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Nuclear actin depolymerisation in transcriptionally active avian and amphibian oocytes leads to collapse of intranuclear structures

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Actin, which is normally depleted in the nuclei of somatic cells, accumulates in high amounts in giant nuclei of amphibian oocytes. The supramolecular organization and functions of this nuclear pool of actin in growing vertebrate oocyte are controversial. Here, we investigated the role of nuclear actin in the maintenance of the spatial architecture of intranuclear structures in avian and amphibian growing oocytes. A meshwork of filamentous actin was not detected in freshly isolated or fixed oocyte nuclei of Xenopus, chicken or quail. We found that the actin meshwork inside the oocyte nucleus could be induced by phalloidin treatment. Actin polymerisation is demonstrated to be required to stabilize the specific spatial organization of nuclear structures in avian and amphibian growing oocytes. In experiments with the actin depolymerising drugs cytochalasin D and latrunculin A, we showed that disassembly of nuclear actin polymers led to chromosome condensation and their transportation to a limited space within the oocyte nucleus. Experimentally induced "collapsing" of chromosomes and nuclear bodies, together with global inhibition of transcription, strongly resembled the process of karyosphere formation during oocyte growth.

Introduction

Actin polymers are highly plastic and dynamic, and they require a significant amount of ATP for remodelling. In addition to the well-known functions of the actin cytoskeleton in the cytoplasm, substantial involvement of actin in a wide spectrum of nuclear processes has been demonstrated.[1-5] In the nucleus, actin associates with all three forms of RNA polymerase facilitating transcription,[6-8] serves as a cofactor in chromatin-remodelling processes,[9] participates in nuclear-cytoplasmic transport,[10,11] and plays a role in the long-range movement of the chromosomal loci.[12-14] In highly differentiated cells such as neuronal and germ cells, which are characterized by a very special organization of the nuclear apparatus, nuclear actin may perform yet unknown roles. Of particular interest are the nuclear functions of actin in growing oocytes.

There have been multiple studies investigating the role of nuclear actin and its dynamic changes in normal oocyte growth and maturation. Considerable enrichment of actin in giant nuclei of amphibian oocytes was established biochemically several decades ago,[15] which was confirmed using nuclear injections of actin-binding proteins and antibodies against actin,[6] as well as by transmission electron and confocal scanning microscopy of fixed oocytes.[16,17] According to the latter work, actin appears in the nucleoplasm of first-stage frog oocytes and is present in the oocyte nucleus (or germinal vesicle, GV) up until fertilisation and nuclear envelope breakdown events.[17] Only recent investigations regarding the discovery of the specific nuclear actin exporting protein exportin-6,[18] have helped unravel the mechanism underlying actin accumulation in the amphibian GV. It has been shown that exportin-6 is lacking in *Xenopus laevis* oocytes, leading to actin accumulation in the oocyte nucleus.[19]

Structures resembling F-actin filaments were found in the karyosphere capsule that surrounds condensed chromosomes in the maturing oocytes of *Rana temporaria* (the common frog).[20] The karyosphere, also called karyosome, is a characteristic feature of the first meiotic prophase or its relatively short period in most animal taxa, although its composition, structure and formation timing may vary, depending on the type of oogenesis.[21-24] Studies in neuropterans also clearly demonstrated participation of nuclear F-actin in the assembly of the karyosome capsule at the late stages of oogenesis.[25]

Although there is some supporting evidence for the importance of actin in maintaining the structural integrity and stability of the giant oocyte nucleus, the functional relevance of actin

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accumulation in the GVs appears to be wider and remains largely unknown. Furthermore, little is known about the polymerisation status of nuclear actin and dynamic equilibrium of actin forms in oocyte development. Moreover, exact molecular mechanisms that govern karyosphere formation at the late stages of oocyte growth remain unknown. The potential involvement of nuclear actin in the process of chromosome gathering during karyosphere formation needs to be specifically tested.

In the present study, we used amphibian (*Xenopus laevis*) and avian (*Gallus gallus domesticus, Coturnix coturnix japonica* and *Fringilla coelebs*) oocytes as model systems to investigate the functions of nuclear actin. Amphibian oocytes, which amass actin in their nucleus, are characterized by high levels of transcriptional activity. In growing amphibian oocytes, due to the high level of transcription, chromosomes decondense and exhibit typical lampbrush morphology during most of the oocyte growth period.[26,27]

Lampbrush chromosomes, with thousands of laterally projecting loops, are also inherent for growing oocytes of several other classes of animals, including some insects, reptiles and birds.[24-28] In this study, we investigated whether large amounts of actin are accumulated in transcriptionally active avian GVs. As opposed to amphibians, birds are warm-blooded animals with much higher rates of energy metabolism that could have considerable impact on intracellular dynamics and nuclear-cytoplasmic diffusion of macromolecules. Therefore, the investigation of actin dynamics in GVs of evolutionally distant and physiologically divergent animals is relevant.

Spatial architecture of amphibian and avian oocyte nuclei has been described in detail, for example see references 20, 29 and 30. In brief, in both avian and amphibian oocyte nuclei, one can see individual lampbrush bivalents distributed in the central part of the nucleus. In amphibians, amplification of the nucleolus organizer region (NOR) during oogenesis leads to the formation of thousands of extrachromosomal nucleoli implementing a high rate of transcription of ribosomal genes.[31] In contrast, egg-laying females of avian species studied so far do not house any functional nucleoli in the nucleus of late-stage oocytes because of inactivation of chromosomal NORs and the absence of amplified NORs.[28,32-34]

This study was undertaken to investigate the role of actin polymerisation in the maintenance of genome architecture in the giant transcriptionally active nucleus of growing oocytes in two classes of animals (amphibians and birds). We found that actin enrichment in the oocyte nucleus is evolutionary conserved among amphibians and aves. We further showed that actin depolymerisation in transcriptionally active nuclei of avian and amphibian oocytes led to dramatic changes in nuclear architecture. These data demonstrate that nuclear actin polymerisation is essential for spatial architecture maintenance of nuclear structures in avian and amphibian oocytes at the lampbrush stage. Moreover, we describe the dynamics of karyosphere formation in oocytes of Galliformes. Taken together, these findings support a model where actin plays an essential role in chromosome gathering and condensation during karyosphere formation in the nucleus of a maturing oocyte.

Results

Actin enrichment in the nuclei of avian and amphibian growing oocytes. In avian and amphibian growing oocytes, nuclear actin was visualized by different approaches including staining with fluorescently labeled phalloidin and immunofluorescent staining with specific antibodies (**Fig. 1**). Our inspection of confocal sections through the paraformaldehyde-fixed chicken and quail oocytes, stained with phalloidin-TRITC, demonstrated that the intensity of the nuclear staining was much greater than that of the surrounding cytoplasm and was comparable with the intensity of oocyte cortex staining. Thus, staining of pre-vitellogenic oocytes with an actin-specific dye has revealed an amassing of actin in avian GVs (**Fig. 1C'**) similar to that in amphibian GVs.[17]

Accumulation of nuclear actin in growing oocytes can also be demonstrated by immunostaining of fixed oocytes with the C4 antibody against actin, which is able to recognize monomeric actin species (**Fig. 1B'**). In oocyte nuclei, the distribution of the fluorescent signal after actin detection with the C4 antibody was almost the same as it was after phalloidin-TRITC staining. The apparent preferential nuclear labeling directly indicates a higher amount of actin in the oocyte nucleus when compared with the ooplasm.

