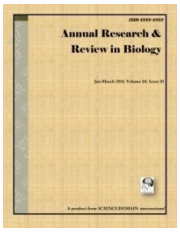

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Interchromatin Granule Clusters of Mouse Preovulatory Oocytes are Enriched with Some Components of mRNA Export Machinery

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Authors' contributions

This work was carried out in collaboration between all authors. Author NPG designed the study, performed the data acquisition, data analysis and wrote the first draft of the manuscript. Authors SSI managed the analyses of the study, manuscript editing and review. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

At the diplotene stage of meiotic prophase, the nucleus of mouse preovulatory oocytes contains multiple and prominent interchromatin granule clusters (IGCs). IGCs, also known as nuclear speckles, splicing factor compartments, or SC35-domains, are one of the most universal nuclear organelles. We have used mouse oocytes as an experimental model to study nuclear distribution of some factors involved in mRNA export: the core protein of the exon-exon junction complex (EJC) Y14, EJC shell protein Aly, and the essential mRNA export factor NXF1/TAP. RNA export proteins were detected by confocal laser microscopy and immunogold-labeling electron microscopy. We found that Y14, Aly and NXF1/TAP are located in the same IGCs together with actin and SC35 used as an IGC marker. The relationships of these proteins with IGCs occur in an RNA-dependent manner, since RNase treatment results in disappearance of the identified antigens from IGCs. After the suppression of transcription by DRB, intranuclear localization of the studied proteins varied considerably: NXF1/TAP, Y14 and Aly were detected only in the nucleoplasm, but not in the IGCs. The concept of the formation and retention of mRNA export complex and nuclear actin in the IGCs of mouse oocyte is discussed.

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Keywords: Mouse oocyte nucleus; interchromatin granule clusters; Y14; Aly; NXF1/TAP; nuclear actin; confocal microscopy; electron microscopy.

ABBREVIATIONS

DRB: 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole; EJC: Exon-exon junction complex; hnRNP: heterogenous nuclear ribonucleoproteins; IGC: interchromatin granule cluster; NXF1: nuclear RNA export factor 1; REF: RNA-binding and export factor; TAP: Tip-associated protein; TREX: TRanscription-EXport.

1. INTRODUCTION

Various multicomponent processes related to each other both physically and functionally provide each step of gene expression. Nascent transcripts of messenger RNA (mRNAs) undergo a complex process of maturation when molecules are prepared to nuclear export. Recent studies have shown that the individual steps of mRNA biogenesis, such as transcription, 5'-end capping, splicing and 3′-end formation, can be reproduced as separate reactions *in vitro*, but all steps of RNA biogenesis are coupled via a complex network of interactions *in vivo* [1–3]. The nuclear export step is fully integrated with other steps of mRNA biogenesis.

NXF1/TAP is one of the best characterized mammalian export receptors which directly binds mRNPs carried to a nuclear pore complex via connections with nucleoporins [4]. NXF1/TAP appeared to participate in the export of 75% of all mRNAs analyzed [5]. In contrast to the small nuclear RNA (snRNA), mRNAs have no unique specific signals for transport receptor encoded in their sequences. Furthermore, mRNAs vary in size, sequence, and structure. Thus, despite NXF1/TAP is able to interact with mRNA *in vitro*, it operates *in vivo* by means of other RNA-binding proteins, named "adaptors". This term was offered, since such proteins were thought to serve as a bridge between mRNA and NXF1/TAP [6].

The canonical adaptor is Aly that belongs to the evolutionary conserved family of hnRNP-like proteins REF (RNA-binding and export factor). It was shown that Aly is preferentially loaded on capped mRNAs and binds very close to the 5'-end. Aly escorts mRNA through the sequential steps of its biogenesis and interacts directly with the receptor NXF1/TAP in the mRNA export complex [7]. Recent studies have shown that Aly is an element of the TREX complex [8–11].

