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Nuage Components and Their Contents in Mammalian Spermatogenic Cells, as Revealed by Immunoelectron Microscopy

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1. Introduction

The nuage is a germ cell-specific organelle that has been studied for more than a hundred years. It was first discovered in spermatogenic cells as a perinuclear granule stained by basic dyes and visualized using a light microscope. Morphological studies using electron microscopes demonstrated that ”chromatoid body (CB)” is a nuage component in spermatids, and that “inter-mitochondrial cement (IMC)” is another nuage component found in spermatocytes. During meiosis, the IMC disappears, but the CB soon reforms in post-meiotic spermatids. Recent morphological and molecular biological studies have identified many components of nuage, and suggest that they play roles in the silencing, decay, and storage of RNA, and in the aggresome system. In this report, we summarize recent findings related to nuage and discuss their functions in spermatogenic cells.

2. Historical aspect of nuage components in germ line cells

By the 19th century, the basic techniques for histology, including preparation of tissue sections and staining of sections with various dyes, had largely been established, and light microscopic studies of tissues and cells from various animals and plants had begun to increase in terms of both number and detail. The subcellular structures in spermatogenic cells strongly attracting histologists’ interest were strongly stained by basic dyes such as safranin, crystal violet, and Heidenhain’s iron-hematoxylin. Two types of structures that today correspond to the chromatoid body (CB) were first described by von Brunn, who termed them “Protoplasmaanhäufungen" (protoplasmic depositions) and followed their fate, concluding that one formed the acrosome cap and the other moved to the caudal portion of spermatids to create the flagellum of spermatozoa (von Brunn, 1876). Afterwards, many histologists described similar structures using various names, such as “Nebenkörper” in spermatogenic cells from salamander and mouse (Herman 1889); “chromatoide Nebenkörper,” derived from “intranuclearkörper” (the intranuclear body), which is different from the nucleolus (von Ebner, 1888; Benda, 1891); “corps chromatoides,” which were stained black by iron-hematoxylin and deep red by safranin and finally degraded to “corps résiduel” (Regaud, 1901); and “chromatoide Körpers,” which appeared as “chromatoiden Nuckeolens” in the nucleus and were dispersed in the cytoplasm (Schreiner & Schreiner, 1905). In the first half of the 20th century, CBs from various animals including insects (Wilson, 1913; Pollister, 1930),

crustaceans (Fasten, 1914), arachnids (von Korff, 1902), fish (Schreiner & Schreiner, 1905), fowl (Zlotnik, 1947), and mammals (Duesberg, 1908; Wodsedalek, 1914; Gatenby & Beams, 1935) were studied extensively by light microscopy. In these classic studies, the potential origins of CBs were discussed. The arguments can be summarized into two ideas: 1) that the CB appears from the beginning in the cytoplasm of spermatocytes or spermatids, and 2) that the CB arises within the nucleoplasm and then moves to the cytoplasm. The fate of CBs can be summarized as follows: the CB exists in a perinuclear area and forms a part of the acrosome or the axis of the flagellum, or enters the bag-like structure that is formed in the cytoplasm at the base of the flagellum, and is degraded there. The studies cited above only involved observing stained tissue sections by light microscopy. Although most of these ideas have been shown to be incorrect, some of them have been accepted.

3. Ultrastructural characteristics of nuage components in mammalian spermatogenic cells

3.1 The CB as a representative nuage structure in mammalian spermatogenic cells

Electron microscopic studies of mammalian spermatogenic cells commenced in the 1950s (Watson, 1952; Challice, 1953). Burgos and Fawcett (1955) were the first to observe the CB by electron microscopy (Figure 1).

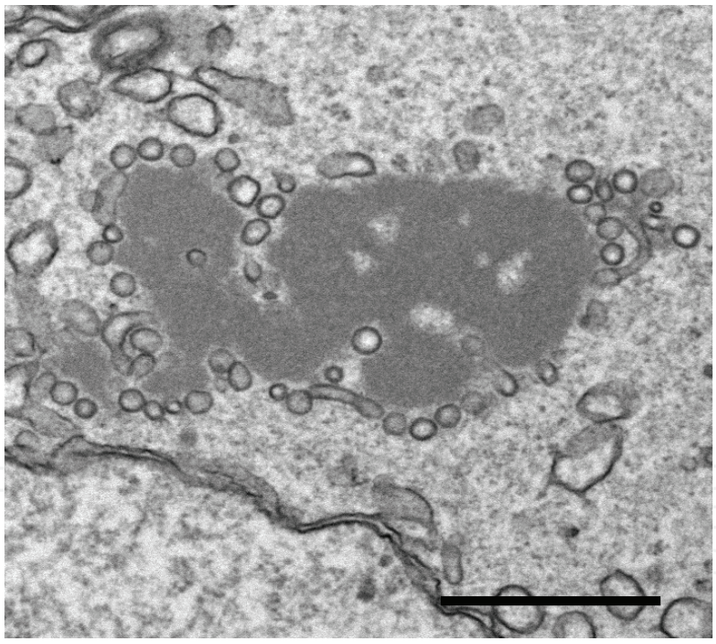

Fig. 1. Electron micrograph of a typical CB of a step 4 spermatid. The CB consists of an electron-dense matrix and is surrounded by small clear vesicles and tubules. The CB has no limiting membrane. Bar = 1 μm.

Burgos and Fawcett described an irregular and osmiophilic component corresponding to the "chromatic body" or "accessory body" detected by light microscopy. This structure was initially found near the Golgi complex, subsequently moving towards the caudal region in late spermatids. In 1955, Minamino described the material as an "idiosome remnant." In 1959, Sasa reported the ultrastructure of the rat testis, and referred to "Chromatoider

Nebenkörper." He suggested that osmiophilic components derived from the cluster of cytoplasmic vesicles might fuse to each other to form the big honeycomb structure.

In 1970, Fawcett et al. studied the origin and fate of CBs and satellite bodies during spermatogenesis in several mammals at the ultrastructural level. They presented evidence that the CB was not derived from the nucleus, as had been reported previously. CBs were not present in spermatocytes. Instead, they were found between clusters of mitochondria, in the dense interstitial material that is today known as inter-mitochondrial cement (IMC) (Figure 2). Because its texture and density were similar to those of CB, the researchers assumed that IMC might be the origin of the CB, and that two CBs might be assembled from IMC and possibly distributed to two daughter cells during meiosis I. In late spermatids, the CB became associated with the base of the flagellum, so they assumed that it might contribute to formation of the "annulus," a ring associated with the plasma membrane in spermatids and subsequently located at the junction of the middle and principal pieces in spermatozoa.

