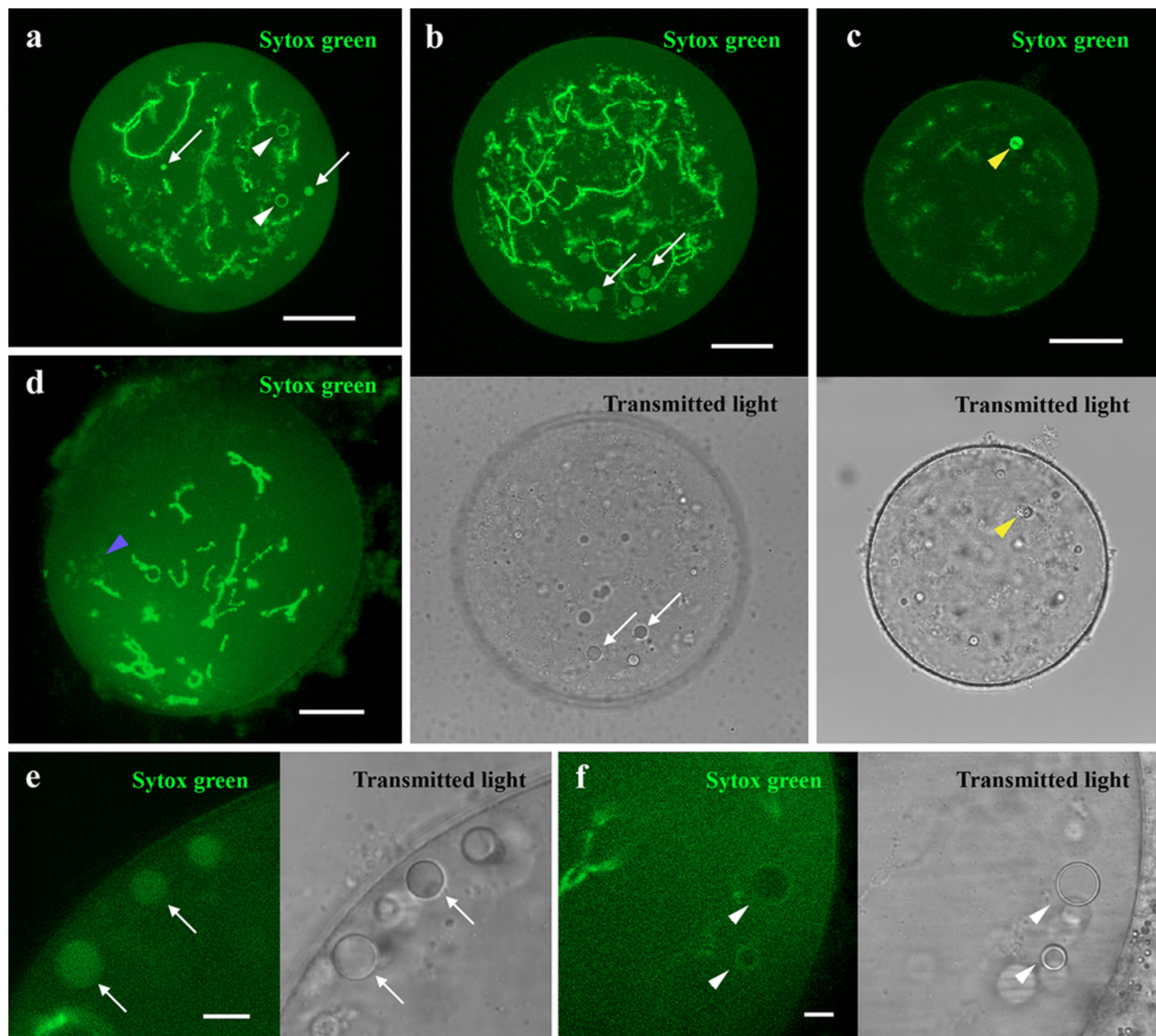

Fig. 2 Extrachromosomal nuclear bodies in large-sized oocytes of egg-laying pigeon females contain nucleic acids. In addition to lampbrush chromosomes, extrachromosomal solid globes (SGs) and hollow spheres (HSs) are stained with the nucleic acid specific fluorescent dye Sytox green. **a**–**d** Nuclei from transcriptionally active oocytes: **a** maximal projection of *XYZ* confocal stack (*Z* distance 20 µm) of intact GV; **b** maximal projection of total nucleus confocal stack; **c** individual *XY* confocal section through the nucleus. *Arrows* point to SGs; *arrowheads* show HSs; *yellow arrowheads* show intermediate structure. *Scale bars* 50 µm. **d** Nucleus from maturing oocyte with highly condensed chromosomes. Extrachromosomal bodies are accumulated independently of bivalents near the nuclear periphery. *Blue arrowhead* indicates grouping of intranuclear spheres at one of the poles of oocyte nucleus. *Scale bar* 50 µm. **e**, **f** Individual confocal sections through part of the isolated nuclei. Both types of spherical bodies contain nucleic acids in their dense matrix. In contrast with SGs (*arrows*), there is a vacuole inside each HS (*arrowheads*). *Scale bars* 10 µm. Images are taken with a laser scanning confocal microscope

the absence of nucleic acids in the vacuole or by dense fibrous material forming a thick peripheral layer in the HS that prevents diffusion of the dye into the vacuole.

The examination of 3D nuclear distribution of extrachromosomal spheres during maturation of pigeon oocytes allows us to consider possible steps of their formation and intercommunication. In larger oocytes with GVs, which contain condensed bivalents, extrachromosomal spheres gather at the periphery of the nucleus (Fig. 2d). At the beginning of karyosphere formation these bodies are grouped close to the nuclear envelope near condensed bivalents. Such close arrangement of extrachromosomal bodies is not specific for early stages of oocyte growth when they are distributed irregularly.