The evolutionarily conserved TREX (Transcription-Export) complex provides a connecting link between transcription and splicing both in yeast and multicellular organisms [12–17]. Biochemical studies carried out on multicellular organisms and genetic analysis in yeast have demonstrated that TREX also acts in mRNA transport [8–11]. The TREX complex comprises proteins involved both in transcription and mRNA nuclear export [11,18]. These proteins bind to pre-mRNA during transcription and accompany mature ribonucleoprotein complexes (mRNPs) into the cytoplasm. Except Aly, the TREX complex contains the multisubunit THO complex, UAP56 and some other factors [19–21]. In mammals, efficient recruitment of the TREX complex to mRNA is splicing-dependent. Specifically, it was previously found that the TREX complex is more efficiently recruited to mRNAs generated by splicing than to cDNA transcripts [22]. This increased recruitment correlates with the increased export efficiency of spliced mRNAs as compared with cDNA transcripts [23].

Another group of proteins associated both with nuclear and cytoplasmic mRNPs represents the exon-junction complex (EJC). The EJCs usually lay about 20–24 nucleotides upstream of mRNA exon-exon junctions and stably bind mRNPs with four core proteins (Y14/Magoh heterodimer, eIF4AIII and MLN51) [24,25]. This core is an essential platform for recruiting of additional peripheral EJC factors among which the TREX proteins UAP56 and Aly have been detected [26]. It is known that Aly acts along with RNA helicase UAP56 within the EJC in the initial steps of spliceosome formation [27]. In mammalian cells, core and peripheral proteins of the EJC participate in numerous regulator mechanisms of mRNA export, translation and degradation. According to the current opinion, the EJC promotes the export of spliced mRNAs possibly due to a close association with the TREX complex [22,28,29].

Despite the fact that the mechanisms of mRNA export and the factors involved in this process have been characterized in detail [30,31], the data on the association of these factors with particular nuclear domains are scanty. In accordance with the present-day understanding, universal nuclear domains, interchromatin granule clusters (IGCs), participate in coordination of the manifold steps of gene expression, including transcription, pre-mRNA splicing and mRNA transport [32–34]. The IGCs, or speckles, are dynamic nuclear compartments enriched with pre-mRNA processing factors: snRNAs and SR-proteins [34].

The SR-proteins are pre-mRNA splicing factors involved both in constitutive and alternative splicing [35–37]. The SR-protein SC35 is one of the essential IGC markers [38,39]. In somatic cells, the interphase nucleus contains about 20-50 speckles. The speckles correspond to IGCs at the ultrastructural level and represent nuclear domains of 1–2 μm in diameter that consist of 20–25 nm granules connected by thin fibrils [40]. Transcriptionally active genes have been shown to localize predominantly close to the periphery of speckles [32,41,42]. Such localization is quite reasonable as pre-mRNA splicing factors are subject to rapid assembly/disassembly in IGCs, and individual factors can therefore shuttle continually between IGCs and active gene loci [34]. Moreover, it is supposed that newly synthesized mRNAs could retain in IGCs for checking their adequacy to nuclear export [43,44]. Recent studies allow believing that IGCs are not only the sites enriched with RNA processing factors but also contain the mRNA export machinery (e.g., the TREX complex) and mRNA surveillance machinery (e.g., the EJC) [45]. Indirect immunofluorescence with the use of antibodies which recognize catalytically active spliceosomes in a combination with transcription down-regulation and splicing arrest has shown that post-transcriptional splicing occurs in the speckles and this process is closely associated with the mRNA export mechanism [46].

Mouse oocytes appear to be a proper model for exploring IGC composition and functions. In their nuclei there are numerous large IGCs that are detected with antibodies against SC35 protein, an IGC marker. During the antral follicle development, essential changes in morphological organization of IGCs are revealed together with the lowering of transcription. In transcriptionally active oocytes from uni/bilaminar follicles, the IGCs are numerous small irregular formations detected in association with the regions of dispersed chromatin [47]. Nuclei of preovulatory oocytes are characterized by a fewer number of IGCs which herein acquire a regular round shape and rather large size. In our previous study the presence of poly(A)$^+$ RNA in mouse oocyte IGCs has been demonstrated with the use of microinjections of 2′-*O*-methyl $(U)_{22}$ probe to reveal RNA poly(A)$^+$ tails [48]. In addition, nuclear actin together with protein A1 that belongs to the A/B hnRNP family and NXF1/TAP, a member of the mRNA export complex, were detectable in mouse oocyte IGCs [49].