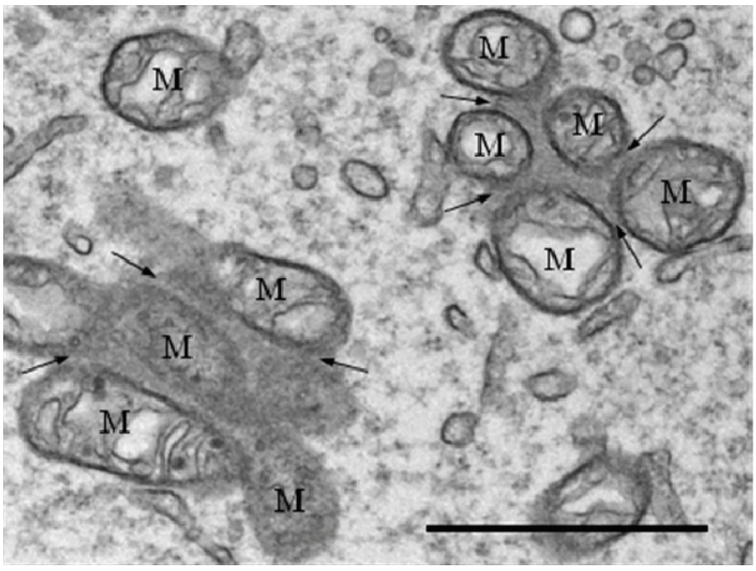

Fig. 2. Electron micrograph of typical IMC in a stage X pachytene spermatocyte. IMC appears as a dense interstitial material (arrows) between the clustered mitochondria (M). Bar = 1 μm.

Susi and Clermont (1970) studied the morphology and fate of CBs by routine electron microscopy. In spermatocytes, the CB was composed of two elements: 1) an electron-dense material with a sponge-like texture; and 2) numerous small vesicles with a diameter of 40–80 nm, located in the cytoplasm near the nuclear envelope. In step 1–7 spermatids, the CB was observed as a single irregular granule with a diameter of 1–2 μm, whose material was more condensed compared with CBs in spermatocytes. It was located proximal to the nuclear envelope, or sometimes near the Golgi apparatus, and was occasionally associated with the "multivesicular body (MB)." The CB and MB were found in the caudal region of the nucleus in step 8 spermatids. In step 9–10 spermatids, the CB was spherical, had a diameter of 1.5 μm, and was surrounded by vesicles positive for glycoproteins (stained by the periodic acid–chromic acid–silver methenamine method). In later spermatids, the CB was fragmented into smaller masses. Susi and Clermont suggested that the CB might be highly active in dramatically changing its form.

Based on the results of indium trichloride staining, Eddy (1970) concluded that no RNA existed in the CB. Similarly, the results of radioautography with isotope-labeled bases suggested that the dense granules observed in oocytes from *Rana* spp. contained neither RNA nor any other nucleic acid (Eddy & Ito, 1971). These results conflicted with previous reports (Daoust & Clermont, 1955).

In 1974, Eddy studied "nuage" (clouds in French) structures in primordial germ cells from rats of both sexes. He identified nuage in both male and female cells, and proposed that the nuage is the characteristic component of mammalian germ cells. He further compared the nuage with the "polar granule" found in insects (Dhainaut, 1970; Mahowald, 1962) and with "germinal plasm" found in amphibians (Nieuwkoop & Faber, 1956). These structures were previously speculated to play a role in germ cell determination. Eddy noted the common morphological profile and distribution of nuage with these components, which led him to suggest that nuage might be involved in the determination of mammalian germ cells, as had been reported for insects and amphibians. In terms of mammals, nuage was identified in guinea pig (Adams & Hertig, 1964), hamster (Weakley, 1969; Fawcett et al., 1970), rabbit (Nicander & Plöen, 1969), mouse (Fawcett et al., 1970), rat (Brökelmann, 1963), and human (Burgos et al., 1970). In germ cells of both sexes, nuage appeared either as a discrete mass or in association with mitochondrial clusters during the different stages of oogenesis and spermatogenesis. Eddy (1974) suggested that that the CB was a component of the nuage in spermatogenic cells.

In the same year, Kerr and Dixon (1974) studied germ plasm, which they regarded as a nuage found in spermatogenic cells, based on its appearance, expression stage, and association with mitochondria. They assumed that germ plasm might be a precursor of the CB. Russell and Frank (1978) classified nuage into six different types based on its form, distribution, and interrelationships with other organelles: 1) 70–90 nm spherical particles: fine fibrous and partly high-dense material found in spermatogonia, secondary spermatocytes, and intermediate forms; 2) sponge bodies: loosely organized masses, not only perinuclear but found throughout the cytoplasm in spermatocytes of all stages, and occasionally in spermatogonia and early spermatids; 3) IMC: 0.1 μm or narrower loosely organized strands, whose density and appearance resemble those of the sponge body, observed mainly in early spermatocytes and occasionally in spermatogonia; 4) 30 nm particles: clusters of dense particles different from ribosomes in the cytoplasm of meiosis I cells and secondary spermatocytes; 5) CBs: located in the cytoplasm proximal to the nucleus in pachytene spermatocytes; and 6) definitive CBs. Temporary CBs had been described previously as large spherical bodies with a diameter of approximately 0.5 μm that were closely associated with the few mitochondria observed in secondary spermatocytes and spermatids. Russell and Frank (1978) first classified the CB into two types that were present during different stages of spermatogenesis, although it was unclear whether the two types were the same in terms of their composition. They further reported that the CBs were not found in diplotene spermatocytes or during meiosis I. During meiosis II, they found numerous CBs in the cytoplasm. These CBs were separated into two components, one of which had a large, round honeycomb structure, and the other of which was a loose network of irregularly shaped dense strands.

The ultrastructural studies of CBs described above can be summarized as follows: 1) the CB is a component of nuage that was specifically detected in spermatogenic cells; 2) CBs displayed irregular shapes, comprising dense fibrous material surrounded by small vesicles, and had no limiting membrane; 3) CBs appeared first in spermatocytes, disappeared during meiosis, and reappeared in spermatids.

3.2 Relationship between the CB and the nuclear envelope

In classical studies, CBs were assumed to be derived from the intranuclear structure, based on the similarity in safranin staining in both structures (Benda, 1891; Regaud, 1901). Sud (1961) first suggested the presence of highly polymerized RNA and arginine-rich basic proteins in CBs based on the results of histochemical studies.

In 1970, Fawcett et al. proposed a new idea: that CB originated not from nucleus, but from the dense materials between clustered mitochondrial, i.e., IMC, which appeared during early spermatocytes. IMC was gradually deposited there as spermatocytes differentiated. Their hypothesis that CB is derived from IMC was supported by the facts that 1) no dense substance was observed on the intranuclear side of nuclear pores when the CB was in contact with the cytoplasmic side, and 2) that CBs already existed in spermatocytes before the CB made contact with nuclear pores. Although the CB is not derived from the nucleus, Fawcett et al. (1970) assumed that some kind of exchange might take place between the nucleus and the CB. On the other hand, in 1972, Comings and Okada observed that a nuclear granule exited to the cytoplasm of primary spermatocytes via the nuclear pore, and therefore concluded that the CB originated from the nucleus. This idea was discussed by Eddy in 1974.