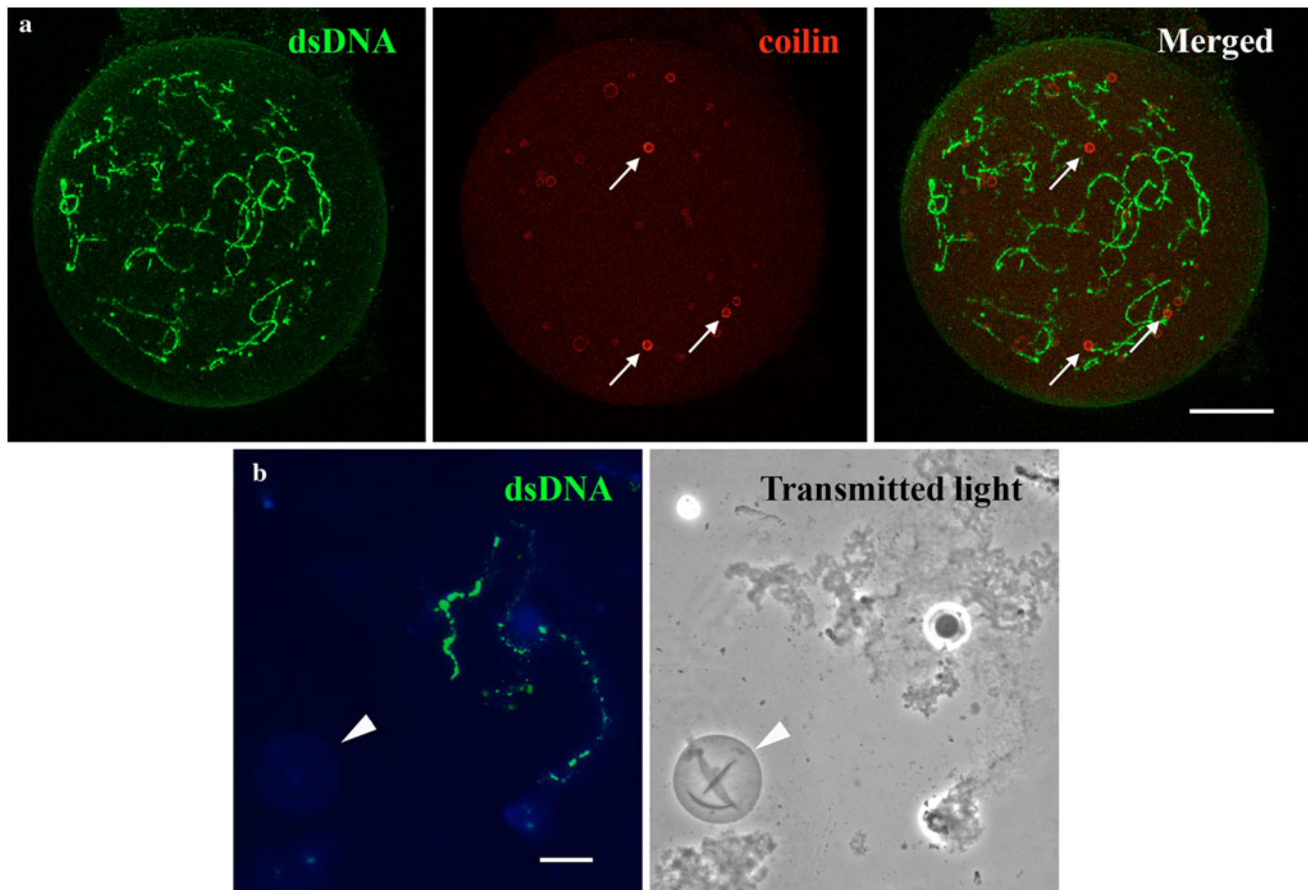

Fig. 3 Extrachromosomal nuclear bodies in pigeon oocytes do not contain DNA. **a** Double immunofluorescent staining of intact pigeon GV with Ab HYB331-01 against double stranded DNA (*green*) and Ab H-300 against coilin (*red*). Maximal projections of *XYZ* confocal stacks of oocyte nucleus are shown. Images are taken with a laser scanning confocal microscope. There is no fluorescence signal in intranuclear bodies after staining with Abs against double stranded DNA. *Arrows* point to some of the extrachromosomal solid globes (SGs) and hollow spheres (HSs). *Scale bar* 50 µm. **b** Immunofluorescent staining of pigeon GV spread with Ab against double stranded DNA. Corresponding phase contrast image is shown. *Arrowheads* indicate HS without fluorescent signal. *Scale bar* 10 µm

Marker components of extrachromosomal spherical bodies in pigeon oocyte nucleus

Extrachromosomal 'solid globes' and 'hollow spheres' in pigeon oocyte nucleus contain RNA but not DNA

Using confocal laser scanning microscopy, we found the dense matrix in both types of extrachromosomal spherical bodies in GVs of *C. livia* to stain brightly with NA-specific fluorescence dye Sytox green. In order to determine the nature of nucleic acids concentrated in HSs and SGs, the GV spreads and intact GVs were immunostained with a monoclonal antibody (mAb) HYB331-01 against double-stranded DNA. We observed no staining in the SGs and HSs on either spreads or the intact GVs (Fig. 3a, b). At the same time, chromomeres and axes of lateral loops of LBCs but not chromosome associated centromere protein bodies produced bright staining, serving as a positive control in the experiments (Fig. 3a, b). These data have led to conclusion that the extrachromosomal bodies in pigeon growing oocytes are not associated with DNA. Except for chromosomes, there were no fluorescent signals in other intranuclear structures. Based on the fact that SGs and HSs are intensively stained with Sytox green (Fig. 2) and do not show any staining with mAb against DNA (Fig. 3), we identified these nuclear bodies as RNA-containing organelles. This result supports our hypothesis that extrachromosomal spherical bodies are indeed the best candidates for CBs in the nucleus of late stage avian oocytes.

Extrachromosomal 'solid globes' and 'hollow spheres' in pigeon oocyte nucleus accumulate coilin

Using immunofluorescent staining of both GV spreads and manually isolated undamaged GVs with 3D-preserved architecture, we investigated the molecular composition of the RNA-containing spherical bodies in pigeon oocyte nucleus.

Coilin is known to be one of the main components of CBs (Andrade et al. 1991) and HLBs (Liu et al. 2006). As expected, extrachromosomal spheres on spreads of GVs from large oocytes of Xenopus fluoresce brightly after immunostaining with any of the polyclonal antibodies (pAb) used against coilin, namely R288 (Wu et al. 1994) and H300 (Fig. 4h). We found that extrachromosomal SGs and HSs in preparations of pigeon GV spreads also fluoresce brightly after immunostaining with the same antibodies (Fig. 4e, f). The specificity of R288 antibodies to avian coilin was demonstrated earlier by western blot analysis (Ochs et al. 1995). The specificity of pAb H300 to avian coilin was confirmed by western blot analysis of pigeon germ cell protein extracts (data not shown) and also by revealing CBs (from 1 to 3) in the interchromatin space of interphase nuclei in somatic cells of pigeon ovary with this antibody (Fig. 4g).

Immunofluorescent staining of undamaged fixed nuclei isolated from pigeon oocytes according to the method described before with either pAbs R288 or pAbs H300 confirmed accumulation of coilin in numerous extrachromosomal intranuclear bodies of different sizes (Fig. 4a–d, and Video a in Online Resource 2). SGs are not stained uniformly with Abs or fluorescent dyes and sometimes contain less bright center on fluorescent images but always have solid homogeneous matrix without vacuoles on transmitted light images. To determine whether coilin-positive bodies belong to SGs or HSs we have carefully analyzed individual transmitted light confocal images. Confocal images of GVs after scanning in transmitted light make it obvious that the bodies stained with pAbs against coilin and the bodies stained with fluorescent dye Sytox green (SGs and HSs) are identical. However, coilin was not identified in LBCs and centromere protein bodies, the results are in accordance with our previous data (Krasikova et al. 2004). There is no any specific locus of the sphere formation on pigeon lampbrush meiotic chromosomes, but sometimes the coilin-positive bodies could be found attached to the terminal regions of ZW-bivalent (Fig. 4f).