Intranuclear distribution of actin revealed by phalloidin and specific antibodies. Supramolecular organization as well as intranuclear distribution of actin accumulated in amphibian and avian GVs were further examined in detail. The actin-binding capacity of phalloidin is restricted to polymeric forms of actin.[35] In whole-mount chicken, quail and Xenopus oocytes stained with phalloidin-TRITC, polymerised actin was distributed evenly throughout the nucleoplasm of the GV (**Fig. 1A' and 1C'**). This form of actin was not detected within lampbrush chromosomes and extrachromosomal bodies (the latter being typical for frog GVs) (**Fig. 1A" and 2C"**). It should be emphasized that the polymeric actin complexes, recognized by phalloidin-TRITC, did not form a network of actin fibers within nuclei of fixed oocytes that could be revealed at the confocal microscopy level. We were not able to observe even individual F-actin fibers within avian and amphibian GVs under normal conditions.

To resolve the intranuclear distribution of actin in nuclei of later avian oocytes (1–1.5 mm in diameter), we stained microsurgically isolated and fixed avian GVs with fluorescently labeled phalloidin. Quick fixation of GVs isolated in '5:1+phosphates' medium allows careful and detailed inspection of actin distribution in the intact oocyte nucleus. Analysis of confocal slice images of stained GVs confirmed our data obtained from whole oocyte examination. Namely, in chicken, quail and chaffinch GVs, polymerised actin was distributed uniformly in the nucleoplasm, and was excluded from spaces occupied by lampbrush bivalents and their laterally projecting loops (**Fig. 1D' and D"**). Despite actin involvement into transcription process, we have not detected actin complexes by phalloidin staining within transcriptionally active chromatin of lateral loops.

The absence of fibers within the GV was also evident from data on immunostaining of whole-mount amphibian oocytes with

the C4 antibody. Intense homogeneous labeling of the nucleoplasm of amphibian GVs with the C4 antibody indicated the presence of monomeric actin forms within the oocyte nucleus (**Fig. 1B'**). Thus, actin species revealed in the avian and amphibian oocyte nucleoplasm by fluorochrome-tagged phalloidin and C4 antibody were actin monomers as well as short actin polymers (oligomers) that did not assemble to form thick actin fibers under normal in vivo conditions.

Formation of an intranuclear actin meshwork. An extended F-actin meshwork in avian and amphibian GVs could be induced by supplying the incubation media with phalloidin, a drug that stabilizes actin filaments by inhibition of filament depolymerisation. Indeed, we were able to induce the formation of actin cables in the Xenopus GV by oocyte incubation in OR2 medium containing phalloidin-TRITC (**Fig. 2A–C**). This meshwork was highly branched in appearance and mostly consisted of actin cables of equal thickness, which were connected to each other by multiple side contacts (**Fig. 2C**). Some cables were associated with the nucleoli surface and other extrachromosomal bodies (**Fig. 2B and C**).

We also addressed whether a similar meshwork of actin cables could be induced in the nuclei of avian oocytes. In general, exposure of dissected unfixed chicken and quail oocytes to phalloidin, as well as microinjection of phalloidin into the ooplasm, did not induce formation of any visible nuclear actin meshwork, which is possibly due to low penetration of phalloidin through the yolky ooplasm and the short period of avian oocyte viability. Nevertheless, actin cable formation could be induced in the isolated GVs. For instance, we observed the formation of a branching actin meshwork in the nuclei isolated from the oocytes of chaffinch (order Passeriformes) after treatment with phalloidin (**Fig. 2D–F and Movie S1**). The fibers detected by fluorochrome-tagged phalloidin were spread throughout the whole nucleoplasm of chaffinch GVs. Interestingly, in phalloidin-treated chaffinch GVs, actin cables had side contacts to

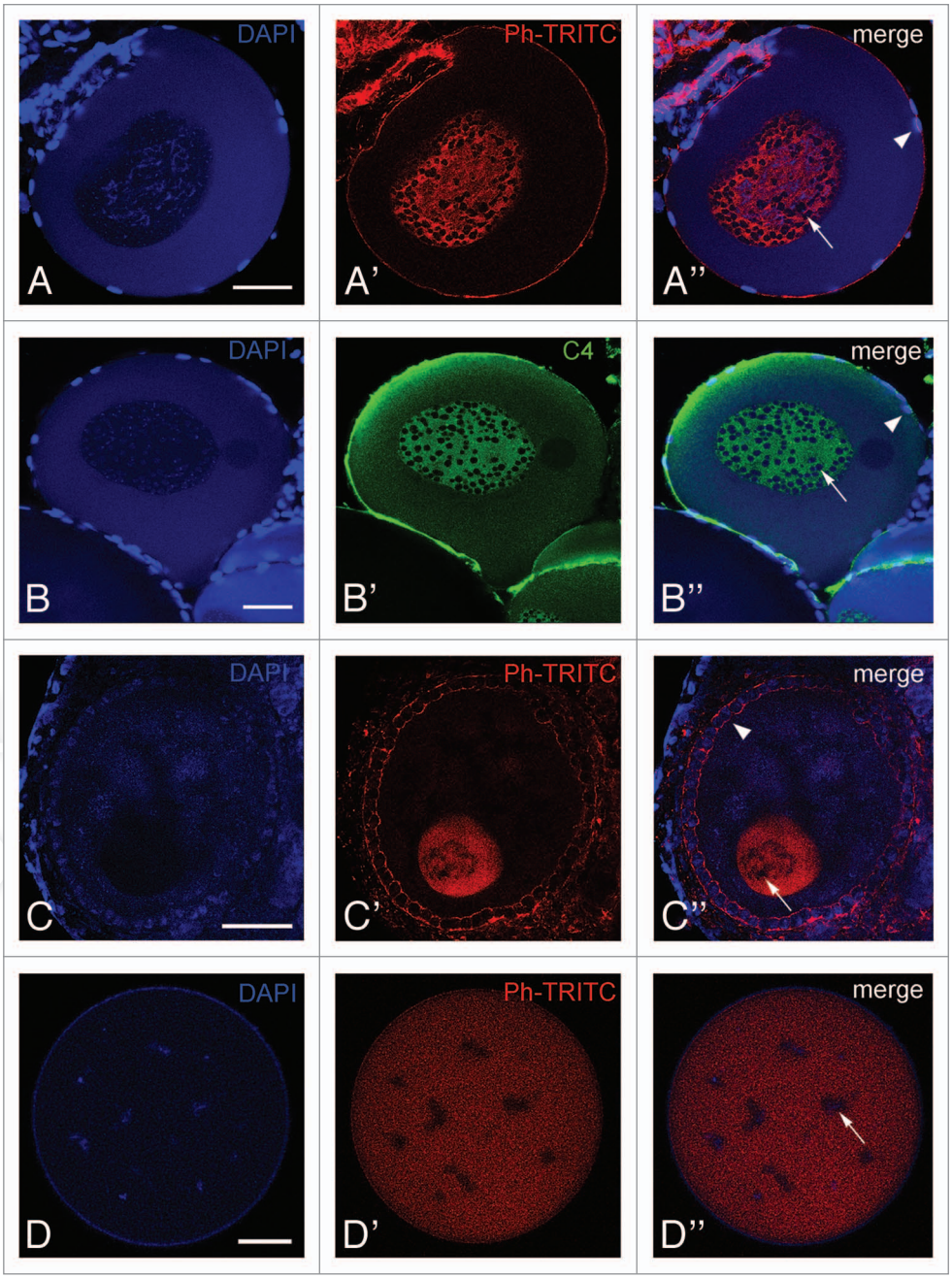

Figure 1. Distribution of actin in amphibian and avian oocyte nuclei (germinal vesicles). Rows represent single confocal sections through Xenopus (A–B″) and quail (C–C″) oocytes at the level of the nucleus and through isolated chicken germinal vesicle (D–D″) stained with actin-specific dye or antibodies. In columns there are images of DNA-counterstaining with DAPI, staining against actin and merged images respectively. Nuclear actin in oocytes is revealed by phalloidin-TRITC staining (A′, C′ and D′) as well as by C4 antibodies against actin (B′). Actin is not detected in nuclei of somatic (follicular) cells neither by phallodin-TRITC nor by C4 antibody against actin (arrowheads). Actin is much more abundant in the nucleoplasm than in the ooplasm with similar character of actin distribution both in amphibian and avian germinal vesicles (A′, C′ and D′). Note the lack of signal from actin in nuclear volumes occupied by intranuclear structures such as nucleoli (A″ and B″-arrows) and lampbrush chromosomes with their lateral loops (C″ and D″ arrows). Scale bars 50 μm.