The movement of CBs in the rat testis was studied using time-lapse cinemicrography, the results of which revealed that the CB rapidly moved around the nuclear envelope and Golgi complex, and occasionally seemed to be detected as an intranuclear particle (Parvinen & Jokelainen, 1974; Parvinen & Parvinen, 1979). Based on these observations, they assumed that the CB played a role in the transport of haploid gene products in early spermatids.

Ventelä et al. (2003) reported that the CB moved from one spermatid to another through the cytoplasmic bridge, and that this movement was inhibited by the microtubule-depolymerizing agents, nocodazole and vincristine. These results demonstrated that the microtubular network is involved in CB mobility.

Communication between the CB and nucleus, suggested by the results of classic light microscopic studies, was confirmed by electron microscopic studies. Cinemicrography studies showing real-time movement of CBs provided clear evidence of CB-nucleus communication (Parvinen & Jokelainen, 1974). Consequently, it was concluded that the CB may not be derived from the nucleus, but communicates with the nucleus via the nuclear pore complex during spermatogenesis.

3.3 Clusters of 30–40 nm particles

Russell and Frank (1978) observed clusters of small, 30–40 nm particles in dividing meiosis I cells and pachytene spermatocytes, which were morphologically different from ribosomes and glycogen particles. These clusters were composed of hundreds of aggregated 30–40 nm particles, and contained a filamentous material. Russell and Frank suggested that these particles might stabilize RNAs encoding proteins required for the subsequent development of spermatids.

3.4 IMC as an origin of the CB

Some concluded that IMC was a precursor of mitochondria because its density was similar to that of the mitochondrial matrix (Odor, 1965; André, 1962). However, this hypothesis was disproved by confirmation of the striking difference in the density of the two components

after improved fixation (Fawcett, et al., 1970). As mentioned above, Fawcett et al. proposed that the CB was derived from IMC (Fawcett, et al. 1970). According to them, IMC gradually increased in size by coalescence as spermatids developed, subsequently dissociated from the mitochondria, and assembled to form the CB.

Russell and Frank (1978) reported that IMC appeared when mitochondria were noticeably clustered in pachytene spermatocytes, and that there was no evidence of IMC during or after the first meiotic metaphase, when the mitochondria dispersed.

3.5 Satellite body (SB, sponge body) and its origin

Sud (1961) first named the structure the "satellite," and described its localization near to, and sometimes in contact with, the CB. The chemical composition of the satellite differed from that of the CB. Sud proposed that the satellite might form the basal body of the axial filament.

Fawcett et al. (1970) described the characteristics, origin, and fate of the satellite body (SB), which they referred it "chromatoid body satellite", and first described the distinction between the SB and the CB at the ultrastructural level. He reported that the SB was present in zygotene and pachytene spermatocytes prior to the appearance of the CB, and had a more regular shape and contained less filamentous material than the CB. In the report of Fawcett et al. (1970), they hypothesized that clusters of small particles of 40–60 nm in diameter, which had previously been suggested to be precursors of centrioles (Stockinger & Cirelli, 1965; Sorokin, 1968), were in fact the source of the SB, because they were the only conceivable precursor of the SB and had a strikingly resemblance to the SB. The fate of the SB was unknown, but it was found to move from the Golgi region to the caudal region of the nucleus in spermatids. In late spermatids, a spherical mass was found near the developing connecting piece, and was assumed to be derived from the SB on the basis of its size.

Russell and Frank (1978) described the SB as a "sponge body" found in the cytoplasm of secondary spermatocytes and young spermatids, and very occasionally spermatogonia. They indicated that the density and appearance of the SB resembled those of IMC.

3.6 Nuage components in meiotic cells

Nuage was found to dissociate from mitochondrial clusters during the pachytene phase of meiosis, forming the CBs in spermatids (Eddy, 1974). Eddy also observed that after meiosis II, the CB was dispersed in the cytoplasm as small dark masses. However, in step 1 spermatids the CB was reconstructed. Russell and Frank (1978) reported that while the CB was rarely seen in dividing meiosis I cells, there was loose, dense material that was irregularly shaped and contained granular materials on its inner surface. As mentioned above, the clusters of 30 nm particles were found in dividing meiosis I cells and were thought to possibly contain mRNAs. Russell and Frank assumed that these clusters might contain RNAs that had been transferred from the nucleus to cytoplasm, and that these RNAs might encode proteins involved in the later stages of spermatogenesis. They also reported that the definitive CB was observed in secondary spermatocytes proximal to meiosis II cells. CB was suggested to be a storage organelle for haploid gene products during meiosis, and to possibly participate in the transport of these products from the nucleus to the cytoplasm (Söderström & Parvinen, 1976; Parvinen & Parvinen, 1979). Söderström (1978) reported that the condensed CB was present during meiotic prophase, and that new materials were formed in the nucleus in cells of meiotic prophase and spermatids, and proposed that the materials might be transported to the cytoplasm and be

added to the CB. We ourselves have shown that the small particles partly associated with mitochondria during meiosis stain positive for the nuage marker protein DDX4. This suggests that nuage components might dissociate into small particles during meiosis, allowing them to be apportioned to the daughter cells evenly (Figure 3).

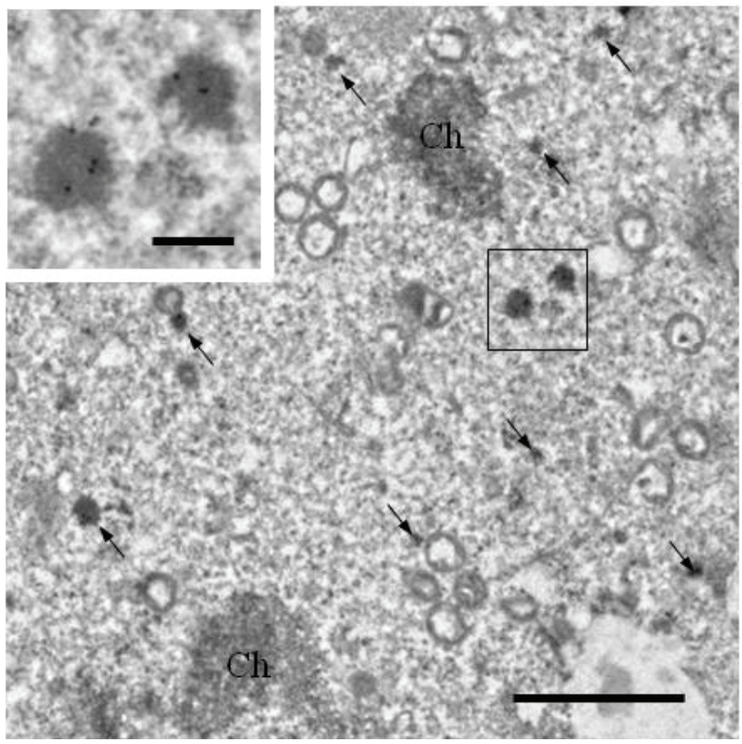

Fig. 3. Rat spermatocyte during meiosis. A dividing chromosome (Ch) is present. No aggregates of particles are seen, but small particles are present (arrows). These particles are positive for DDX4 (inset). Bar = 2 μm (main image) and 0.5 μm (inset).