To compare the relative concentration of coilin molecules in intranuclear structures and nucleoplasm within the same GV of pigeon late-stage oocytes, we estimated the fluorescence intensity in different nuclear structures after immunostaining with pAbs against coilin. Using program LAS AF we plotted the graphics of fluorescence level and determined that SGs and HSs but not other intranuclear structures are highly enriched with protein coilin if compared with its distribution in nucleoplasm. Given that these experiments have demonstrated SGs and HSs in nuclei of pigeon growing oocytes to accumulate the protein coilin, we assumed that these structures are possible analogues of CBs in late stage oocytes of adult birds.

Number and size distribution of extrachromosomal spherical bodies in pigeon oocyte nucleus

In studies of pigeon oogenesis it was observed that larger oocytes have a bigger nucleus and there are more intranuclear bodies present in this GV. In addition to NA-containing extrachromosomal HSs and SGs, there are numerous chromosome associated centromere protein bodies in pigeon GVs (Khutinaeva et al. 1989; Solovei et al. 1996); therefore it was almost impossible to distinguish these bodies without accurate molecular markers.

In this work we have quantified and measured dimensions of NA-containing extrachromosomal HSs and SGs in 57 nuclei isolated from pigeon oocytes at different stages (starting from the stage of LBCs and ending at the stage of oocyte maturation, the latter coinciding highly condensed bivalents). Extrachromosomal bodies in the GV were identified using the fluorescent dye Sytox green (Fig. 2) or via immunofluorescent staining with antibodies against marker proteins specific for the SGs and HSs (Fig. 4a–d, and Video a in Online Resource 2). The diameters of analyzed nuclei varied from 100 to 287 μm, while the number of analyzed extrachromosomal bodies varied from 1 to 34 per nucleus (Online Resource 1). The diameter of HSs and SGs in different nuclei ranged from 5 to 28 μm, and from 1 to 10 μm respectively. SGs that do not have any vacuoles or cavities inside are usually smaller than HSs. It is important to note that there were bodies with intermediate structure: their diameter was smaller than that of HSs and they contained from 2 to 4 small vacuoles (Fig. 2c). This allowed us to suggest that during oocyte growth SGs gradually convert into HSs due to vacuolization. The appearance and gradual growth of vacuoles in HSs could be caused by accumulation of hydrophobic components in the spheres.

Interestingly, there were individuals with numerous extrachromosomal bodies (from 18 to 34) within the oocyte nucleus, and individuals with only one prominent extrachromosomal body within the GV (Fig. 4a–d). Results of quantification of HSs and SGs numbers in oocytes nuclei of different diameter from five egg-laying females grouped by individuals can be found in Online Resource 1 table. This variation of extrachromosomal sphere number in pigeon GVs might be the consequence of either female age or intraspecies polymorphism.

Molecular composition of coilin-positive spherical bodies

Extrachromosomal coilin-positive organelles in pigeon oocyte nucleus contain TMG-capped snRNAs but not splicing factor SC35

The presence of snRNPs typical for CBs in pigeon coilin-containing oocyte nuclear organelles (SGs and HSs) was