The present study has been carried out to proof the concept of formation and retention of an integrated mRNA — export factors — nuclear actin complex in the mouse oocyte IGCs. We have analyzed conjoint distribution of the proteins actin, NXF1/TAP, Y14 and Aly in the nuclei of preovulatory mouse oocytes under standard conditions and after down-regulation of transcription by DRB with the use of confocal laser scanning and electron microscopy. Furthermore, the RNA-dependence in distribution of these proteins has been studied.

2. MATERIALS AND METHODS

2.1 Animals and Tissue Preparation

Objects of the study were preovulatory oocytes from antral follicles of sexually mature Balb/C mice (days 21-28 of postnatal development). Inbred BALB/c mice were obtained from the animal nursery “Rappolovo” of the Russian Academy of Medical Sciences. Mice were housed in a group of 2 or 3 mice per cage. The animals were maintained at 23±3ºC, 50±10% relative humidity and 12-hr light and dark cycle (07:00–19:00). All mice had ad libitum access to food and water. All procedures were performed according to guidelines by the European Communities Council Directives of 24 November 1986, 86/609/EEC.

The cumulus-enclosed oocytes were isolated from antral follicles in M2 medium [50] containing 100 mg/ml dibutyryl cAMP (Sigma, St. Louis, MO) to prevent spontaneous maturation. To inhibit transcription, some oocytes were treated with 500 μM DRB (Sigma) for 2 h. Some preparations were treated with 20 mg/ml RNase A (Sigma) for 2 h at 37ºC before immunostaining.

2.2 Antibodies

The following primary antibodies were used: mouse monoclonal antibody (mAb) 1501R to highly conserved amino acids 50-70 at the N-terminus of actin molecule (Chemicon; dilution 1:50); mAb against non-snRNP splicing factor SC35 (Sigma, USA, cat. no. S4045; dilution 1:50); mAb 11G5 against recombinant human REF/Aly fusion protein (Abcam, Cambridge, UK; cat. no. ab 6141; dilution 1:20); rabbit polyclonal antibody (pAb) against recombinant protein fragment with a sequence corresponding to a region within amino acids 1-203 of human Aly (AAH52302) (Abcam, Cambridge, UK; cat. no. ab95962; dilution 1:20); rabbit pAb H-120 against amino acids 1-120 of NXF1/TAP of human origin (Santa Cruz Biotechnology, Calif., USA; cat. no. sc-25768; dilution 1:20); and goat pAb C-20 against peptide mapping near the C-terminus of Y14 of human origin (Santa Cruz Biotechnology, Calif., USA; cat. no. sc-23444; dilution 1:20). The antibody mixture was used for double staining of oocyte preparations [51].

2.3 Immunofluorescent/Confocal Microscopy

Immunofluorescence experiments were performed on whole-mount preparations of oocytes using the method described previously [52,53]. Oocytes were fixed in 4 % formaldehyde freshly prepared from paraformaldehyde in PBS and then oocytes were incubated in 0.1 % Triton X-100 in PBS for 10 min. The incubation in first antibody solution was carried out overnight in a moist chamber at 4ºC. After rinsing in PBS, the preparations were incubated with secondary antibodies for 1.5 h at room temperature. After rinsing in PBS, the preparations were additionally stained for 1 min with To-Pro-3 dye (Molecular probes; dilution 1:1000) to reveal DNA and mounted in Vectashield medium (Vector Laboratories).

Secondary antibodies were FITC (Sigma) or Alexa-568 (Molecular probes) conjugated goat anti-mouse, goat anti-rabbit or rabbit anti-goat immunoglobulins (IgGs) (dilution 1:200). Preparations were analyzed in a Leica TCS SP5 confocal microscope equipped with argon (488 nm) and helium-neon (543 and 633 nm) lasers and 40×objective (NA 1.25). Merged images were obtained using ImageJ 1.37a. Relative brightness/contrast was adjusted with Adobe Photoshop.