4. Immunoelectron microscopic localization of nuage proteins in spermatogenic cells

4.1 Argonaute/Piwi and Tudor families in nuage components

The Argonaute/Piwi family of proteins can be split into CB and nuage components. Members of the Argonaute/Piwi family commonly have PAZ and PIWI domains, and are classified into the Ago1 and PIWI subfamilies based on their sequences. Ago 1–4 belong to the Ago1 subfamily, are highly expressed in various tissues, and function in the RNA-induced silencing complex (RISC) pathway (Meister et al., 2004). In contrast, members of the PIWI subfamily are specifically expressed in germ cells, with MIWI and MILI performing particularly important roles in spermatogenesis (Sasaki et al., 2003; Deng & Lin, 2002). MIWI was localized to the CB in spermatids. In a MIWI-null mouse model, the fully compacted CB was not found, with a non-compacted and diffuse CB instead being observed (Kotaja et al., 2006b), which suggests the importance of MIWI for the construction of the compacted CB. The Tudor family proteins Tudor repeat domain containing protein (TDRD)-1, -4, -5, -6, -7, and -9 were reported to be expressed in germ cells, and were shown to co-localize with Piwi family proteins in nuage. TDRD-1 has been detected in nuage in spermatogonia, in IMC in spermatocytes, and in CBs in

round spermatids (Chuma et al., 2003). The analysis of a TDRD-1-null mouse model revealed that TDRD-1 was essential for piRNA biogenesis, formation of IMC, and the correct localization of DDX4 in the CB (Hosokawa et al., 2007). In pachytene spermatocytes, TDRD-5 co-localized in IMC and the CB with TDRD-1, MIWI, MILI, and DDX4. In mid-pachytene spermatocytes, TDRD-5 was partly co-localized with TDRD-6 and -7; in diplotene spermatocytes, it was mainly co-localized with TDRD-6. In round spermatids, TDRD-5 co-localized in the CB with DDX4, MIWI, TDRD-1, -6, and -9, and interestingly, at the same time, TDRD-7 localized to another perinuclear structure distal from the CB (Yabuta et al., 2011), which seemed to be the second nuage component. In a TDRD-6-null mouse model, CB was not condensed, but rather was dispersed (Vasileva et al., 2009).

4.2 RNF17/TDRD-4 in nuage components

RNF17/TDRD-4 was first described as a germ cell-specific gene that contains repeated Tudor domains and a RING finger motif, which is present in ubiquitin E3 ligase. Yin et al. (1999, 2001) reported that RNF17 interacted with Mad family proteins and repressed transcription of the gene encoding the oncoprotein Myc. RNF17 contributes to activation of the transcription of Myc-responsive genes by supplying Mad proteins. In RNF17-null mice, which are sterile, spermatogenesis completely stops at the round spermatid stage (Pan et al., 2005), which indicates the importance of RNF17 in the development of spermatids. Pan et al. (2005) described a new dense granule that is positive for RNF17 and located in the perinuclear region near to the CB throughout spermatogenesis, and named it the "RNF17 granule." The morphological appearance of the RNF17 granule closely resembles that of the SB. If the SB contains RNF17, it would be one of the first SB components to be identified. Our preliminary data show that MAELSTRÖM (MAEL) is also localized to the SB, as well as other nuage components (Fig. 4).

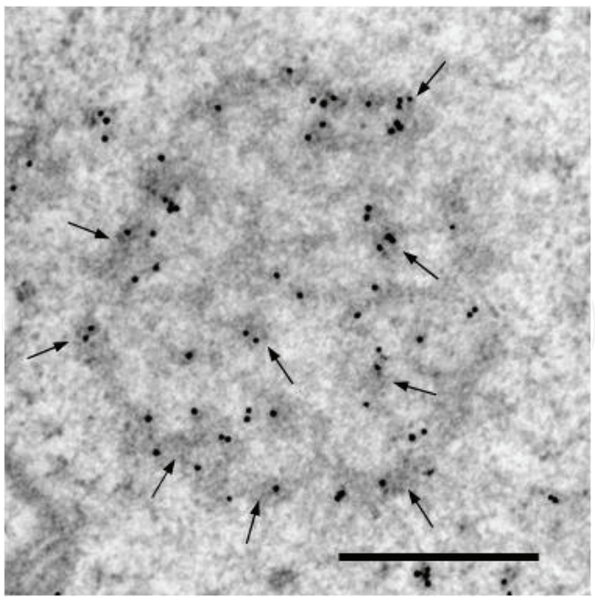

Fig. 4. Typical SB of a stage X pachytene spermatocyte stained for MAEL by immunoelectron microscopy (IEM). Gold particles showing MAEL antigenic sites are present on denser patches (arrows). The SB is 0.5–1.5 μm in diameter, and consists of a network of fibrils overlaid by denser patches of amorphous material. Bar = 0.5 μm.

4.3 DDX4 in nuage components

DDX4, the mouse homolog of the *Drosophila* vasa gene MVH, is an ATP-dependent RNA helicase belonging to the DEAD box family. DDX4 has been studied as a CB marker protein (Noce et al., 2001; Kotaja et al., 2006a; Kotaja & Sassone-Corsi, 2007; Onohara et al., 2010). In DDX4-null mice, spermatogenesis arrests at the prophase of meiosis, and most spermatocytes undergo degeneration. Furthermore, the amount of IMC in the spermatocytes is greatly reduced (Tanaka et al., 2000; Chuma et al., 2006). DDX4 was reported to interact with MIWI and MILI. Moreover, MILI$^{-/-}$ and DDX4$^{-/-}$ mice have similar phenotypes, which suggest that these proteins work cooperatively (Kuramachi-Miyagawa et al., 2004). In MILI-null mice, DDX4 was not detected in the nuage components (Kuramachi-Miyagawa et al., 2004), which suggests that DDX4 requires MILI to localize to the nuage components.

We previously reported the ultrastructual localization of DDX4 in nuage components during rat spermatogenesis (Onohara et al., 2010). Our report showed the wide expression of DDX4 in nuage components and other structures. In pachytene spermatocytes, DDX4 mainly localized to the surface of IMC, while loose aggregates were observed in the juxtanuclear area (Figure 5). The loose aggregate consisted of 70–90 nm particles and the material was dissociated from the IMC. In meiosis, structures positive for DDX4 disappeared, but small particles were identified in the cytoplasm (Figure 3). In spermatids, DDX4 mainly localized to the CB until step 6, after which the signal became gradually weaker, which suggests that the expression of CB proteins was dependent on spermatid step. No DDX4 signal was observed in the SB.