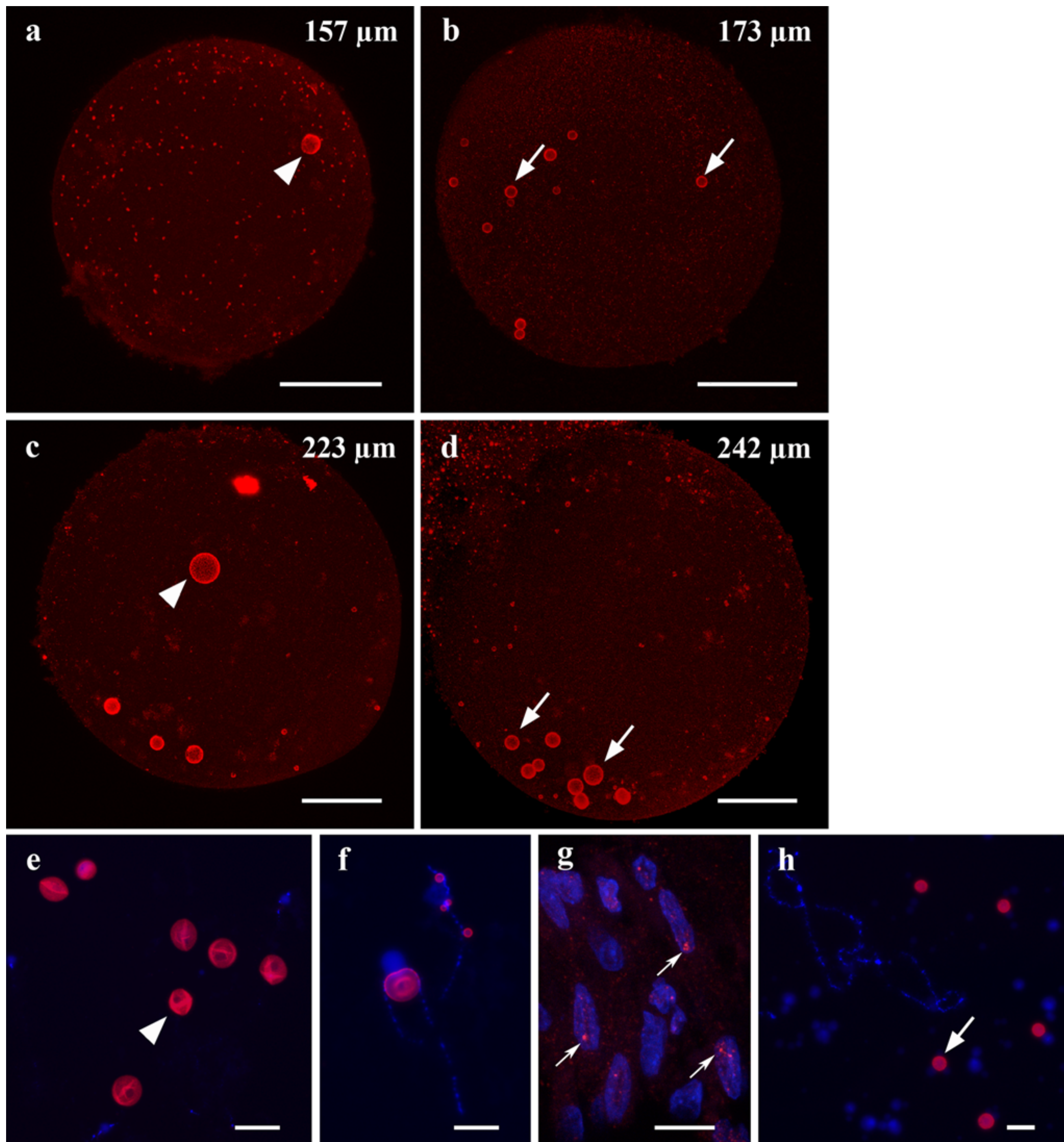

Fig. 4 Extrachromosomal nuclear bodies in pigeon large-sized oocytes accumulate protein coilin. **a–d** Distribution of protein coilin in intranuclear structures of pigeon growing oocyte. Immunofluorescent staining of intact germinal vesicles (GVs) with Abs H-300 against coilin. Images are taken with laser scanning confocal microscope. Maximal projections of *XYZ* confocal stacks of oocyte nuclei are shown. The diameter of each nucleus is indicated. In pigeon GVs coilin is accumulated in extrachromosomal solid globes (SGs) and hollow spheres (HSs). Images also demonstrate three-dimensional distribution and variation in number and diameter of extrachromosomal coilin-positive spheres in pigeon oocyte nuclei from oocytes of different individuals and stages of growth. Usually SGs (*arrows*) are a smaller size than HSs (*arrowheads*). *Scale bars* 50 µm. Related video: Video a in Online Resource 2. **e**, **f**, **h** Immunofluorescent staining of *X. laevis* (**h**) and *C. livia* (**e**, **f**) GV spreads with Abs H-300 against coilin. Arrows indicate SGs and HSs from pigeon oocytes and coilin-positive bodies from late-stage frog oocytes. *Scale bars* 10 µm. In pigeon GV spreads coilin-positive spheres can be found in association with ZW bivalent (**f**). **g** Somatic cells of pigeon ovary contain coilin-positive bodies (*arrows*) detected by immunofluorescent staining with Abs H-300 against coilin. *Scale bar* 10 µm

tested with mAb Y12 against symmetrical dimethylarginine, a secondary modification of some of the Sm proteins, and with mAb K121 against TMG cap of most snRNAs both on GV spreads and intact oocyte nuclei. We found that both types of spheres along with RNP-matrix of chromosomal simple lateral loops and terminal giant loops do react with mAb Y12 (Fig. 5d, e). Lateral loops of LBCs represent sites of active transcription where co-transcriptional events of nascent RNA processing take place. On western blots of pigeon germ cells protein extracts, mAb Y12 recognizes one band with a molecular weight corresponding to snRNP Sm B/B′ proteins (Online Resource 3e). Similar to Sm-proteins of snRNPs, TMG-capped snRNAs were localized in SGs, HSs and RNP-matrix of lateral loops (Fig. 5a, b). We also analyzed the distribution of TMG-capped snRNAs and Sm-core proteins of snRNPs in the intact pigeon oocyte nucleus and found that while nucleoplasm is stained moderately, polarized RNP-matrix of lateral loops of lampbrush bivalents fluoresces more brightly, and considerable accumulation of antigens is seen in coilin-rich SGs and the dense matrix of HSs (Fig. 5f, g, and Video b in Online Resource 2). The content of internal vacuoles of HSs does not fluoresce or fluoresces less brightly than the nucleoplasm (Fig. 5c, g). In addition to SGs and HSs there are numbers of small granules in the interchromatin space of oocyte nuclei, in which the TMG cap of snRNAs and Sm proteins can be also revealed (Fig. 5f, g). Taken together, these results demonstrate that TMG-capped snRNAs and Sm-proteins with symmetrically-dimethylated arginine are present in a dense matrix of coilin-containing nuclear bodies in pigeon late-stage oocytes.

Using double immunofluorescent staining of intact fixed oocyte nuclei with Abs against coilin and splicing factor SC35, we detected SC35 accumulation only in LBCs but not in coilin-positive organelles (SGs or HSs) (Fig. 6). Bright fluorescence of fluorochrome used for detection of splicing factor SC35 was observed on chromosomal lateral loops. Based on measurements by LAS AF Software, the lower level of the fluorescence was found in the nucleoplasm, however, the level of fluorescence in coilin-containing bodies was at least four times lower than in the nucleoplasm. The specificity of Ab αSC35 to corresponding avian protein was earlier verified using western blotting (Krasikova et al. 2004). We conclude that neither SGs nor HSs contain any noticeable levels of splicing factor SC35.

Extrachromosomal coilin-positive organelles in pigeon oocyte nucleus are not equivalents of histone locus bodies

To determine whether intranuclear coilin-positive organelles (SGs and HSs) in growing pigeon oocytes are the CBs' analogues or the equivalents of HLBs, we performed indirect immunofluorescent staining of pigeon GV contents using mAb against symplekin that react with corresponding chicken protein (Online Resource 3a). Symplekin is known to be the temperature-sensitive component of the heat-labile factor necessary for 3′-end maturation of histone pre-mRNA (Kolev and Steitz 2005; Millevoi and Vagner 2010) and is one of the noticeable components of HLBs (Sullivan et al. 2009).

After double immunofluorescent staining of preparations of GV content and intact fixed nuclei from large size oocytes, no discernible fluorescence signals from mAb against symplekin were detected within extrachromosomal coilin-positive organelles (SGs and HSs) (Fig. 7a, b). At the same time, RNP-matrix of lateral loops of LBCs contains protein symplekin (Fig. 7a, b), which is a common component of two different cleavage complexes one being required for processing of histone pre-mRNA and another one being required for processing of all polyadenylated pre-mRNA (Sullivan et al. 2009; Millevoi and Vagner 2010). This result definitely shows the lack of symplekin accumulation in both SGs and HSs. At the same time somatic cells of the pigeon ovary, used as a control in this experiment, were found to contain symplekin-rich bodies, which apparently represent functional HLBs.

Double immunofluorescent staining of small pigeon oocytes (with nucleus diameter <90 µm) from the ovary of egg-laying females with Abs against coilin and symplekin allowed us to reveal two different types of nuclear bodies (Fig. 7). There were bodies stained only with pAbs against coilin and bodies stained only with Ab against symplekin in the nuclei of small pigeon oocytes. Therefore in small oocytes, coilin-containing bodies do not accumulate symplekin and vice versa (Fig. 7d, e). However, in larger oocytes (with nucleus from 90 µm in diameter and bigger), there were no nuclear bodies stained with antibodies against symplekin, while coilin-containing bodies having the appearance of both SGs and HSs were still present (Fig. 7b, c, see also Video c in Online Resource 2). Evidently, symplekin-containing nuclear bodies in early pigeon oocytes are neither SGs nor HSs.

To check SGs or HSs from large oocytes for the presence of U7 snRNA, which is known to be specific for HLBs (Wu and Gall 1993), we used FISH with biotinylated U7 snRNA antisense oligomer. The antisense oligomer was chosen according to available data for the chicken U7 snRNA sequence (Marz et al. 2007). The pieces of somatic tissues of *C. livia* and chicken somatic cells were used as a control. After FISH we identified one or two nuclear domains concentrating U7 snRNA in somatic cells (Online Resource 3b), but there were no any detectable signals in SGs and HSs on preparations of GV contents from large oocytes. The results demonstrated that coilin-rich SGs and HSs not to be the equivalents of HLBs.