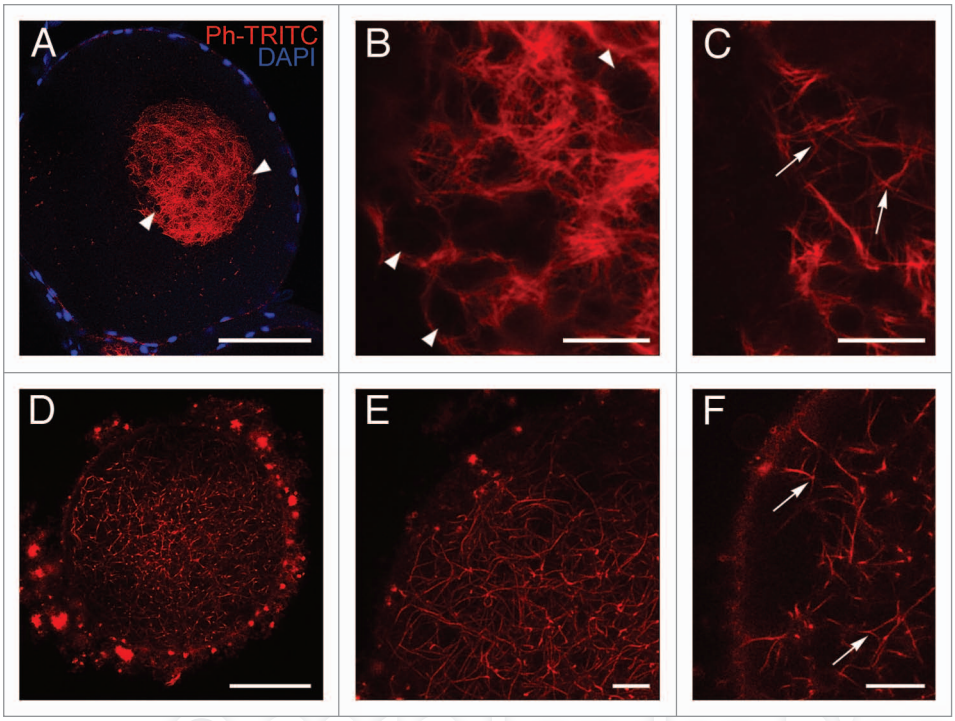

Figure 2. Phalloidin-induced meshwork of intranuclear actin cables in amphibian and avian germinal vesicles. Single confocal slices through Xenopus oocyte (A–C) or chaffinch oocyte nucleus (D–F) after incubation in the medium containing phalloidin and staining with phalloidin-TRITC. Actin filaments labeled by phalloidin-TRITC are clearly distinguishable in the oocyte nucleoplasm where they form a branching meshwork (B and E) with multiple side contacts between individual cables (C and F arrows). In Xenopus germinal vesicles actin filaments also surround or end at the surface of amplified nucleoli (arrowheads). Scale bar A and D-100 μm; B, C, E and F-10 μm.

chromosome-associated centromere protein bodies and were observed in their vacuoles. Near the nuclear envelope, actin fibers were found adjacent to the inner membrane, rather than connected to it.

The data presented here suggest a striking resemblance of a branched meshwork of actin cables formed in the avian and frog GVs upon phalloidin treatment. Moreover, in its morphology, the nucleoplasmic meshwork of branched cables was very similar, if not identical, to the one experimentally induced in the newt.[16] The cables appeared to have equal thickness with a roughly measured diameter of about 0.3 μm.

Cytochalasin D- and latrunculin A-induced collapse of nuclear structures. It has been repeatedly suggested that the reason for actin accumulation in the amphibian oocyte nucleus is to provide mechanical support for the giant GV.[16,19,36] However, there was no direct evidence for a relationship between the polymerisation of actin and maintenance of the 3D-architecture of intranuclear structures within the oocyte nucleus. In a further series of experiments, we investigated the consequences of the global inhibition of actin polymerisation on the spatial organization of chromosomes and nuclear bodies in amphibian and avian intact GVs at the lampbrush stage of oogenesis. Cytochalasin D (CD), a drug that interferes with actin polymerisation by capping the barbed end of actin filaments,[37] was used to inhibit nuclear actin polymerisation.

In an amphibian model system, CD treatment was performed by incubation of Xenopus oocytes in CD-containing OR2 medium. Nuclear structures were revealed by either DAPI whole-mount staining of fixed oocytes or DAPI and Sytox Green staining of microsurgically isolated GVs. Under such conditions, the effect of oocyte exposure to 1 μM CD on the relative positions of intranuclear structures could be observed in isolated GVs within 2 h after starting the incubation. In contrast to nuclei of control Xenopus oocytes, where thousands of amplified extrachromosomal nucleoli were located at the nuclear periphery, in nuclei from CD-treated oocytes nucleoli had changed their position (**Fig. 3A and A'**). One of the initial signs of a disrupted topography of nuclear structures in isolated frog GVs was the detection of the crowding of nucleoli and other bodies, which usually occurred in one or several centers near the nuclear envelope, although a large number of nuclear bodies seemed to exhibit normal topography.

After a longer incubation time, global rearrangements of nuclear bodies within the GV occurred. After 10 h of incubation, crowded structures were found to aggregate with each other, forming large masses of fused bodies in the nuclear volume of CD-treated oocytes (**Fig. 3A"**). The process of fusion of intranuclear bodies was coupled with gradual loss of transcriptional activity in the frog GV. Indirect evidence for global inactivation of transcription was indicated by the altered staining of extrachromosomal nucleoli with Sytox Green. In untreated GVs amplified nucleoli were completely stained with Sytox Green, while in the GVs isolated from CD-treated oocytes the nucleoli appeared to be vacuolated entities. In addition, dramatic changes in the morphology of lampbrush chromosomes incompatible with active transcription were also detected in amphibian GVs (**Fig. 3A' and A"**). Condensed chromosomes were seen frequently in intimate association with each other or fused nuclear bodies (**Movie S2**). In Xenopus, progressive converging of nuclear bodies and chromosomes led to their collapse and formation of a compact irregularly shaped vacuolated karyosphere-like structure at the periphery of the oocyte nucleus (**Fig. 3A"' and Movie S3**). The speed with which nuclear structures lost their normal topography depended on the CD concentration in the incubation medium. A 2-fold increase in CD concentration in the oocyte incubation medium accelerated the collapse of nuclear bodies and chromosomes.