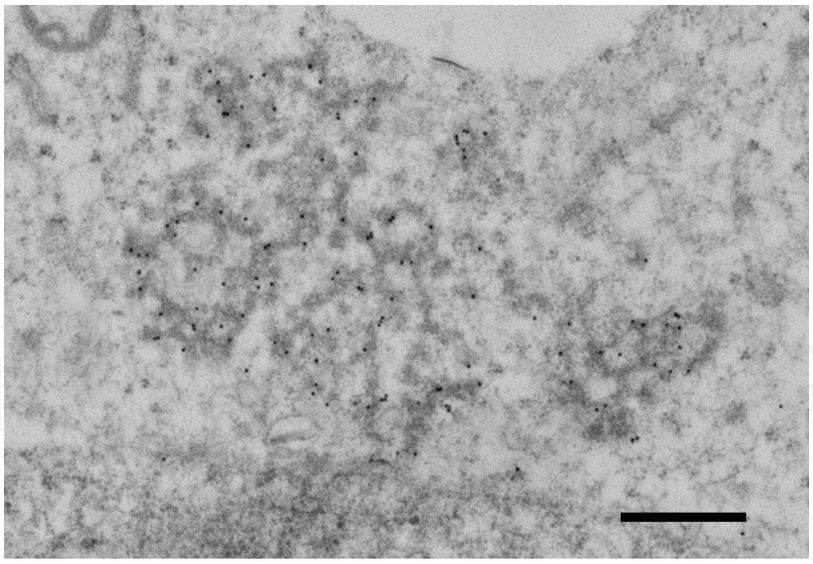

Fig. 5. IEM image of the loose aggregate consisting of 70–90 nm particles. Note that the particles contain gold particles used to label DDX4. Bar = 0.5 μm.

4.4 DDX25/GRTH in nuage components

DDX25 belongs to the DEAD-box family, and is essential for the completion of spermatogenesis. DDX25 acts as a hormone-dependent RNA helicase in Leydig cells and

germ cells, and is referred to as a gonadotropin-regulated testicular helicase (GRTH) (Tang et al., 1999). DDX25-null mice, which are sterile, showed arrest of spermatogenesis at the round spermatid stage (Sheng et al., 2006). Sato et al. (2010) treated spermatogenic cells with inhibitors of RNA polymerase and nuclear protein export. As a result, the amount of DDX25 in the nucleus increased, while the amount in the cytoplasm decreased. Furthermore, after treatment, the CB became smaller and condensed, as observed in DDX25-deficient mice. The results of co-immunoprecipitation studies using an anti-DDX25 antibody revealed that DDX25 bound to mRNAs, including DDX25 mRNA, in both the nucleus and cytoplasm. This suggested that the main function of DDX25 is to transport mRNA from the nucleus to cytoplasm, and to transport DDX25-ribonucleoprotein (RNP) complexes essential for controlling CB construction.

We ourselves found that DDX25 was abundant in small particles and loose aggregates of 70–90 nm particles in spermatocytes, but was less abundant in IMC (in press). The loose aggregate is closely associated with the nuclear envelope (Onohara et al., 2010). In spermatids, DDX25 is also localized to CB, but rarely or not at all to the SB.

4.5 Other proteins in nuage components

Haraguchi et al. (2005) reported the localization of the ubiquitin-conjugating enzyme cytochrome oxidase subunit I (COXI), Hsp70, and phospholipid hydroperoxide glutathione peroxidase (PHGPx) to the CB, and the localization of vimentin, the 20S proteasome subunit, and Lamp1 in the region around the CB. The protein composition of the CB overlapped with that of the aggresome, which indicates that the CB has a role not only in protein synthesis, but also in degradation, as an aggresome system.

Dicer, a key protein in the biogenesis of mature microRNAs and small-interfering RNAs (siRNAs), has a highly dynamic expression pattern in the CBs of meiotic spermatocytes and round spermatids, and interacts with DDX4 at its C-terminus, as revealed by immunoprecipitation (Kotaja et al., 2006a). The localization of Ago2, -3, Dcp1a, and GW182 to the CB has been reported. Because these proteins are known to be markers of the processing body (P-body), a dense granule present in the cytoplasm in somatic cells (Kedersha et al., 2005), the CB may have functions in common with the P-body.

The Kinesin motor protein KIF17b is involved in the nucleus-to-cytoplasm transport of RNA, and was reported to co-localize with MIWI in the CB of round spermatids (Kotaja et al., 2006b). It may therefore be involved in the microtubule-mediated transport of CBs.

Brunol2/CUG-BP1/CELF1 is a homolog of the *Drosophila melanogaster* protein Bruno, and is a member of the CELF family that controls the splicing of pre-mRNAs. Bruno was found in nuage in ovaries and embryos (Snee & Macdonald, 2004). Spermatogenesis was arrested in step 7 spermatids in Brunol2-deficient mice (Kress et al., 2007). Recently we showed that Brunol2 localizes to the CB and other nuage components during rat spermatogenesis (in press).

HuR, an RNA-binding protein in somatic cells that controls the stability or translation of AU-rich mRNAs, was found in the CB and co-localized with DDX4 (Nguyen et al., 2009). HuR is involved in the shuttling of mRNAs from the nucleus to the CB for storage and protection against degradation.

MAEL, which is an ortholog of the *Drosophila* protein, MAELSTROM, is localized to the CB and unsynapsed chromosomes in spermatocytes, and has been implicated in the silencing of unsynapsed chromatin in spermatocytes during meiosis (Costa et al., 2006). MAEL co-localizes with DDX4, MIWI2, TDRD-9, and the P-body components Dcp1a, DDX6, GW182, and Xrn1 in the CB, but does not localize to the IMC. In MAEL-knockout mice, Piwi-interacting RNA (piRNA) production and post-transcriptional transposon silencing are perturbed in fetal gonocytes, suggesting that MAEL may be involved in these processes (Aravin et al., 2009).

GASZ, a germ cell protein with ankyrin repeats, a sterile alpha motif, and a leucine zipper, is localized to the CB. In GASZ-null mice, levels of MILI/piRNAs in the CB were dramatically reduced, and spermatogenesis was arrested before meiosis (Ma et al., 2009).

In 2010, MOV10L1, a member of the DEAD-box RNA helicase family essential for retrotransposon silencing in mouse germ cells, was reported to be expressed in spermatogonia, pachytene spermatocytes, and all intermediate forms in the mouse testis, and to interact with MILI, MIWI, and heat shock 70 kDa protein 2 (Frost et al., 2010). MOV10L1 appeared in a complex termed "MIWI-MILI block," which may be identical to IMC.

MitoPLD, a member of the phospholipase D superfamily, is abundant in the outer membrane of mitochondria, and degrades cardiolipin, yielding the lipid signaling molecule phosphatidic acid (PA). In MitoPLD-knockout mice, the nuage was arranged like a donut in the perinuclear region, and γ-tubulin was detected in the center of the aberrant nuage in spermatogonia, suggesting that MitoPLD may be involved in microtubule-dependent nuage transport. Furthermore, in MitoPLD-null mice, spermatogenesis arrests in late or post-meiotic spermatocytes, and the IMC is absent from spermatocytes. In addition, TDRD-1 and mitochondria aggregate to the pericentriolar region instead of the perinuclear region in gonocytes. In mice lacking Lipin1, which metabolizes PA, nuage increases in size and density, and the localization of TDRD-1 is altered. These results suggest that MitoPLD and/or PA may be involved in the generation of IMC and the correct localization of nuage and maintenance of its components (Huang et al., 2011; Watanabe et al., 2011).