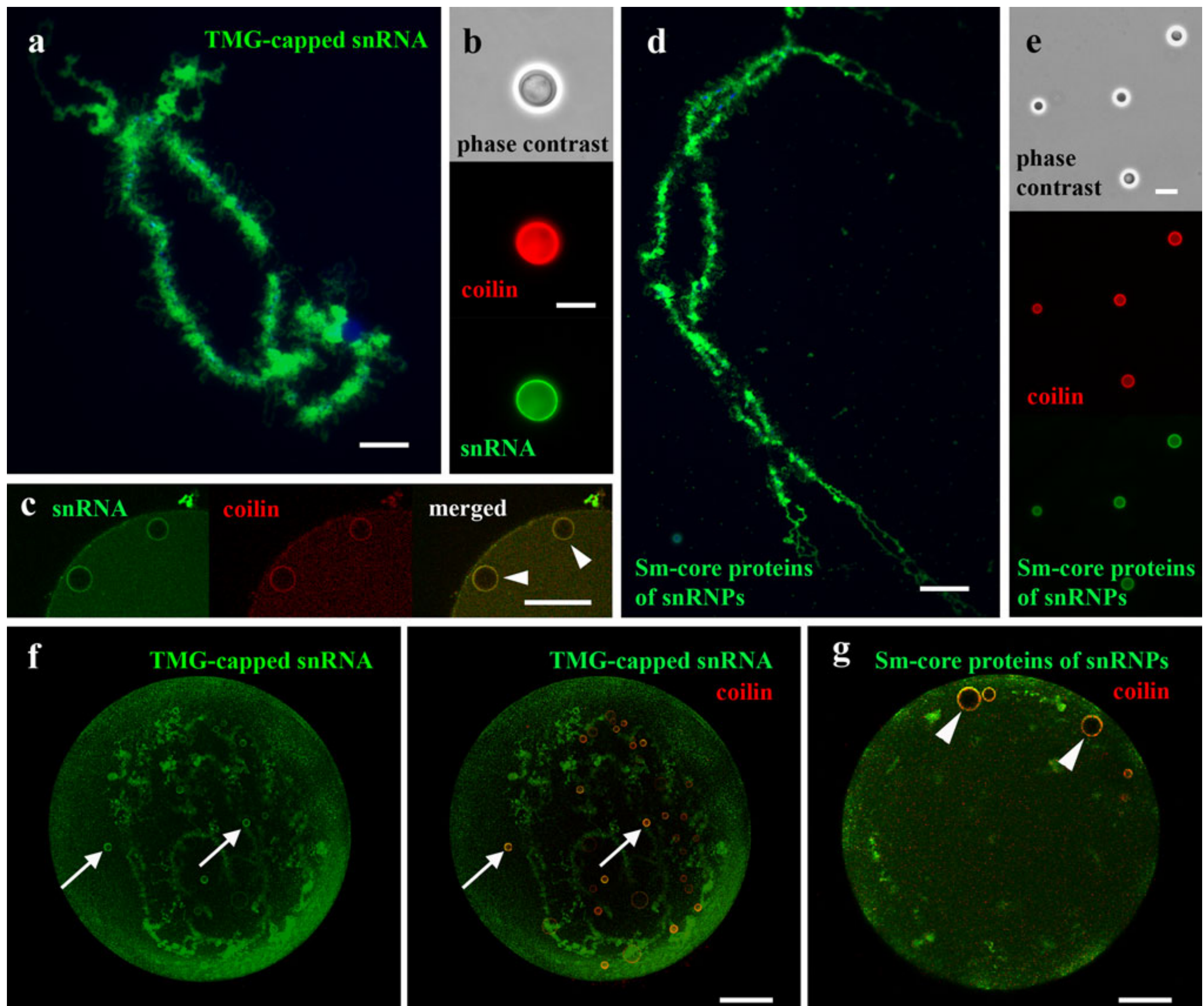

Fig. 5 Distribution of splicing factors in intranuclear structures of pigeon growing oocyte. **a**, **b** Double immunofluorescent staining of pigeon germinal vesicle (GV) spreads with Ab K121 against TMG-cap of small nuclear RNAs (*green*) and Abs H-300 against coilin (*red*). **a** Isolated lampbrush chromosome; **b** individual solid globe (SG). RNP-matrix of lampbrush chromosomes and coilin-positive SGs fluoresce brightly after staining with Ab against TMG-capped small nuclear RNAs. *Scale bars* 10 μm. Related video: Video b in Online Resource 2. **c**, **f** Double immunofluorescent staining of intact pigeon GV with the same Abs. **c** Individual *XY* confocal section. **f** Maximal projections of *XYZ* confocal stacks of oocyte nucleus. Coilin-containing bodies accumulate TMG-capped small nuclear RNAs. Arrows point to hollow spheres (HSs). Images are taken with laser scanning confocal microscope. *Scale bars* 50 μm; LSCM. **d**, **e** Immunofluorescent staining of GV spreads with Ab Y12 against Sm-core proteins of snRNPs (*green*) and Abs H-300 against coilin (*red*). RNP-matrix of lampbrush chromosomes (**d**) and coilin-positive SGs (**e**) fluoresce brightly after staining with Ab against Sm-proteins. *Scale bars* 20 μm (**b**, **d**) 10 μm (**e**). **g** Double immunofluorescent staining of intact GV with the Ab Y12 against Sm-proteins of snRNPs (*green*) and Abs H-300 against coilin (*red*). Individual XY confocal section. Coilin-containing bodies accumulate Sm-proteins of snRNPs. Arrowheads point to HSs. Images are taken with laser scanning confocal microscope. *Scale bar* 50 μm

Extrachromosomal coilin-positive organelles in pigeon oocyte nucleus do not contain nucleolar proteins

Nucleolar proteins such as Nopp140, which interacts with coilin, the main protein of snoRNP fibrillarin that is involved in early processing and methylation of pre-ribosomal RNA (rRNA) and snRNA and the main factor of assembling of ribosome subparticles protein NO38 were shown to accumulate in coilin-positive nuclear bodies of large amphibian oocytes as well as in mammalian CBs (Bellini 2000; Gall et al. 2004; Kiss et al. 2002). To find out whether coilin-positive bodies in the pigeon oocyte nucleus recruit these proteins, we carried out immunofluorescent staining of GV spreads with mAbs 17c12 or 38F3 against fibrillarin, mAb No114 against Nopp140 and mAb No185 against NO38 (Online Resource 3c). Neither SGs nor HSs