2.4 Immunogold Electron Microscopy

Oocyte fixation and embedding for electron microscopy were performed using the routine technique [54]. Oocytes were prefixed for 1.5 h in a solution containing 4% formaldehyde (Ted Pella, Redding, Calif., USA) and 0.5% glutaraldehyde in PBS and then fixed overnight in 2% formaldehyde at 4ºC. After rinsing in PBS containing 0.05 M NH_4Cl (Sigma) and subsequent dehydration in an ethanol series, oocytes were embedded in LR White medium grade resin (Polyscience, Warrington, Pa., USA). Ultrathin sections were incubated for 10 min in a blocking buffer containing 0.5% fish gelatin (Sigma) and 0.02% Tween-20 (Sigma) in PBS, pH 7.4. After that, sections were incubated in the primary antibody solution overnight in a moist chamber at 4ºC. After rinsing in PBS containing 0.1% fish gelatin and 0.05% Tween-20, the sections were incubated with secondary donkey anti-mouse IgGs conjugated with 15 nm gold particles, donkey anti-goat IgGs conjugated with 10 nm gold particles, donkey anti-rabbit IgGs conjugated with 6 nm gold particles (Electron Microscopy Sciences, US). As a control, additional sections were incubated only in secondary antibodies. Ultrathin sections were contrasted with 1% uranyl acetate water solution and examined in an electron microscope Libra 120 operated at 80 kV.

3. RESULTS

The most prominent bodies in the nucleus of mouse antral oocytes are the so-called nucleolus like bodies (NLBs) and IGCs. The latter are round structures brightly stained with antibodies against the splicing factor SC35 and actin [49].

In the present study, we revealed round fluorescent structures and diffuse glow of the nucleoplasm after the treatment of the specimens with antibodies against the export factor NXF1/TAP and SC35 used as a IGCs marker (Fig. 1a). Double staining of the specimens with antibodies against actin and the proteins Aly and Y14 was applied to confirm the localization of these antigens in the IGCs. Obvious colocalization of actin and Aly in the IGCs was seen at the background of a diffuse glow of the nucleoplasm in the merged confocal images (Fig. 1b). The same results were also obtained for the protein Y14 (Fig. 1c).

The transcription inhibitor DRB caused distinct alterations in the nuclear appearance. The number of IGCs in DRB-exposed oocytes decreased in several times as compared to the intact cells, but the size of these structures increased significantly (Fig. 2). Distribution pattern of the studied proteins has also been altered. DRB treatment did not affect the distribution of SC35 in IGCs, as expected. However, anti-NXF1/TAP (Fig. 2a), anti-Aly (Fig. 2b) and anti-Y14 (Fig. 2c) signals disappeared completely from IGCs.

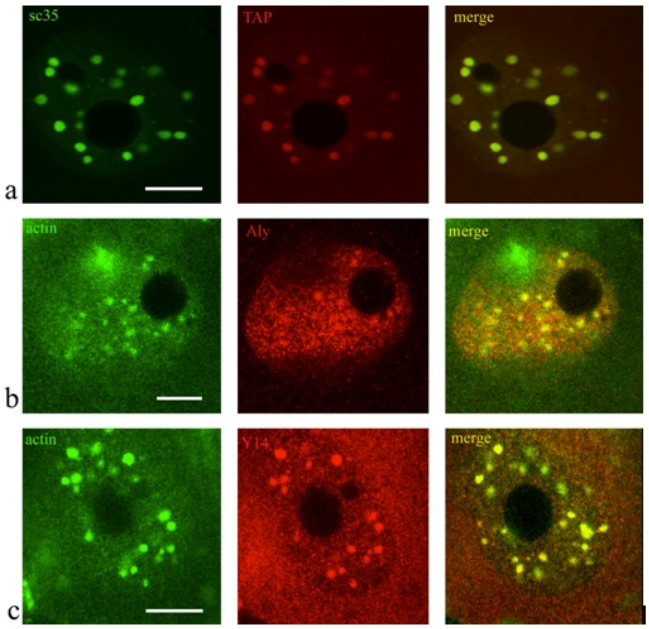

Fig. 1. Detection of mRNA export receptor NXF1/TAP (a), peripheral EJC protein Aly (b) and core EJC protein Y14 (c) in the nuclei of preovulatory mouse oocytes. Each panel corresponds to the same nucleus