NANOS, which is essential for primordial germ cell (PGC) in *Drosophila*, plays a critical role in spermatogenesis, particularly during meiosis. NANOS2 blocks meiosis by suppressing Stra8, an inducer of meiosis (Saga, 2008). NANOS1, which is known to regulate the translation of specific mRNAs, is found in the CB and co-localizes with PUMILIO protein, as well as the microRNA biogenesis factor GEMIN3 (Ginter-Matuszewska et al., 2011).

During the last decade, many nuage constituent proteins have been identified. Above all, it is noteworthy that RNA-binding proteins and RNA-regulating proteins are abundant in nuage, indicating that nuage may contribute to RNA silencing and RNA decay during spermatogenesis. Many nuage proteins co-localize simultaneously with other proteins in the same compartment, suggesting that most nuage components may function cooperatively during germ cell development. Functional nuage proteins are listed in Table 1.

Table 1. Functional nuage-related proteins that play roles in RNA silencing, RNA decay, and nuage morphogenesis.

Component	Domain/Function	Where found
TDRD-1	Tudor proteins; contain Tudor domains; essential for piRNA biosynthesis	Spermatogonia, spermatocytes, round spermatids
TDRD-5		Spermatocytes, spermatids
TDRD-6		Spermatocytes, spermatids
TDRD-7		Spermatids
TDRD-9	Tudor protein; ATPase/DExH-type helicase; essential for piRNA biosynthesis	Spermatocytes
TDRD-4/RNF17	Tudor protein; contains a RING finger motif	Spermatogonia, spermatocytes, round spermatids
Ago2/Ago3	Argonaute family protein; component of the RISC	Spermatids
MILI	Piwi family proteins; associate with piRNA	Spermatocytes
MIWI2		Spermatids
DDX4/MVH	DEAD box RNA helicases	Spermatocytes, spermatids
DDX6		Spermatocytes
DDX25		Spermatocytes, spermatids
MOV10L1	DEAD box RNA helicase family; silencing retrotransposons in germ cells	Spermatogonia to pachytene spermatocytes
Dicer	RNase III; major protein in the biogenesis of small RNAs	Spermatocytes, round spermatids
Dcp1a	P-body markers	Pachytene spermatocytes, spermatids
GW182		Spermatids
Xnt1		Spermatids
Brunol2/CUG-BP1	CELF family protein; controls the splicing of pre-mRNAs	Spermatocytes, spermatids
GASZ	Essential for fertility in females and spermatogenesis	PGCs, gonocytes, spermatogonia, spermatocytes
HuR	RNA-binding protein in somatic cells; controls the stability or translation of AU-rich mRNAs	Spermatids
KIF17b	Kinesin motor protein	Round spermatids
MAEL	HMG box protein	Gonocytes, meiosis, spermatocytes, round spermatids
NANOS1	Suppression of meiosis; regulates the translation of specific mRNA	PGCs, gonocytes, meiosis, spermatids
PUMILIO2	RNA-binding protein; translational regulation	Round spermatids
GEMIN3	MicroRNA biogenesis factor	Round spermatids

5. Functions of nuage proteins and their relationships to morphological features

5.1 A transcription context specific for spermatogenic cells

Proteins such as Dicer and PIWI family proteins that are involved in the posttranscriptional regulation of microRNAs during spermatogenesis are highly concentrated in nuage. Dicer processes the precursors of both siRNAs and microRNAs (miRNAs) into small mature RNAs in the cytoplasm in somatic cells. These small mature RNAs are assembled into an RISC, which contains Argonaute family proteins and causes translational repression or mRNA cleavage in the cytoplasm in somatic cells (Stefani & Slack, 2008). Ago family proteins, Dicer, miRNAs, and miRNA-repressed mRNA are localized to the P-bodies of somatic cells, suggesting that RNA silencing and RNA disruption may occur there (Sen & Blau, 2005). Some P-body markers are also expressed in the CB, suggesting that the CBs of germ cells may be involved in RNA silencing and RNA disruption, as in the P-bodies of somatic cells (Kotaja et al., 2006a). The function of the P-body in somatic cells has been studied extensively (Sen & Blau, 2005). The CB has many features in common with the P-body, strongly suggesting that the CB may play similar roles to the P-body.

5.2 Function of the CB in mRNA storage and transport

Söderström and Parvinen (1976) studied RNA synthesis during spermatogenesis by autoradiography using tritiated uridine. In step 1–8 spermatids, the rate of RNA synthesis was low in the cell as a whole, but very high in the CB, and was arrest in step 8 spermatids in both places. Therefore, it is likely that CBs in spermatids contain RNAs, which may encode proteins involved in the regulation of spermiogenesis in late spermatids.

Kotaja et al. (2006a) performed *in situ* hybridization to determine whether or not the CB contains miRNA, and demonstrated the localization of miRNAs in the CB. HuR is involved in the transport of mRNAs from the nucleus to the CB so that they can be stored and protected against degradation (Nguyen et al., 2009). Kotaja et al. (2006b) detected KIF17b, known to bind to mRNAs, in the CB, and suggested that the CB might be involved in the transport of mRNAs in early spermatids.

These studies strongly suggest that the CB is also a site for the storage and transport of mRNAs that encode proteins with roles in late spermatids.

5.3 The function of nuage components other than the CB

During the differentiation of spermatogonia and spermatocytes, several nuage structures with specific shapes appear, persist for a period of time, and then disappear, as described above. The other organelles, including mitochondria, the Golgi complex, and lysosomes, do not undergo marked changes in morphology. However, the differentiation of spermatids to spermatozoa is dynamic and dramatic. Spermatids transform into spermatozoa, whose role is to enable transport of DNA to the target cell, the oocyte, through an extraordinarily intricate process of cell differentiation termed spermiogenesis, which lasts approximately 20 days in rats (Clermont et al., 1959) and 24 days in humans (Heller & Clermont, 1964). Major events that occur during spermiogenesis include

formation of the acrosome, nuclear condensation, formation of the tail, trimming of the cytoplasm, and organelle reorganization (de Kretser & Kerr, 1988; Clermont et al., 1993). Ultrastructural studies have described various specialized structures, including the radial body-annulate lamellae complex; small puffs of a fine, filamentous, fuzzy material; a granulated body composed of fine, dense granular materials; a reticulated body consisting of several dense anastomosed cords; and a large, dense granule surrounded by crescentic mitochondria (Clermont et al., 1993), which we termed the mitochondria-associated granule (MAG).

Our own IEM studies provide evidence that some of these structures contain nuage proteins.