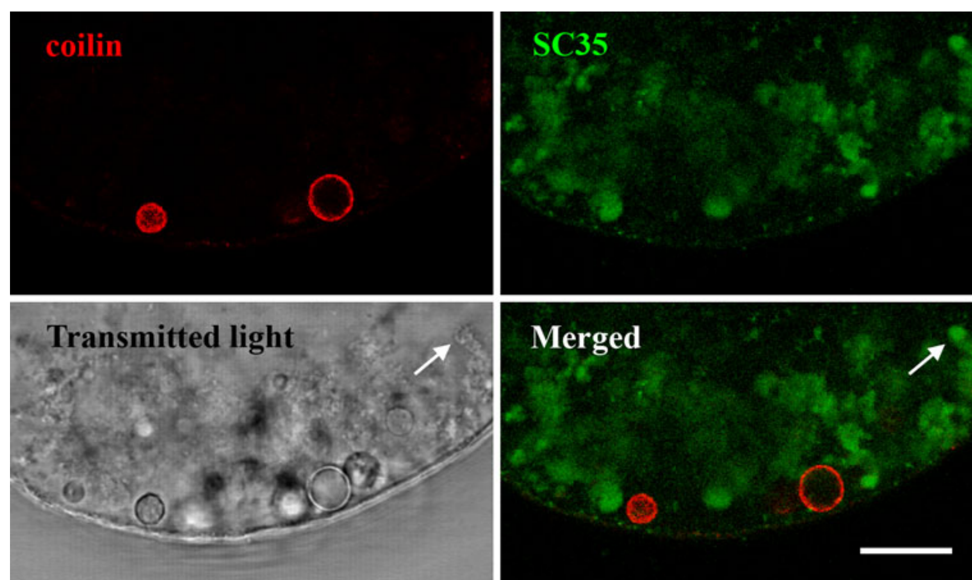

Fig. 6 Extrachromosomal nuclear bodies in late-stage pigeon oocytes do not accumulate splicing factor SC35. Double immunofluorescent staining of intact pigeon oocyte nucleus with Ab against SR-protein SC35 (*green*) and Abs H-300 against coilin (*red*). Coilin-positive bodies (SG at the *left* and HS at the *right*) do not show presence of splicing factor SC35. Example of loop stained with Ab against SC35 is shown by an *arrow*. Images are taken with laser scanning confocal microscope. *Scale bar* 20 μm

were stained with any of these Abs (Table 1). However, these Abs were earlier shown to detect nucleolar subcompartments in avian somatic cells (Online Resource 3d) (Krasikova et al. 2004). Thus, we can conclude that intranuclear extrachromosomal spheres (SGs and HSs) of pigeon growing oocyte do not contain nucleolar proteins Nopp140, fibrillarin and NO38.

Discussion

It is well established that there are certain common RNA-containing bodies in the oocyte nucleus, such as the nucleolus and interchromatin granule clusters (IGCs) (Mintz et al. 1999; Morgan 2002; Gall et al. 2004; Bogolyubov and Parfenov 2008; Bogolyubov et al. 2009). Nevertheless, growing oocytes of adult birds of the studied orders were shown to lack functional nucleoli (reviewed in Gaginskaya et al. 2009). This deficiency is suggested to be compensated by rRNA transportation from cells of follicular epithelium to the oocyte cytoplasm.

In this work we have characterized two types of nuclear extrachromosomal spherical bodies named 'hollow spheres' (HSs) and 'solid globes' (SGs), observed in late-stage oocytes of egg-laying females of the rock pigeon. It was evident that SGs and HSs could not represent active amplified nucleoli. We also established that they do not correspond to residual non-functional nucleolus substructures since they lack nucleolar proteins Nopp140, fibrillarin and NO38. Moreover, neither SGs nor HSs were shown to accumulate splicing factor SC35, which is known to be the universal marker for IGCs.

Apart from nucleoli and IGCs, CBs are also evolutionarily conserved intranuclear organelles. Importantly, the morphological connection between nucleolus and CBs was noticed when the CBs were firstly described, which was reflected in CB's original name, the "accessory body" (Gall et al. 2004; Lafarga et al. 2009). Up to now, a number of proteins have been found that belong both to nucleolus and CB such as Nopp140, GAR1, Nap57, nucleolin, NO38 and fibrillarin, as well as various basal transcriptional factors and kinases (Bellini 2000; Cioce and Lamond 2005). This was not surprising, because CBs were found to participate in snoRNP biogenesis and, what is more, small CB-specific RNPs (scaRNPs) share some proteins with snoRNPs (Matera 2006; Lemm et al. 2006; Kaiser et al. 2008; Shaw et al. 2008). However it was not known whether CBs could form at all in a transcriptionally active nucleus which lacks a functional nucleolus.

Earlier investigations directed to identify CBs in growing oocyte nucleus of adult birds revealed that there are no CBs or even any coilin-positive bodies in nucleolus-free GVs of adult females at least in three species of birds (Krasikova et al. 2004; Krasikova 2007). In contrast, this study describes extrachromosomal nuclear bodies (SGs and HSs), identified only in pigeon GVs, in which coilin is a specific component. In somatic cells of plants and animals, including chicken, coilin was shown to concentrate in distinct nuclear bodies, which correspond to CBs (Ochs et al. 1995; Tucker et al. 2001; Bogolyubov et al. 2009). Using