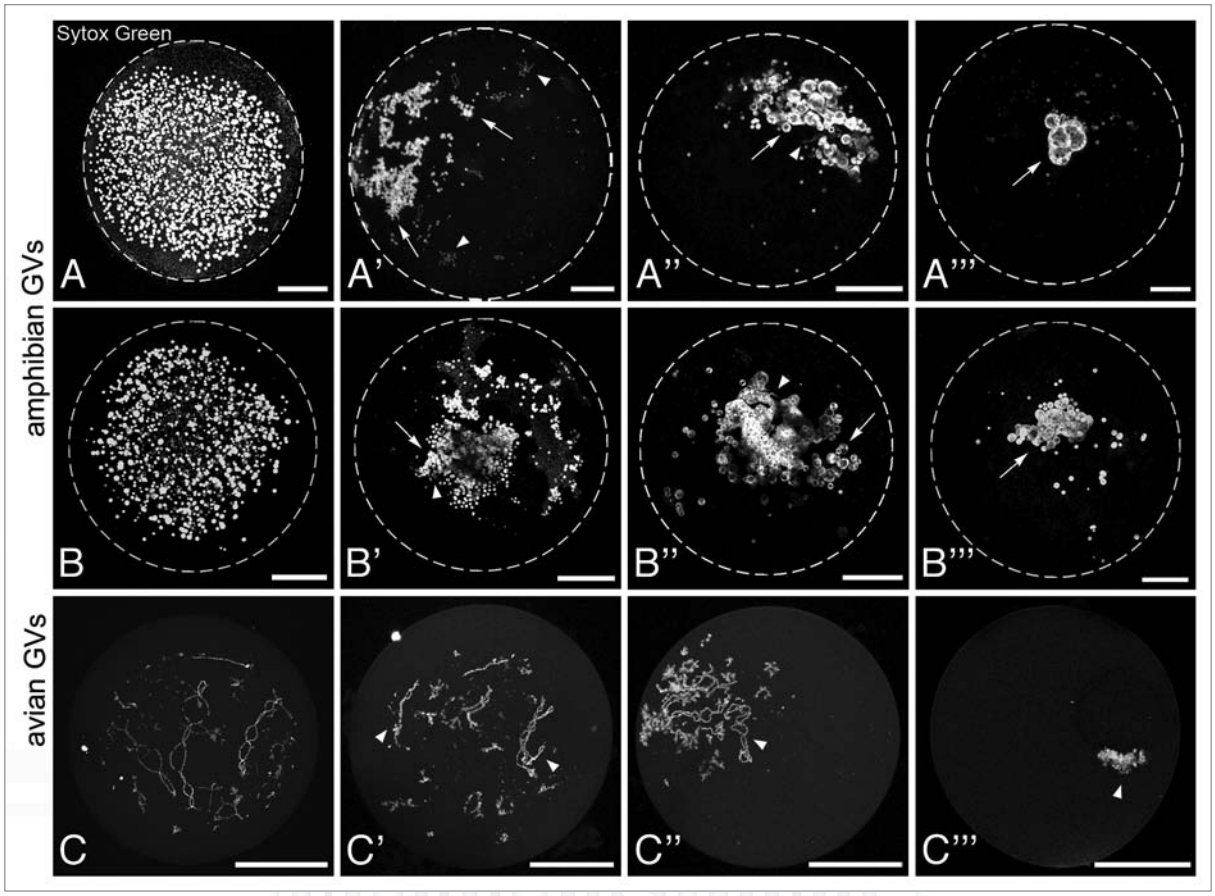

Figure 3. Progressive collapse of nuclear structures in oocytes after treatment with cytochalasin D (CD) or latrunculin A (LA). Step by step loss of normal for that stage of oocyte growth spatial topography of intranuclear structures after CD (A–A‴, C–C‴) or LA (B–B‴) treatment is shown for Xenopus (upper parts) and chicken (lower part) oocyte nuclei, stained with Sytox Green. Maximum projections of Xenopus and chicken nuclei isolated from control untreated oocytes are presented at (A–C). Initial changes are evident with condensation of lampbrush bivalents (A′–C′-arrowheads) and in addition with crowding of amplified nucleoli in frog germinal vesicles (A′ and B′ arrows). Further gathering of chromosomes (A″–C″ and C‴ arrowheads) and fusions of nucleoli (A″ and A‴, B″ and B‴ arrows) after prolonged CD or LA treatment led to collapse of nuclear structures to a compact irregularly-shaped mass (A‴–C‴). In (C″ and C‴) confocal slices through the oocyte nuclei at the level of entangled chromosomes are shown. Scale bars 100 μm.

Similar changes in nuclear architecture were observed in Xenopus oocytes after treatment with 0.5 μg/ml latrunculin A (LA), another actin-depolymerising agent (**Fig. 3B–B‴**). LA treatment led to more rapid collapse of lampbrush chromosomes and nuclear organelles within frog GVs. Thus two different inhibitors of actin polymerisation with different mechanisms of action have similar effect on spatial genome architecture in frog GVs.

To investigate changes in nuclear actin supramolecular organization in oocytes exposed to an inhibitor of actin polymerisation, we performed whole-mount staining of CD-treated oocytes with phalloidin-TRITC. As expected, the intense labeling of the entire nucleoplasm by phalloidin-TRITC was not observed in GVs after CD treatment. At the same time, rare aggregates of polymerised actin were observed near fused nuclear bodies in frog GVs even after 12 h of treatment with actin-depolymerising drugs (**Fig. 4B**). These intranuclear, brightly stained actin aggregates could represent residual capped or bundled forms of actin. These remarkable actin aggregates were not detected in the GVs of Xenopus oocytes immunostained with the C4 antibody against actin. Conversely, the overall nuclear pattern of C4 staining, did not change in the oocytes exposed to CD compared with control oocytes (**Fig. 4D and E**). Nuclear actin, recognized by the C4 antibody, was also present within cavities and vacuoles of fused nuclear bodies in frog GVs.

In avian GVs, isolated from oocytes microinjected with CD, more obvious and rapid loss of typical lampbrush morphology was observed. Initial signs of this event included retraction of lateral loops, possibly due to inhibition of transcription (**Fig. 3C**'). As in case of Xenopus GVs, in avian GVs we also observed condensation of lampbrush bivalents within 1 h after injection of CD. In addition, the normal positions of individual bivalents in the nucleoplasm of both chicken and quail oocytes were altered. Analysis of confocal slices and 3D-reconstructions of isolated nuclei showed that some of the remaining distinguishable bivalents were located in close proximity to the nuclear envelope, while others were randomly distributed in the limited nucleoplasmic volume (**Fig. 3C**"). Increasing the incubation time or concentration of CD injected into oocytes resulted in rapid collapsing of all macro- and microchromosomes in avian GVs, with