1. The radial body-annulate lamellae complex, a membrane-bound structure that is continuous with the endoplasmic reticulum (ER), is observed in the cytoplasmic lobe of late spermatids, and is believed to be the site at which resorption of the ER membrane occurs (Clermont & Rambourg, 1978). No nuage proteins have yet been detected in this structure. Therefore, it seems that it is not a structural site for the function of nuage proteins.
2. Small puffs have no limiting membrane, are attached to the cytoplasmic surface of the ER, and are observed in step 8–10 spermatids. The only nuage protein to have been detected in the puffs so far is Brunol2 (in press).
3. Granulated bodies appear in the cytoplasmic lobes of step 14 spermatids, are most abundant in step 17 spermatids, and decrease in number during subsequent stages of spermatogenesis. It was reported by Clermont et al. (1990) that they contain outer dense fiber (ODF) proteins. It is assumed that ODF polypeptides are temporarily stored in these bodies (Clermont et al., 1993). However, their function remains unclear. Our preliminary IEM studies detected BRUNOL2, DDX25, NANOS1, and MAEL in these bodies, but not DDX4 (unpublished data). Gold particles that were used to label these nuage proteins were confined to the fine granular matrix.
4. The reticulated body first appears in the cytoplasm of step 14 spermatids and completely disappears in step 18 spermatids (Clermont et al. 1990). The body is characterized by several dense, anastomosed cords with a width of 80–100 nm. We detected BRUNOL2, DDX25, NANOS1, and MAEL in this structure by IEM, but not DDX4. Other than the nuage proteins, only cathepsin H has so far been detected in the structure (Haraguchi et al., 2003). The nature and function of this structure remains unclear.
5. The MAG is 1–2 μm in diameter, is composed of a fine granular material, and appears in the elongated cytoplasmic lobes of step 9–17 spermatids (Figure 6). Its strongest characteristic is its close association with mitochondria (Clermont et al., 1990). We detected the nuage proteins BRUNOL2, DDX4, DDX25, NANOS1, and MAEL in this structure in our preliminary studies (unpublished data). Although dense materials in the MAG were assumed to be a source of ODF proteins, the results of IEM experiments that detected no ODF protein signals in the MAG indicates that this is not the case (Clermont et al., 1990). No proteins other than the above five nuage proteins have so far been detected in this structure. Although the nature and function of the MAG are unknown, the existence of nuage proteins may provide important clues about the function of the MAG.

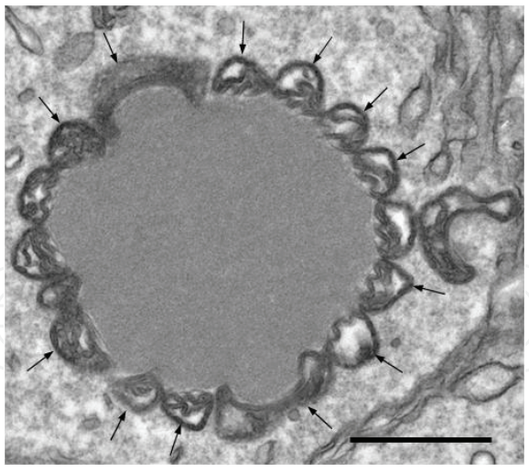

Fig. 6. Electron micrograph of an MAG in a step 16 spermatid. The MAG is a large spherical mass with a diameter of 1–2 μm, composed of granular material and surrounded by mitochondria. Bar = 1 μm.

In the neck cytoplasm of step 11 spermatids, small, discrete, dense masses are frequently observed (Fawcett & Phillips, 1969), closely associated with other structures or free in the cytoplasm. One, which is associated with the plasma membrane and subsequently moves to a position at the junction of the middle and principal regions of the tail, is known as the annulus. It was assumed that the annulus was derived from the CB (Fawcett et al., 1970). However, our study (Onohara et al., 2010) and preliminary results (unpublished data) indicate a lack of signals in the annulus for the nuage proteins listed above, indicating that the CB and the nuage proteins are not involved in the formation of the annulus. Spherical masses are frequently observed at the base of the flagellum of late spermatids (Fawcett & Phillips, 1969). Our IEM studies show that these masses are positive for Dicer1 and MAEL (unpublished data). Interestingly, these Dicer1-positive particles are maintained in epididymal sperm, suggesting that they are carried into the cytoplasm of the ovum. Furthermore, a less electron-dense mass composed of fine filamentous materials surrounds the connecting piece in late spermatids (Clermont et al., 1993). This structure is positive for BRUNOL2 (unpublished data). In the neck region, small dense particles are present. These particles contain small amounts of the nuage proteins BRUNOL2, DDX25, NANOS1, and MAEL. In addition, BRUNOL2 and MAEL have been detected in large aggregates of free ribosomes and other unknown materials in the residual bodies of step 19 spermatids (unpublished data). Thus, nuage proteins are associated with various structures that are unrelated to the nuage. Although it is not clear whether the nuage proteins function there, it is likely that they are active in some of the structures mentioned above.

6. Conclusion - Future nuage research

In classic reports, the CB was detected in either the nucleus or the cytoplasm, but there was no obvious evidence to confirm either location. More recent studies have suggested that the IMC is a source of the CB. However, in TDRD-1-null mice, CBs were present in spite of the fact that the IMC disappeared (Chuma et al., 2006). It therefore appears that IMC is not essential for the formation of the CB. Recently, Meikar et al. (2010) purified the CB by immunoprecipitation and found that it contained more than 100 proteins. MitoPLD is essential for the formation of IMC, while PA, which is produced by MitoPLD, affects the

architecture of the CB (Huang et al., 2011; Watanabe et al., 2011), suggesting that various proteins and factors are involved in the construction of nuage. Nuage has various forms and is associated with other subcellular organelles such as the nucleus, mitochondria, and the Golgi apparatus (Parvinen, 2005), suggesting that communication between nuage and the other organelles is important for the completion of spermatogenesis. More detailed studies of the properties of nuage will elucidate its function and origin.

One big question remains to be answered: during meiosis, after chromosome recombination has occurred, where do the nuage components go, how are they divided between the daughter cells equally, and how is the nuage reformed? It is also unclear how nuage or nuage components contribute to meiosis. MAEL suppresses transcription via the small RNA pathway during meiosis (Costa et al., 2006). Defects in several nuage components cause the arrest of spermatogenesis before meiosis, suggesting that the correct function of these components is essential for spermatogenesis. Microtubule-dependent movement of the CB is critical to enable communication with other organelles (Kotaja et al., 2006b; Huang et al., 2011; Watanabe et al., 2011).

Frost et al. (2010) suggested that the piRNA silencing complex may be constructed hierarchically. In other reports, nuage protein immunofluorescence staining patterns in some cases overlap completely, but in others only partially, even in the same stage of spermatogenesis (Figure 7). These observations suggest that nuage is formed hierarchically by the coalescence of small complexes assembled from a few proteins. To confirm whether this is indeed the case, we need to clarify the precise relationships among nuage components.