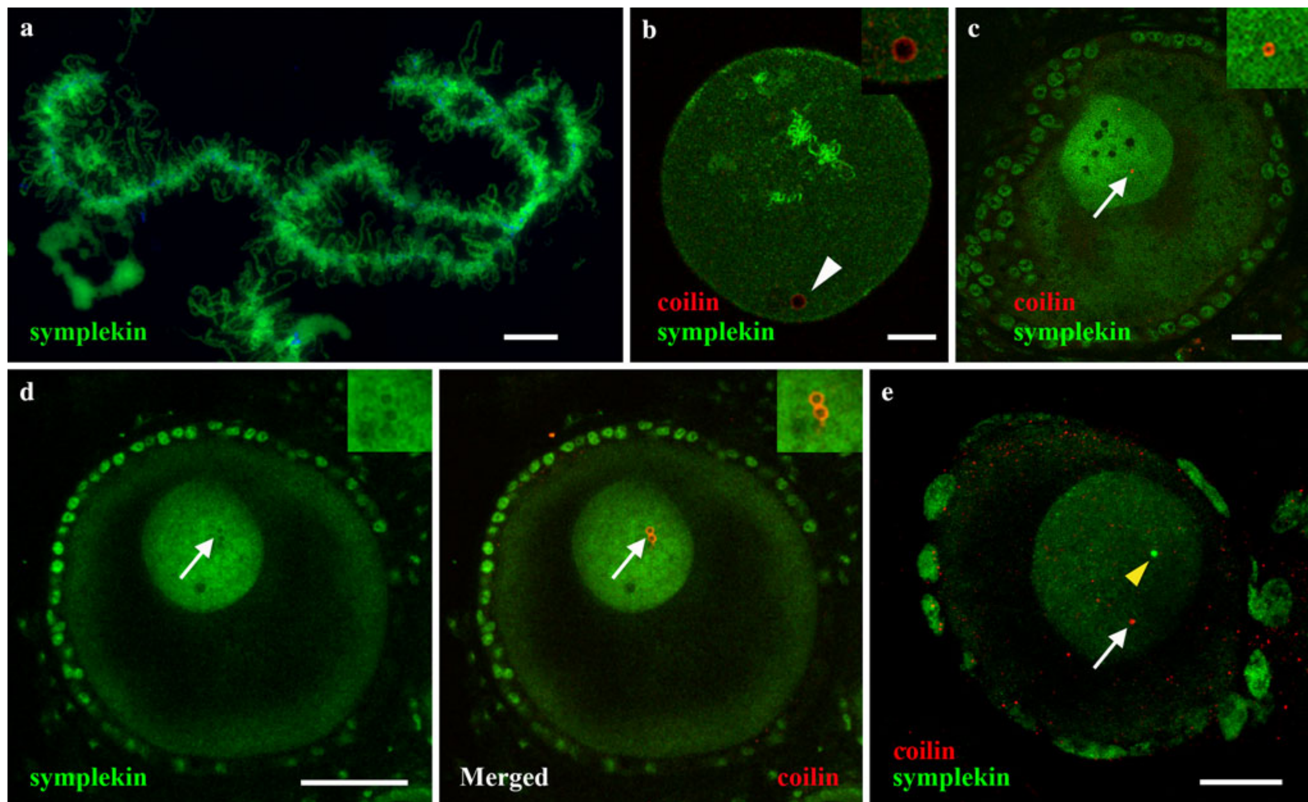

Fig. 7 Distribution of protein symplekin in subnuclear stuctures of pigeon early and late-stage oocytes. **a** Immunofluorescent staining of pigeon germinal vesicle (GV) spreads with Ab against symplekin (*green*). Symplekin is associated with RNP-matrix of lateral loops of lampbrush chromosome. *Scale bar* 100 µm. **b**–**d** In pigeon oocyte nuclei of different diameters, symplekin (*green*) does not accumulate in coilin-containing bodies (*red*). **b** Double immunofluorescent staining of intact pigeon GV with Abs against symplekin (*green*) and coilin (*red*); individual confocal section showing one hollow sphere (*arrowhead*). *Scale bar* 50 µm. Symplekin is localized in RNP-matrix of lateral loops of lampbrush chromosomes, but not in coilin-positive bodies. **c**–**d** Double immunofluorescent staining of large collagenase-treated oocytes with Abs against symplekin (*green*) and coilin (*red*); individual XY confocal sections. *Arrows* point to coilin-positive solid globes. *Scale bar* 20 µm (**c**) and 50 µm (**d**). Related video: Video c in Online Resource 2. **e** Small oocyte immunostained with Abs against symplekin (*green*) and coilin (*red*). There are distinct coilin-containing (*arrow*) and symplekin-containing (*yellow arrowhead*) bodies in small oocytes from sexually mature pigeon females. *Scale bar* 10 µm. Images (**b**–**e**) are taken with laser scanning confocal microscope. Inserts in (**b**–**d**) demonstrate enlarged images of nuclear bodies from the same oocyte sections

molecular composition analysis of the RNA-containing spheres (both HSs and SGs) in pigeon nucleolus-free GVs we found them to accumulate coilin, TMG-capped snRNAs and proteins containing symmetrically-dimethylated arginine presumably Sm-proteins of snRNPs that are typical for CBs in different species and tissues (Table 1). That has allowed us to include the coilin-containing extrachromosomal spheres of growing pigeon oocytes with a family of CBs.

In a recent study of nuclear organelles in Xenopus and Drosophila oocytes of different stages of development, coilin-rich bodies were subdivided into two major types according to their molecular composition (Nizami et al. 2010b). Namely in early-stage Xenopus and Drosophila oocytes, there are coilin-positive nuclear bodies, which are presumably the analogues of the CBs, and in late-stage oocytes, there are numerous coilin-positive nuclear bodies that have a molecular composition similar to HLBs (Nizami et al. 2010b). It was concluded that HLBs in amphibian oocytes in its turn can either be attached to transcriptionally active histone gene loci or can be extrachromosomal (Gall et al. 1981; Callan et al. 1991; Gall et al. 2004; Nizami et al. 2010b). Both types of HLBs concentrate components such as U7 snRNA (Wu and Gall 1993), stem-loop-binding protein SLBP (Abbott et al. 1999), symplekin (Hofmann et al. 2002) and FLASH (Nizami et al. 2010b). At the same time, it is still unclear whether 3′-processing of histone pre-mRNA can occur in extrachromosomal HLBs (Nizami et al. 2010b).

To determine whether coilin-positive extrachromosomal spherical bodies in pigeon late-stage oocytes (SGs and HSs) are CBs or HLBs we checked them for presence of two molecular components of HLBs. We found out that the coilin-containing spheres in pigeon oocyte nucleus do not accumulate U7 snRNA and protein symplekin (Table 1; Fig. 8). Moreover, we have recently shown that clustered

Table 1 Comparison of molecular composition of extrachromosomal coilin-containing nuclear organelles in Xenopus and pigeon late-stage oocytes

	Coilin-containing spheres in large oocytes of *Xenopus laevis*[a]	Coilin-containing solid globes and hollow spheres in large oocytes of *C. livia*
RNA	+	+
DNA	–	–
Coilin	+	+
TMG-cap of small nuclear RNAs	+	+
Sm core proteins of small nuclear RNPs	+	+
U7 small nuclear RNA	+	–
Symplekin	+	–
SC35	+/–	–
Fibrillarin	+	–
Nopp140	+	–
NO38	+	–

[a] According to the published data Wu and Gall (1993), Wu et al. (1994), Gall et al. (1999), Gall et al. (2004), Nizami et al. (2010a, b)

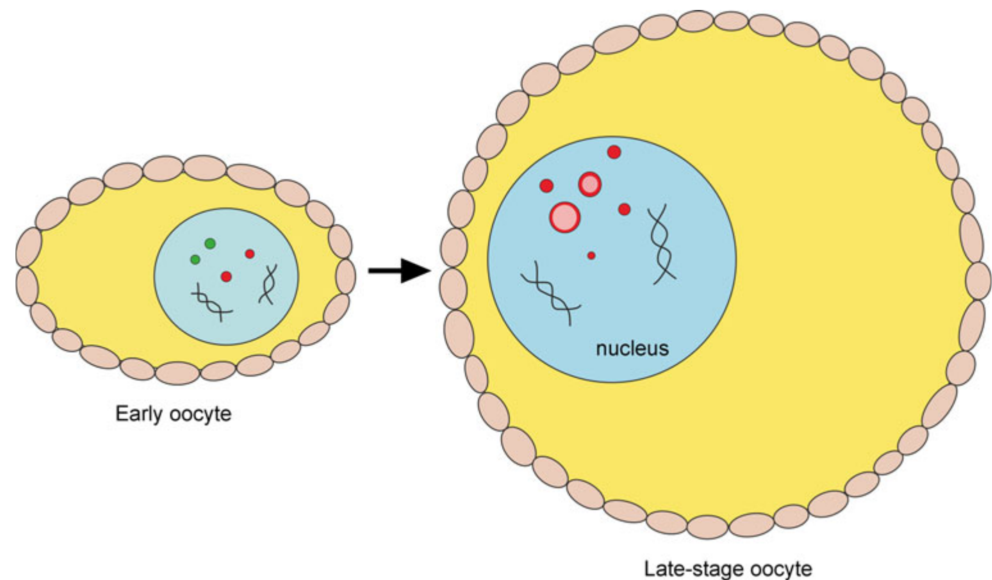

Fig. 8 Intranuclear structures in pigeon growing oocytes. Hypothetical scheme of transition from small oocytes containing coilin-positive (*red*) and symplekin-positive (*green*) nuclear organelles (presumably Cajal bodies and histone locus bodies respectively) to large oocytes housing only coilin-positive (*red*) nuclear organelles (non-canonical Cajal bodies) during oocyte growth in the ovary of egg-laying pigeon females