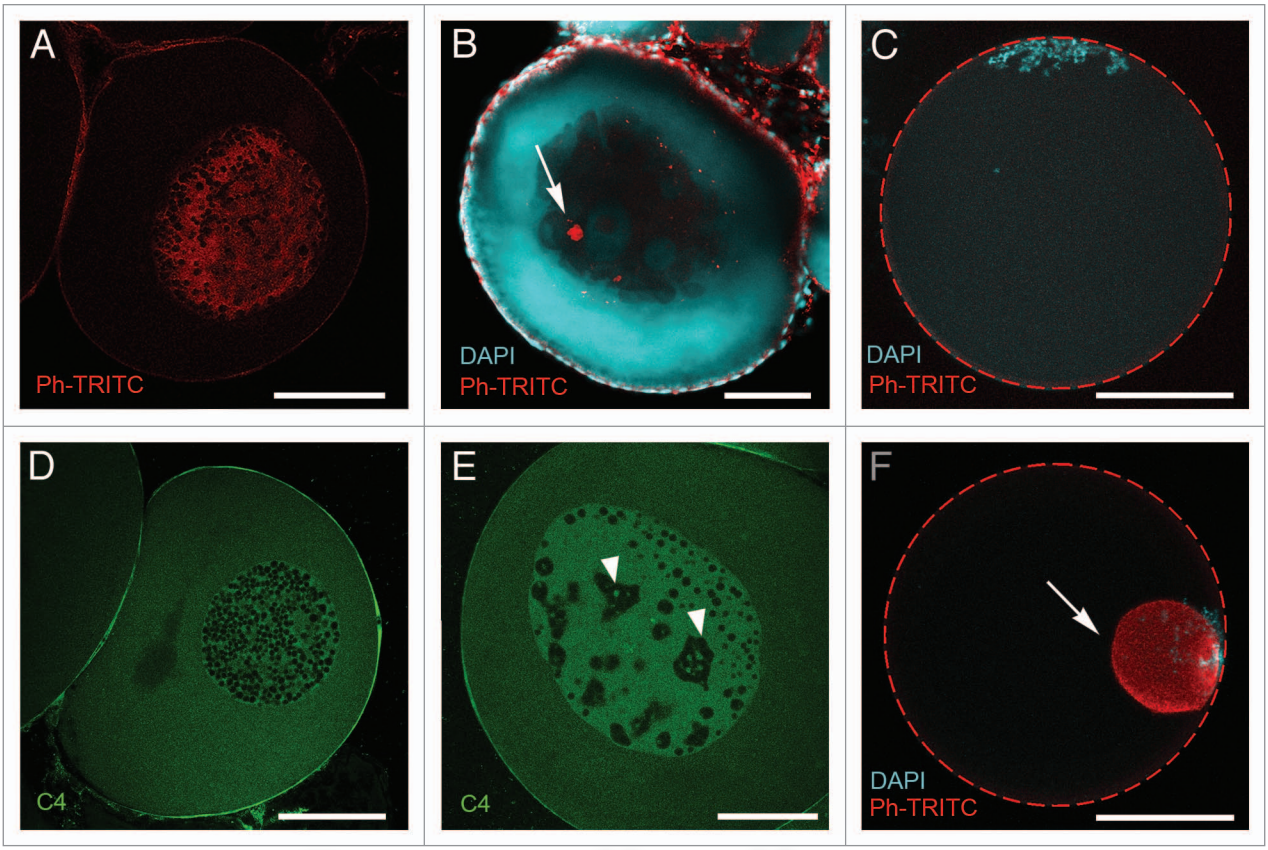

Figure 4. Distribution of actin in amphibian and avian oocyte nuclei after exposure to cytochalasin D (CD). Single confocal slices through control (A and D) and CD-treated (B and E) Xenopus oocytes stained with phalloidin-TRITC (A and B) or antibody against actin (D and E). Note disappearance of homogeneous nucleoplasmic staining in the nucleus of CD-treated oocyte (B), indicating almost total absence of actin polymers, and a few brightly stained aggregations of polymerised actin (B-arrow) in the nucleoplasm. Nuclear actin recognized by C4 antibody is still abundant in the oocyte nucleus after exposure to CD (E). Note the presence of actin in vacuoles of fused nucleoli within Xenopus germinal vesicles (E arrowheads). Single confocal sections at the level of entangled chromosomes through chicken germinal vesicles after exposure to CD (C and F). Isolated chicken germinal vesicles are stained with phalloidin-TRITC and counterstained with DAPI. Polymeric actin is either completely absent in isolated nucleus (C) or is confined to a small volume of nucleoplasm within the germinal vesicle (F). Scale bars 100 μm.

entangled bivalents found near the nuclear envelope or at the nuclear periphery (**Fig. 3C‴ and Movie S4**). Separate bivalents were not observed in such intact oocyte nuclei. In some nuclei isolated from the chicken oocytes within 1 h after injection, we also observed a spherical volume of congressed nucleoplasm, which appeared to be denser than the surrounding nucleoplasm. The general absence of polymerised actin in a contracted nucleoplasm of GVs isolated from CD-microinjected avian oocytes was confirmed by phalloidin staining (**Fig. 4C and F**). However, phalloidin-positive actin clusters were rarely found in the isolated avian GVs (**Fig. 4F**). Thus, inhibition of transcription at lampbrush chromosomes, their condensation, and gathering induced by CD argue for the importance of the ongoing process of nuclear actin polymerisation in both avian and amphibian growing oocytes.

Changes in genome architecture during karyosphere formation. Substantial changes in the distribution of intranuclear structures within GVs naturally occur during oocyte maturation accompanied by yolk accumulation (**Fig. 5**). In chicken and quail oocytes, transcriptionally active lampbrush chromosomes occupy a large part of the nucleus (**Fig. 5A and E**). At the stage of functional lampbrush chromosomes, the nucleoplasmic area, occupied by chromosomes, grows in proportion to the increasing nuclear size, and reaches a maximum. When RNA synthesis on the lateral loops of lampbrush chromosomes ceases and bivalents progressively condense, the nucleoplasmic zone occupied by chromosomes gradually decreases (**Fig. 5B and F**). Condensed chromosomes approach each other to form a karyosphere and are finally found in a very limited nuclear space (**Fig. 5C and G**). Uniform distribution of actin complexes revealed by phalloidin was observed in the nucleoplasm of nuclei obtained from the germinal discs of untreated yellow yolk oocytes. Unstained spherical areas seen in confocal optical slices corresponded to condensed post-lampbrush chromosomes (**Fig. 6** and compare with **1D'**). Nuclei isolated from large growth stage oocytes were more elastic than the nuclei from germinal discs, the latter being easily deformed during isolation. Nucleoplasmic fibers were not detected in the nuclei isolated from large growth-stage oocytes or in nuclei isolated from rapid growth-stage oocytes.

Considering the strong similarities in processes of naturally occurring karyosphere formation and experimentally induced