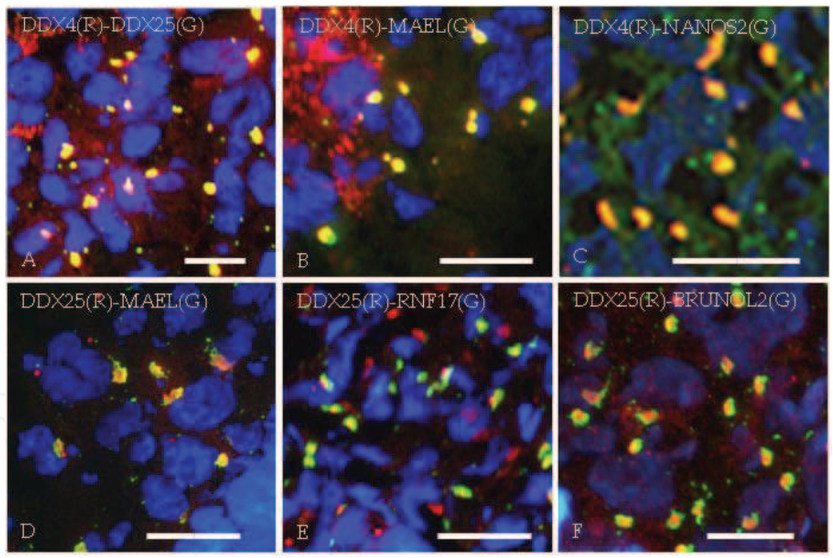

Fig. 7. Immunofluorescence staining of rat seminiferous epithelium with a combination of two different antibodies. Different antigens were stained using Alexa Fluor® 488 and Alexa Fluor® 568. The images were merged using Adobe Photoshop®. Staining for (A) DDX4 and DDX25, (B) DDX4 and MAEL, (C) DDX4 and NANOS2, (D) DDX25 and MAEL, (E) DDX25 and RNF17, and (F) DDX25 and BRUNOL2. All proteins examined were localized to CBs and two staining patterns were seen: 1) the patterns for both proteins completely overlapped within the CBs (A–C); and 2) the patterns for both proteins partially overlapped within the CBs (D–F). This clearly indicates that some nuage proteins distribute homogeneously, while others are segregated within CBs. Bar = 50 μm.

Figure 8 summarizes the possible functions of nuage in molecular biology and morphology. While the function and significance of nuage in spermatogenic cells have begun to be elucidated, they still remain largely unclear. Collecting information on nuage components and clarifying the function of other nuage-like structures may reveal insights into the significance of nuage in the development and maintenance of germ cells.

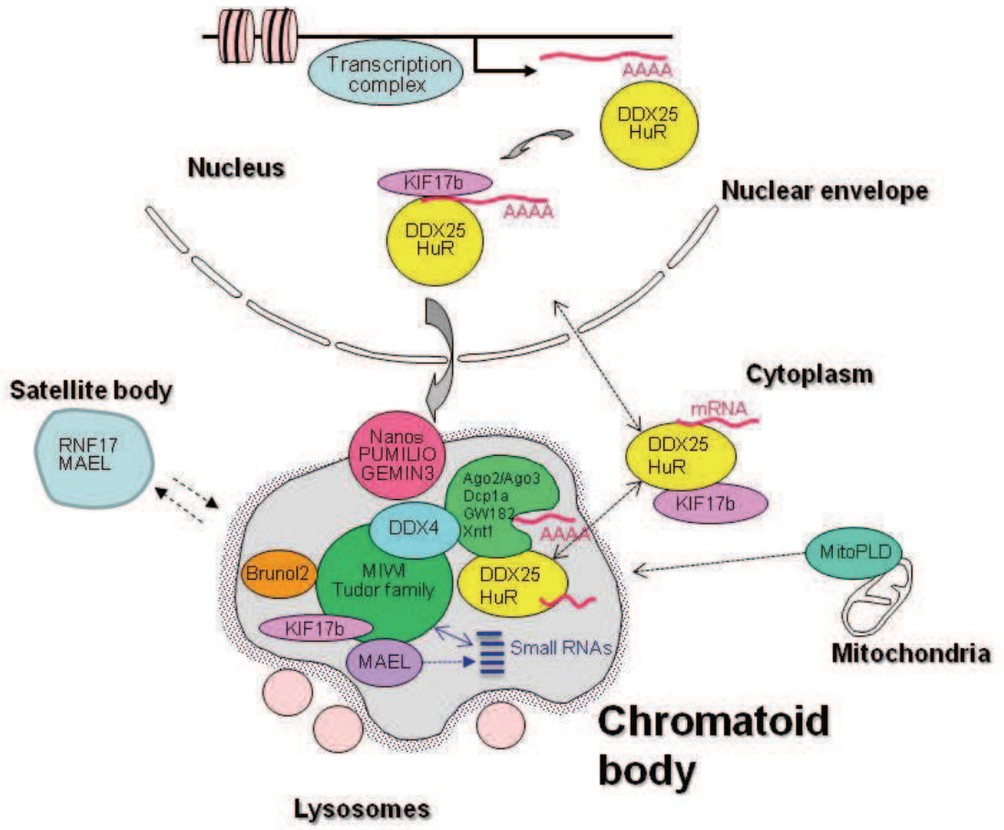

Fig. 8. Possible functions of nuage. Haploid gene products, including RNA binding proteins such as DDX25 and HuR, and microtubule-associated proteins such as KIF17b, bind to RNP. The mRNA-RNP complex is transported from the nucleus to the cytoplasm via the nuclear pore complex, or directly, to the CB. The movement of this complex is known to be supported by the microtubule network. In the cytoplasm, the CB frequently associates with the nuclear pores to interact with mRNAs. The CB contains RNA-binding and RNA-processing proteins such as DDX4, DDX6, and DDX25, and components of the RNA silencing and RNA decay pathways, such as small RNAs, Dicer, MAEL, Piwi family proteins, and Tudor family proteins. Furthermore, the CB contains P-body components such as Ago2, Dcp1a, GW182, and Xnt1, and thus, like the P-body, functions as a post-transcriptional regulator. The CB also contains an RNA-protecting protein, HuR. mRNAs stored in the CB are released into the cytoplasm in response to appropriate stimuli and are translated. Dicer produces small RNAs from their precursors in the CB. The mitochondrial membrane protein MitoPLD is involved in the movement and construction of the CB. The CB also contains the ubiquitin-conjugating enzyme E2. Some CB proteins are polyubiquitinated and degraded by proteasomes located on the surface of the CB (dots). Other components are degraded by lysosomes in close contact with the CB.

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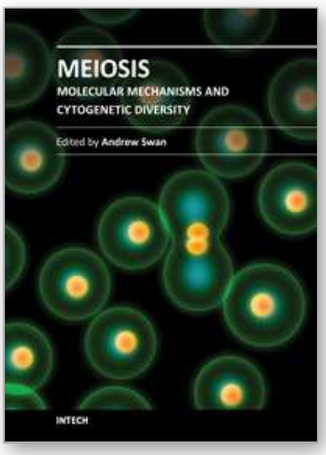

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Meiosis, the process of forming gametes in preparation for sexual reproduction, has long been a focus of intense study. Meiosis has been studied at the cytological, genetic, molecular and cellular levels. Studies in model systems have revealed common underlying mechanisms while in parallel, studies in diverse organisms have revealed the incredible variation in meiotic mechanisms. This book brings together many of the diverse strands of investigation into this fascinating and challenging field of biology.

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