Large non-stained "holes" in all images correspond to NLBs. Scale = 10 μm

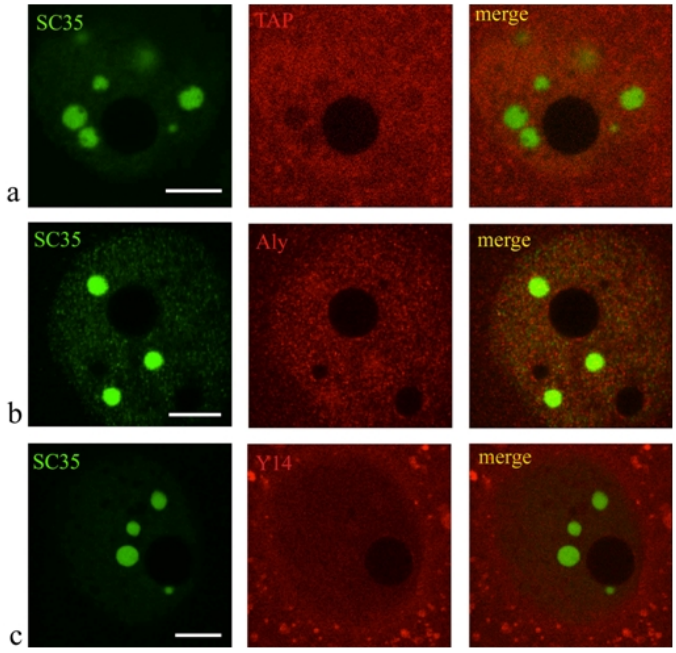

Fig. 2. Immunofluorescent revealing of proteins NXF1/TAP (a), Aly (b) and Y14 (c) in mouse oocyte nuclei after DRB-treatment

Scale = 10 μm

RNAse treatment did not affect SC35 localization in IGCs. No distinct anti-Aly (Fig. 3a), anti-NXF1/TAP (Fig. 3b) and anti-Y14 (Fig. 3c) signals were observed in the IGCs.

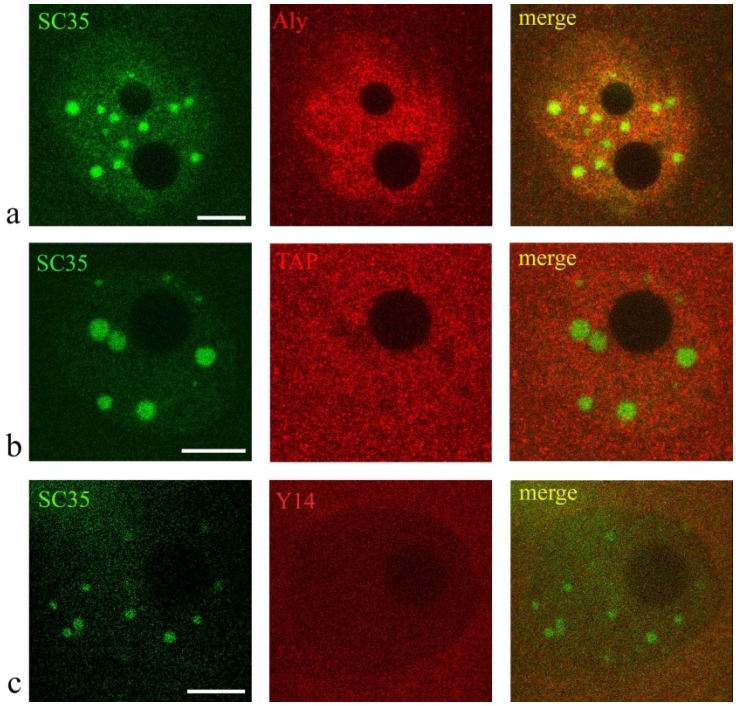

Fig. 3. Detection of Aly, NXF1/TAP and Y14 in nuclei of preovulatory mouse oocytes after RNase treatment

Scale = 10 μm

The data on accumulation of some pre-mRNA export factors in IGCs obtained using fluorescent microscopy we confirmed by immunogold labeling microscopy. A conjoint distribution of NXF1/TAP, Aly and Y14 in the IGCs was documented after triple immunolabelling of mouse oocyte ultrathin sections with a mixture of antibodies against these proteins (Fig. 4a). At the ultrastructural level, IGCs of the studied mouse oocytes were uniform roundish structures 1–2 μm in diameter. They consist of the aggregates of 10–15-nm interchromatin granules. A characteristic feature of mouse oocyte IGCs is the presence of 1-3 prominent fibrillar areas (0.2–0.5 μm in size) that are located at the periphery of the IGC. After immunogold labeling IGCs were evenly filled with different-sized labels, and it should be noted that NXF1/TAP tracer (6 nm) was predominantly located at the IGC periphery, and 10 nm tracer of the splicing factor Y14 was localized within the fibrillar zones. At higher magnification we could observe that the distance between the most of different gold particles does not exceed 10–15 nm (Fig. 4b).