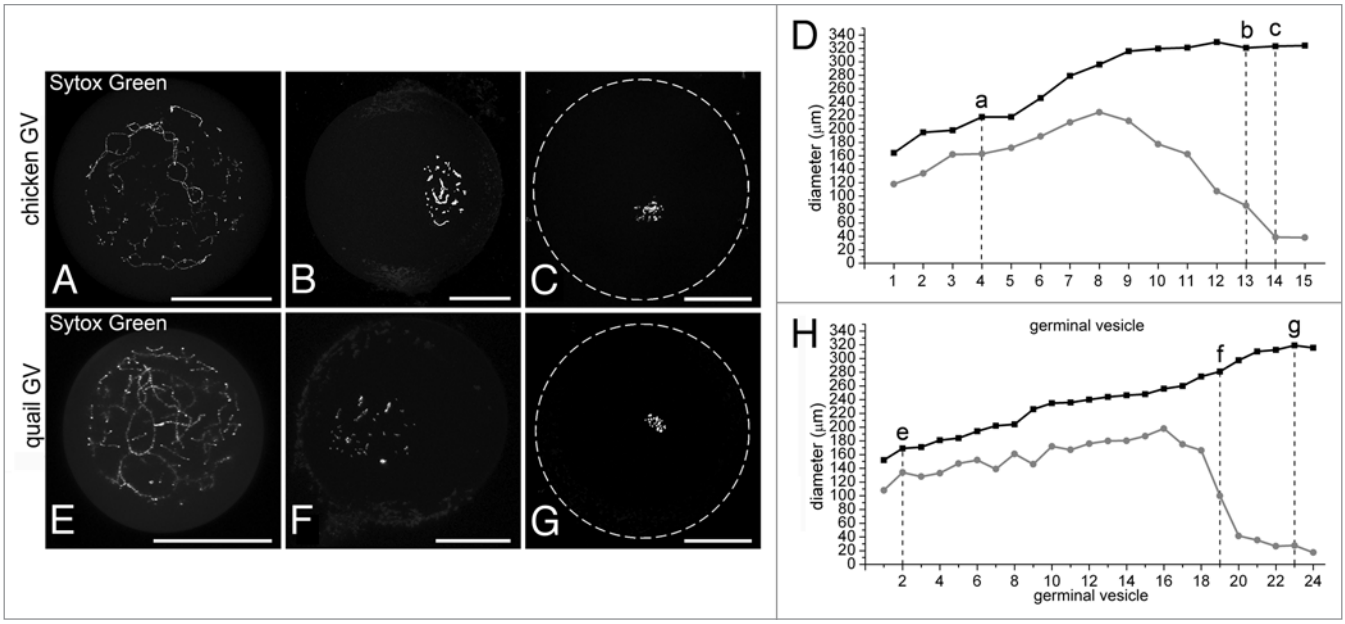

Figure 5. Consequent stages of chromosome condensation in avian oocytes during the period of large growth. Maximum projections of chicken (A–C) and quail (E–G) nuclei isolated from oocytes and stained with Sytox Green. Three representative germinal vesicles with different degree of chromosome condensation are shown for each species. Partially decondensed and transcriptionally active meiotic bivalents exhibiting lampbrush appearance are seen to occupy the large part of the nucleus (A and E). Gradual inactivation of transcription at later stages of oocyte growth is accompanied by chromosome condensation and transportation to a smaller volume of the nucleoplasm (B and F). Highly condensed chromosomes, located in a limited nuclear space, form a karyosphere (C and G). Scale bars 100 μm. Curves represent the changes in the diameter of nuclear volume, occupied by chromosomes (gray curves), and the diameter of the nucleus (black curves) for chicken (D) and quail (H) oocytes during large growth stages. Nuclei shown on (A–C) and (E–G) are indicated by vertical dotted lines.

gathering of nuclear structures inside the intact oocyte nucleus, we propose that dynamic changes in supramolecular organization of nuclear actin may drive directed transportation of chromosomes and nuclear bodies during oocyte growth.

Discussion

Actin participates in a variety of nuclear processes. Nevertheless, its amount in the interphase nucleus of somatic cells required for regular functioning of the genome is relatively low. However, in oocytes of certain animals such as Xenopus, actin accumulates in the nucleoplasm in high amounts due to inactivation of the actin-exporting process.[19] We have demonstrated that actin is accumulated in transcriptionally active giant nuclei of avian oocytes similar to that in Xenopus oocytes. The mechanism of actin accumulation in avian GVs has remained unexplored likely being the same as in amphibian oocyte nuclei. The enrichment of actin within the nucleus (up to 2 mg/mL) and its ability to bind phalloidin could be considered to be a distinguishing feature of avian and amphibian growing oocytes and oocytes of other animals with the hypertranscriptional type of oogenesis.[38]

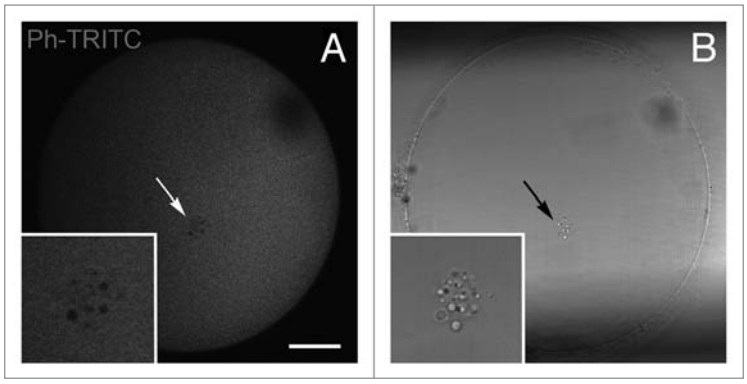

Figure 6. Detection of polymerized actin in quail oocyte nucleus at the postlampbrush stage. Single confocal section through isolated quail germinal vesicle stained with phalloidin-TRITC (A) at the level of karyosphere-like structure (arrow) and corresponding transmitted light image (B). Polymeric actin detected by phalloidin-TRITC distributes uniformly throughout the nucleoplasm excluding volumes occupied by condensed spherical chromosomes (insert). Scale bar 50 μm.

We demonstrated the presence of two major forms of actin within avian oocyte nucleus, which is similar to results previously reported for amphibian GVs. It should be emphasized that polymeric actin detected in oocyte nuclei of the species under investigation is represented predominantly by short actin polymers (oligomers), since we did not detect long actin filaments in the intact GVs. Oligomeric actin species in avian and amphibian GVs have the potential to polymerise further, and associate with each other in certain experimental conditions. We found that

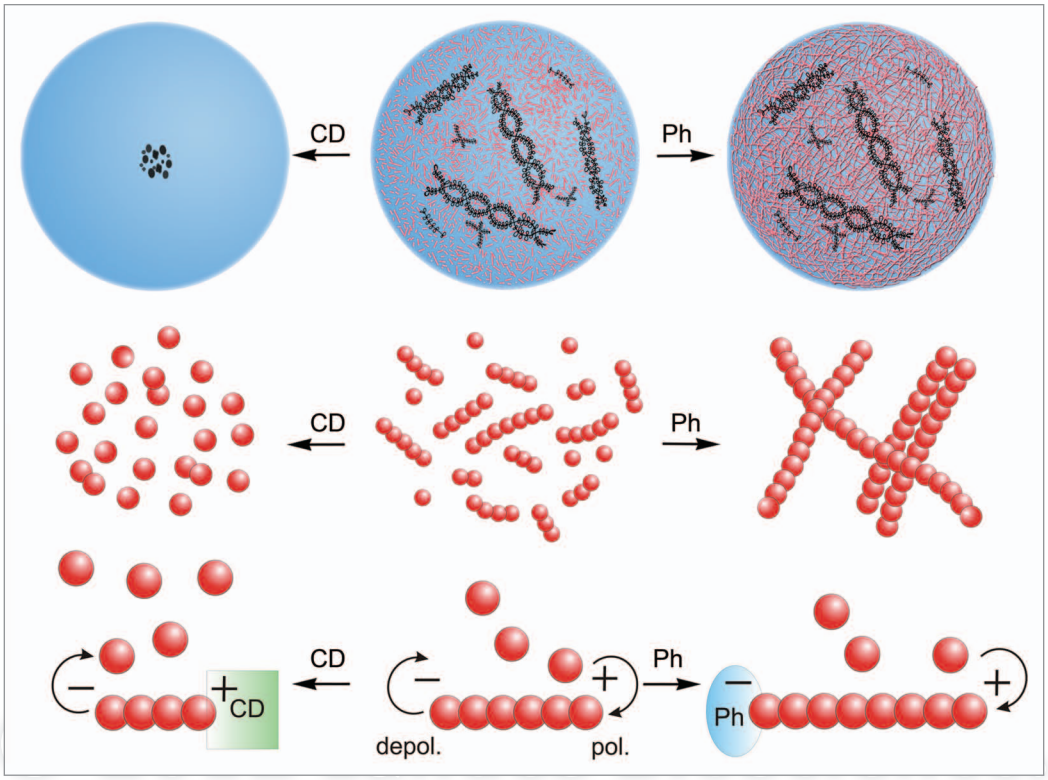

Figure 7. The scheme of changes in nuclear architecture occurring in avian and amphibian oocytes (upper row) under experimental stabilization of actin filaments or inhibition of actin polymerisation (middle and bottom rows). In growing avian and amphibian oocytes, transcriptionally active lampbrush chromosomes and nuclear bodies are radially distributed throughout the nuclear space (central column). According to the described results, in the nucleoplasm of these germinal vesicles, actin exists in monomeric and olygomeric forms. High dynamics of actin polymer turnover in the nucleoplasm limits the length of actin polymers and their ability to form filaments. The inhibition of actin depolymerisation by phalloidin (Ph) results in the artificial formation of the branched meshwork of F-actin cables within the oocyte nucleus (right column). The inhibition of actin polymerisation in the oocyte nucleus by Cytochalasin D (CD) leads to the irreversible collapse of nuclear structures, chromosome condensation and their gathering in a limited nuclear space to a karyosphere-like structure (left column). Similar changes in spatial genome architecture were induced by another actin-depolymerising drug latrunculin A that sequesters G-actin monomers.