histone pre-mRNA genes on chicken LBCs do not transcribe, and late-stage oocytes of chicken, quail and chaffinch do not contain HLBs (A.K., unpublished observations). It was proposed that in contrast to amphibian oogenesis, avian oogenesis is generally characterized by the absence of any HLBs in late-stage oocytes. According to the results of comparative analysis of chicken and pigeon karyotypes with high probability, we can expect pigeon histone gene cluster to be situated on one of the macrochromosomes, most probably the chromosome 1. There is no any association between coilin-containing bodies and LBCs 1 or 2 according to our analysis of 3D coilin-containing bodies distribution in undamaged pigeon GVs. This is additional evidence in favor of SGs and HSs not to be analogous to HLBs in pigeon growing oocytes. However it is not known precisely whether the histone gene cluster is transcribed in late-stage pigeon oocytes. At the same time, HLBs that accumulate protein symplekin were observed in small pigeon oocytes at the early stages of development (Fig. 8).

Coilin-positive nuclear bodies in late stage pigeon oocytes seem to be of a different nature if compared with coilin-positive bodies (HLBs) in Xenopus and Drosophila late stage oocytes (Table 1). At the same time, by their morphology, coilin-rich SGs and HSs in large pigeon oocytes strongly resemble the "ring-like" or "crescent-shaped" coilin-rich bodies recently discovered in small Xenopus oocytes (Nizami et al. 2010b). The molecular composition of the latter bodies is not known in details and precise comparative analysis is currently impossible. Taken our results altogether, we conclude that SGs and HSs in GVs of pigeon belong to a group of CB-like nuclear organelles.

Thus, the situation with coilin-rich nuclear bodies' morphogenesis in pigeon ovary is not analogous but probably opposite to the situation with coilin-rich bodies' morphogenesis in Xenopus and Drosophila ovaries. In the case of pigeon oocytes, it seems that CB-like bodies initially formed in small oocytes do not disappear during oocyte growth, but instead increase in size and number (Fig. 8).

Depending on cell needs the molecular composition of nuclear bodies belonging to a family of CBs can change, which is reflected in different combinations of macromolecular complexes inside CBs. Usually, in CBs there are several sets of RNA processing factors for distinct small RNA processing pathways (molecular modules) including snRNP module, snoRNP/scaRNP module as well as certain components of telomere maintenance pathway (Lemm et al. 2006; Matera 2006; Matera and Shpargel 2006). Moreover, in plants CBs are involved in siRNA processing (Pontes and Pikaard 2008). Demonstrated differences in molecular composition of CB-like bodies in pigeon oocytes relative to CBs in somatic cells give an opportunity to address some important questions concerning CB modular structure and functions. We have found by fibrillarin immunostaining experiments that in SGs and HSs some important factors responsible for snoRNP biogenesis and traffic are not present. In pigeon late stage oocytes, the fact that nucleolar factors are absent in coilin-positive bodies likely reflects the fact that there is no need for the rRNA processing machinery to be present due to inactivation of nucleolar organizer region. This fact is an interesting example of natural confirmation of CB modular organization and variation in molecular composition of these nuclear bodies.

Although the coilin-positive organelles in pigeon GVs with inactivated nucleolar organizing region could be considered as a subtype of CBs, they have specific and unusual molecular composition (Table 1), which is atypical of any kind of CBs known so far. Moreover, the ultrastructure of the dense matrix of SGs and HSs is very different from the EM structure of canonical CBs that are known to appear as aggregates of dense coiled threads embedded in a low dense material (Cioce and Lamond 2005). Along with coilin, the bodies accumulate TMG-capped snRNAs and Sm core proteins of snRNPs, but in spite of canonical CB components, they do not contain some nucleolar proteins (Nopp140, NO38 and fibrillarin). The lack of fibrillarin, which is known to be one of the core components of C/D-snoRNPs and scaRNPs essential for guided 2′-O-methylation of Sm snRNAs (Galardi et al. 2002), in RNPs accumulated inside non-canonical CBs of pigeon GVs is puzzling. It is not clear yet whether the function of CB-like bodies in growing pigeon oocytes is consistent with certain steps of spliceosomal snRNAs modifications, since they do not recruit the protein fibrillarin. However, the high concentration of partly mature Sm snRNAs (but not U7 snRNA) in these non-canonical CBs gives us evidence that in pigeon oocytes certain steps of spliceosomal snRNAs biogenesis or recycling might occur in these nuclear structures.

Interestingly, different species of birds appear to demonstrate different strategies in coilin-rich body formation, which can be determined by a variety of conditions of oocyte development including pattern of transcribed sequences, levels of expression and modifications of particular CB components. In the case of the rock pigeon but not chicken, quail or chaffinch, the conditions of its oocyte development probably results in aggregation of coilin and other CB components into discrete and prominent bodies. It is obvious that the formation of the CBs or CB-like bodies is not obligatory in this cell type (Nizami et al. 2010b; and our unpublished observations), so we can hypothesize that their presence in pigeon GVs could be explained by self-organisation and aggregation of a limited set of participants of conservative snRNP biogenesis mechanism. In pigeon large-sized oocytes, neither SGs nor HSs have obvious chromosomal organizers and a limiting membrane. Coilin-rich nuclear bodies in pigeon oocytes form freely in the nucleoplasm and according to classification suggested by Dundr and Misteli (2010) belong to a group of activity-independent nuclear bodies in terms of the mechanism of their formation. Moreover, it is not likely that coilin-positive bodies in avian GVs require a specific template for their formation, since they form irrespectively of chromatin and other nuclear bodies such as HLBs and nucleoli. In addition, our results demonstrate that CB-like structures could form without recruitment of any notable amount of snoRNP/scaRNP protein fibrillarin.

We conclude that in contrast to transcriptionally active nuclei in late stage oocytes of Xenopus and several species of birds, nuclei in late stage oocytes of adult pigeon females contain non-canonical CBs or CB-like structures. These structures form in the absence of a nucleolus, have unusual molecular composition and may be involved into certain steps of spliceosomal snRNP biogenesis or recycling. Although to understand the biological significance of the coilin-containing nuclear bodies in *C. livia* oocyte nucleus further experiments need to be done, we have described here a specific type of CBs in growing oocytes of adult birds. Avian growing oocytes with naturally switched off nucleolar organizer regions can thus be considered as a new model to study the mechanism of CB formation and functional relevance of these structures.

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