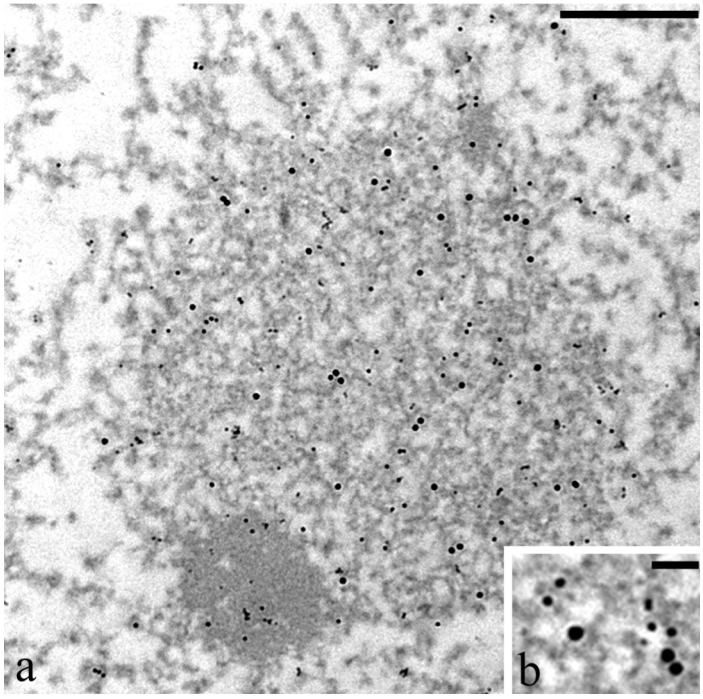

Fig. 4. Simultaneous detection of Y14, Aly and NXF1/TAP in IGCs of mouse oocytes using immunogold labeling technique

a - Triple immunolabeling in a mixture of antibodies against the proteins Y14, Aly and NXF1/TAP. 6 nm colloidal gold particles label NXF1/TAP, 10 nm gold particles label Y14 and 15 nm gold particles label protein Aly. IGCs are filled with labels of different size. Scale = 500 nm; b – A fragment of an IGC at higher magnification after the same labeling as in 4a. Scale = 50 nm.

4. DISCUSSION

Our results have demonstrated that: (i) the IGCs of preovulatory mouse oocytes comprise the proteins of the pre-mRNA export complex, namely NXF1/TAP, Y14 and Aly; (ii) the association of these proteins with the IGCs is RNA-dependent; (iii) the nuclear localization of the studied proteins significantly alters after transcription down-regulation with DRB.

All preovulatory mouse oocytes are commonly divided into two groups, active and inactive, in accordance with a degree of synthetic chromatin activity [55,56]. In the first group of oocytes, the so-called non-surrounded nucleolus (NSN) oocytes, the nuclei are characterized by disperse chromatin with several chromocenters. In these cells, IGCs are numerous and vary in size. In the second group of oocytes, the so-called surrounded nucleolus (SN) oocytes, a dense chromatin ring is detected around NLBs. The remaining chromatin is also condensed and arranged in small chromocenters. The perinucleolar rim in the SN oocytes is also known as the karyosphere [57,58]. IGCs in mouse SN oocytes are obviously larger, but their number decreases significantly as compared with NSN oocytes. However, these differences in nuclear organization seem not to affect the IGC composition. Oocyte IGCs in both groups comprise all antigens we have detected: actin, A1, NXF1/TAP [49] as well as Y14 and Aly (present study).