in amphibian and avian GVs, the actin-stabilizing drug phalloidin initiates the formation of thick cables that form a branched 3D-meshwork, which stained positive with phalloidin-TRITC. In contrast, oocyte treatment with the actin-depolymerising drug CD led to the disappearance of detectable F-actin within the nucleus. Taken together, these data indicate that within the giant oocyte nucleus of frogs and birds, actin molecules are in a dynamic equilibrium between monomeric and polymeric forms (**Fig. 7**). Moreover, these results imply that the process of actin polymers formation and disassembly in avian and amphibian GVs is highly dynamic.

Currently, there are two central hypothesis concerning actin supramolecular organization in amphibian transcriptionally active oocyte nucleus. One hypothesis considers actin to be involved in the formation of a rigid nucleoskeletal meshwork needed for mechanical support of the giant GV structure. This hypothesis is based on investigations of the F-actin form and fibrillar 'nucleoskeleton' structures within the amphibian oocyte nucleus. For example, the nuclear meshwork of thick actin filaments was revealed by confocal laser scanning microscopy of sections through frozen Xenopus oocytes stained with anti-actin antibodies and fluorescently labeled phalloidin.[19] In addition, the complex and dense meshwork of actin-containing fibers protruding from nuclear pores to the nuclear interior was revealed by scanning electron microscopy of nuclear content, obtained from isolated Xenopus GVs.[39] Recently, by using new strategies for the direct visualization of F-actin, the Gurdon group obtained additional evidences in favor of the existence of an F-actin meshwork inside the Xenopus oocyte nucleus.[40] However, taking into consideration other observations, some precautions should be considered when interpreting the data on the amount of filamentous actin and the existence of a fibrillar nucleoskeleton within the GV in vivo.

A second hypothesis generally states that in amphibian oocytes actin does not form a rigid nucleoskeletal meshwork and that the abundance of nuclear actin leads to the formation of a stable actin meshwork during manipulation of oocytes and isolated GVs. F-actin filaments were shown to be a structural component of the solidifying nuclear gel formed in amphibian GVs after exposure to saline.[15,41] Furthermore, Gounon and Karsenti[16] have demonstrated that the nuclear content of GVs isolated from newt (*Pleurodeles waltl*) oocytes, and its filamentous fraction, in particular, could be reversibly changed by varying the concentration of divalent cations in the isolation

medium.[16] Importantly, freshly isolated newt GVs lacked filamentous structures. The depletion of Ca^{2+} in the isolation medium promoted the formation of filaments, while incubation of isolated nuclei with phalloidin resulted in the formation of thick cables consisting of filament bundles.[16] It was also demonstrated that the actin meshwork formed upon drug-induced inactivation of transcription on lampbrush chromosomes of *Pleurodeles*.[6] Thus, it cannot be ruled out that at least some of the filaments seen in the preparations of oocyte nucleus material arises from rapid polymerisation of nuclear actin, resembling the gelation of the nuclear content of GVs isolated in buffer. In Xenopus, the formation of nuclear gel occurs in several seconds when the nucleus is isolated from oocytes in saline.[42] However, isolation in mineral oil results in a more 'fluid' GV, which can be easily deformed.[43] Thus, it is unlikely that the polymerised fraction of nuclear actin forms a rigid meshwork in the Xenopus oocyte nucleus.[42] Our results provide evidence in favor of the second hypothesis demonstrating that, in the nuclei of birds and frogs, polymerised actin in growing oocytes is represented mainly by short polymeric forms, but not by a rigid meshwork of F-actin cables.

In the oocyte nucleus, actin polymers can be recruited to processes other than mechanical support of GV shape. Our experiments demonstrated that in oocytes of frogs and birds, nuclear actin is involved in establishing and maintaining the spatial arrangement of the intranuclear structures. After treatment of amphibian and avian growing oocytes with actin-depolymerising agents, the loss of normal nuclear architecture correlated with the disappearance of the nuclear fraction of actin recognized by tagged phalloidin (**Fig.** 7). As the most prominent effect of CD on actin is inhibition of its polymerisation,[44] we concluded that interference with this process is the main cause of distortions in the 3D architecture of the intranuclear structures. Moreover, the effect of LA, that binds to monomeric G-actin,[45] on oocyte genome architecture was similar to that of CD. Previously, LA treatment was shown to disrupt the cytoplasm and nucleoplasm of Xenopus oocytes but no data on changes in spatial organization of intranuclear structures was presented.[46]

One common principle of altered distribution of nuclear bodies within the GVs of birds and Xenopus induced by inhibition of actin polymerisation were aggregations and fusions of nuclear structures that were accompanied by the loss of their activity. This phenomenon, especially in its final stages, was reminiscent of the process of karyosphere formation, which takes place in the late prophase I of oogenesis. Spatial proximity of chromosomes in the voluminous nucleus established at late prophase is supposed to be inevitable for meiotic progression.[47] However, the molecular mechanisms responsible for bringing chromosomes together in a large nucleus, such as avian or amphibian GVs, are still unknown.

Our results suggest that in avian and amphibian oocytes chromosome gathering during karyosphere formation could involve an intranuclear actin depolymerisation step. In avian and amphibian maturing oocytes, we observed karyospheres at the nuclear periphery, which suggests asymmetrical transport of chromosomes in the earlier stages. Similar characteristics of chromosome transportation were reported for starfish oocytes, in which chromosome congression began a few minutes after nuclear envelope breakdown and was proposed to be driven by cytoplasmic actin and microtubules.[48] However, avian and amphibian oocytes differ from starfish oocytes in terms of karyosphere formation that occurs prior to nuclear envelope breakdown. Moreover, our results on phalloidin staining of isolated avian GVs at the karyosphere stage argue against the presence of any F-actin cables in the nucleoplasm or adjacent to karyospheres. We found that in intact GVs, the actin network can be induced by inhibition of actin depolymerisation even in small, and middle-sized oocytes, in which chromosomes and nuclear bodies are distributed throughout the nuclear volume, and do not change their position after phalloidin treatment (**Fig.** 7). Thus, the appearance of an actin filament meshwork does not correlate with karyosphere formation.

Strikingly, GVs with intact nuclear envelope membranes from earlier avian and amphibian oocytes containing elongated transcriptionally active chromosomes may be directed to rapid chromosome condensation and movement by just cytochalasin or latrunculin treatment (**Fig.** 7). Even the smallest avian microchromosomes (3 μm in diameter after condensation) can be transported to karyosphere-like structures during actin depolymerisation. Contraction of a specific nuclear sol-gel consisting of actin oligomers and/or short actin polymers, rather than contraction of the actin meshwork, is a more likely mechanism for chromosomes and nuclear body transportation in avian and amphibian growing oocytes. This mechanism differs from that recently suggested for starfish oocytes, namely, the homogeneously contracting meshwork model.[48] Although depolymerisation is also compulsory for contraction of nuclear content in the model suggested for avian and amphibian oocytes, this model does not include the requirement for homogeneous actin meshwork formation. We suggest that a nuclear sol consisting of monomeric and oligomeric actin is required for homogeneous radial distribution of chromosomes and other nuclear structures inside the oocyte during the high transcriptional activity stage. Our data confirm one of the hypotheses suggested by Scheer et al.[6] who stated that within the amphibian GV 'nuclear actin in such high concentrations contributes to the formation of a colloidal protein milieu in the nucleoplasm, which is essential for the transcriptional process and the three-dimensional organization of the chromosome loops'. We also suggest that during karyosphere formation, the actin sol, together with all suspended nuclear structures, is contracted; this process can be simulated by actin depolymerising drugs (**Fig.** 7).