The detection of NXF1/TAP, Aly and Y14 in mouse oocyte IGCs is in accordance with the proposed hypothesis on the participation of IGCs in mRNP export; moreover, we have found that these proteins are located in IGCs in a RNA-dependent manner. Similar results were obtained for mouse early embryos [59,60]. NXF1/TAP, which is a shuttle protein, might be able both to couple with and to dissociate from the IGCs. At the ultrastructural level, this protein is predominantly detected at the IGC periphery and apparently does not persist in transcription down-regulation as not being a resident IGC protein. This is in agreement with the concept of the continuous protein exchange between IGCs and the nucleoplasm [61]. Thus, an IGC shape and size might reflect a dynamic equilibrium between mobile components of IGCs entering these domains or moving from them into the nucleoplasm. It is obvious that this equilibrium shifts towards the IGCs with inhibition of the transcription activity (natural or artificial) that causes an enlargement of these structures. The occurrence of large round IGCs in mouse preovulatory oocyte nuclei reflects a general tendency of increasing the number and shape of these nuclear domains in cells of various types at low level of transcription activity [34,47,62–64]. Mammalian oogenesis is characterized by a physiological decline of a level of total RNA synthesis and the onset of transition of the oocyte nucleus in an inactivated state that occurs in mice in multilayer follicles in a period of their extensive growth [65,66,67]. One of the reasons of an IGC size enlargement in this period may be due to the return into these structures of factors excluded from the processes of transcription and co-transcriptional splicing and from other steps of pre-mRNA processing. A cyclic recruitment of IGC factors, which provide these crucial steps of gene expression, is substantiated by the hypothesis stating that the IGCs play a role as peculiar reservoirs for storage and/or recycling of a variety of nuclear factors [34, 68]. An additional inhibition of transcription in mouse oocytes with DRB causes not only a critical reduction of IGC number but also affects the IGC composition. Almost complete disappearance of the proteins involved in mRNP export from IGCs might be associated with the fact that new mRNP transcripts do not enter the IGCs resulting from the inhibition of chromatin synthetic activity.

There are scarce data on nuclear localization of the proteins comprising the mRNA export complex. However, a molecular composition of the complex which mediates mRNA export from the nucleus to the cytoplasm through the nuclear pores has been studied in detail [6,69,70]. The dominating role of NXF1/TAP in the export of cellular poly(A)$^+$ RNA was confirmed in a large-scale analysis of mRNA of different cells. NXF1/TAP appeared to participate in the export of at least 75% of 6,000 mRNAs analyzed [71]. An accumulation of a nuclear pool of poly(A)$^+$ RNA is shown to occur with an inhibition of this transport receptor [8,72,73]. However, the interaction of NXF1/TAP with the adaptor mRNA-binding protein Aly is required for NXF1/TAP to be bond to the exported mRNA molecule that results in the formation of export-competent mRNA-protein complexes [74]. On the other hand, Aly in association with the helicase UAP56 is known to participate in the formation of the active TREX complex [75]. Besides, Aly is also a peripheral EJC factor that provides a functional link between splicing and mRNA export within the TREX along with the core EJC proteins [76]. The EJCs structurally comprise of more than 10 proteins which are divided into two groups such as core and peripheral factors [77,78]. Four EJC core protein, the heterodimer Y14/Magoh, DEAD-box RNA helicase eIF4A3 (eukaryotic initiation factor 4AIII) and MLN51 (metastatic lymph node 51), form a platform for peripheral factors which are capable for the dynamic coupling and disassociation with mRNPs during export. These are the core proteins presented by the factor Y14 in our present study that provide a stable interrelation between mRNPs and EJCs in the export complex [77]. In mouse oocyte IGCs, Y14 is localized along with actin and Aly (Figs. 4a, b). Immunogold labeling electron microscopy has revealed rather close distance between gold labels masking these proteins.

According to the modern notion, IGCs serve not only as functional centers combining active genes but also represent the sites where pre-mRNA may acquire the export competency [79]. The detection of a number of proteins in mouse oocyte IGCs that play an important role in the organization of the TREX and EJC, namely the export factor NXF1/TAP, the adaptor protein Aly and the splicing factor Y14, contributes its share into the development of the understanding the functioning of these nuclear domains in the latest stages of gene expression. It is quite possible that it is the IGCs that may be a missing structural element allowing to prove the aspect on intranucler retention of mRNA which determines the mRNA export rate and to provide a qualitative surveillance mechanism of this export [61,80].

5. CONCLUSION

Our findings reveal that mouse oocyte ICGs simultaneously contain the components of mRNA export machinery such as Y14, Aly, and NXF1/TAP. Interaction of these proteins with the IGCs occurs in an RNA-dependent manner. Artificial inhibition of transcription using DRB causes dynamic redistribution of the NXF1/TAP, Y14 and Aly proteins in the nucleoplasm.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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