We consider "switching" of the actin polymerisation state and/or its dynamics as a possible mechanism for the movement of intranuclear structures, including chromosomes within the avian and amphibian GVs during oocyte growth (**Fig.** 7). Inhibition of actin polymerisation and a shift in the equilibrium of actin forms toward monomeric actin could itself drive the fusion of intranuclear structures, resulting in compaction of the nuclear contents to a lower volume. Our results highlight the special role of nuclear actin in the maintenance and transformation of genome architecture in growing oocytes with large nuclei.

Materials and Methods

Experimental objects. Oocytes of clawed frog (*Xenopus laevis*), domestic chicken (*Gallus gallus domesticus*), Japanese quail (*Coturnix coturnix japonica*) and chaffinch (*Fringilla coelebs*) were used in this study. All manipulations with animals were performed in accordance with the "Guide for the Care and Use of Laboratory Animals".[49] Prior to ovary isolation, frogs were anaesthetized with MS222 1.5 g/l (Sigma). A small part of ovary surgically removed from the frog was incubated at 18°C in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM$MgCl_2$, 1.0 mM Na_2HPO_4, 5.0 mM HEPES) according to the standard procedures for not more than 60 h.[50] Ovaries, obtained from chicken, quail or chaffinch were placed into a small weighing bottle with a piece of filter paper, moistened with "5:1+phosphates" medium (83.0 μM KCl, 17.0 μM NaCl, 6.5 μM Na_2HPO_4, 3.5 μM KH_2PO_4, 1 μM $MgCl_2$, 1 μM dithiothreitol), and were kept at 4°C (http://projects.exeter.ac.uk/lampbrush/protocols.htm).[51] As for birds, only freshly isolated oocytes or oocytes after several hours after dissection were used.

Phalloidin treatment. To stabilize actin polymers inside the nucleus, amphibian and avian unfixed oocytes were incubated in OR2 or "5:1+phosphates" medium containing 1 μM phalloidin-TRITC. Amphibian oocytes were kept in the medium for 10 h at 18°C and avian oocytes were incubated no longer than 1 h at 10°C. After a short wash in phalloidin-free incubation medium at room temperature (RT) oocytes were mounted as described below and examined by confocal laser scanning microscopy.

Some freshly isolated avian GVs were also exposed to phalloidin treatment. GVs were transferred to the "5:1+phosphates" medium containing 1 μM phalloidin-TRITC and maintained there for about 1 h at RT. Than nuclei were carefully removed from the medium containing phalloidin-TRITC and placed into a chamber for confocal microscopy, that was preliminary filled with fresh "5:1+phosphates" medium.

Cytochalasin D and latrunclulin A treatment and microinjections into oocytes. In the used experimental system, amphibian oocytes (stage 1–6) were incubated in cytochalasin D-containing or latrunculin A-containing OR2 medium at 18°C. Cytochalasin D (CD, Sigma) was diluted to a final concentration of 1–4 μM while latrunculin A (LA, Sigma) was diluted to a final concentration 0.5 μg/ml. Incubation timing varied from 2 to 12 h. Due to short lifespan of isolated avian oocytes and weak penetration of CD, chicken and quail oocytes (1–1.5 mm in diameter, large growth stage) were microinjected with 27.6 nl of 10 μM CD and maintained at 10°C during 1 h. After incubation, oocytes were either fixed or used for nucleus isolation.

Nuclei isolation and staining. Amphibian and avian GVs were isolated according to the standard procedures in the "5:1+phosphates" medium from oocytes of 0.7–1.5 mm in diameter.[51,52] Small hole in oocyte envelopes and cell membrane was made by thin tungsten needles in the case of avian oocytes and by fine jewelry forceps in the case of amphibian ones. Squeezed nuclei were carefully cleaned out of yolk granules and ooplasm. During all manipulations oocytes and nuclei were inspected under stereomicroscope Leica MZ16. To investigate the content of intact GV by means of confocal microscopy, isolated nucleus was placed in a chamber filled with "5:1+phosphates" medium, containing 0.07 μM NA-specific dye SYTOX Green (Molecular Probes). Chamber for microscopy was made of a plastic square with a hole stuck to the cover glass of 24 x 50 x 0.16–0.19 mm (Deckgläser, Menzel-glaser) by paraffin with vaseline (1:1).

For detection of polymerized actin, avian germinal vesicles, isolated from oocytes of 1–20 mm in diameter (large growth and rapid growth stages) were fixed in 2% PFA during 30 min at RT immediately after isolation, than washed in PBS and stained with 10 μM phalloidin-TRITC (Sigma) during 15 min at RT. Then nuclei were transferred into the chamber, containing DAPI on 1x PBS for DNA counterstaining and covered with coverglass. After mounting, nuclei were immediately examined by confocal laser scanning microscopy.

Total staining of oocytes. For staining procedures, individual CD-treated or untreated oocytes of 200–600 μm for frogs and 100–300 μm for birds were fixed in 2% PFA on PBS during 2 h. Before fixation, avian oocytes were pretreated with 1 mg/ml collagenase (Sigma) on PBS during 30 min and rinsed in PBS. To reveal F-actin, fixed oocytes were incubated overnight in PBS containing 1 μg/ml phalloidin-TRITC (Sigma). For immunofluorescent staining, fixed oocytes were permeabilized in 0.1% Triton in PBS and incubated with 1% blocking agent (Roche) in PBS during one hour at RT followed by overnight incubation with primary antibody at 4°C. For detection of actin, monoclonal antibody C4,[53] diluted to 1:300 (Abcam) was used. After incubation with primary antibody oocytes were washed in several changes of PBS during 15 min at RT and then treated overnight by secondary antibodies at 4°C. Goat anti-mouse Alexa-488-coupled antibody (Molecular Probes) diluted to 1:500 was used to reveal C4 antibody. Oocytes were counterstained with DAPI on PBS at 4°C for not less than 24 h. After several washes in PBS whole oocytes were transferred onto cover glass in the mounting medium containing 65% glycerine, 0.023 g/ml antifade DABCO (Merk) and 1.6 μg/ml DAPI.

Confocal laser scanning microscopy and image processing. Specimens were examined using confocal laser scanning system Leica TCS SP5 (Leica-microsystems) with inverted fluorescent microscope. Diode 405 nm, argon 488 nm and helium-neon 543 nm laser lines were used for fluorochrome excitation. Sequential scan was always applied for double-stained objects to ensure proper channel separation. Apochromatic objectives with magnification of 20x and 40x were used for image acquisition. Optimal voxel parameters were obtained by choosing Z-step size and scanning format matching objective characteristics. Confocal stacks of optical slices acquired in the format of 1,024 x 1,024 or 512 x 512 pixels and transmitted light images were captured by LAS AF Software (Leica-microsystems). Image maximum projection views, 3D-reconstructions and videos were obtained by applying appropriate options in LAS AF Software. Digital images were cropped, measured and overall enhanced if necessary using LAS AF, ImageJ and Adobe Photoshop software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at: http://www.landesbioscience.com/journals/nucleus/article/20393/

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