

Review

Phase Separation during Germline Development

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Phase separation has emerged as a new key principle of intracellular organization. Phase-separated structures play diverse roles in various biological processes and pathogenesis of protein aggregation diseases. Recent work has revealed crucial functions for phase separation during germline development. Phase separation controls the assembly and segregation of germ granules that determine which embryonic cells become germ cells. Phase separation promotes the formation of the Balbiani body, a structure that stores organelles and RNAs during the prolonged prophase arrest of oocytes. Phase separation also facilitates meiotic recombination that prepares homologous chromosomes for segregation, and drives the formation of a liquid-like spindle domain that promotes spindle assembly in mammalian oocytes. We review how phase separation drives these essential steps during germline development.

When the Germline Meets Phase Separation

The cells that will become eggs and sperm in sexually reproducing organisms are called germ cells. They are set aside early during embryo development to ensure that they develop into germ cells instead of activating somatic differentiation programs. In some species, the so-called germ granules comprising RNAs and RNA-associated proteins determine where the germ cells will form [1–5]. How germ granules are organized has long been unclear. Pioneering work in *Caenorhabditis elegans* revealed that germ granules are organized as biomolecular condensates via phase separation [6]. Subsequently, many other structures were found to form by phase separation, including those that have key functions in later stages of germline development. In this review we summarize and discuss how phase separation facilitates germline development from germ cell fate determination to the maturation of an **oocyte** (see [Glossary](#)) into a fertilizable egg.

What Is Phase Separation?

Classical organelles such as the nucleus, the endoplasmic reticulum, and mitochondria are compartments that are surrounded by membranes. However, P granules, and many other cellular compartments, lack a physical barrier that separates their internal components from the surrounding environment. They are biomolecular condensates formed by phase separation. In simple terms, phase separation can occur when the interactions between molecules (e.g., proteins) are more thermodynamically favorable than the interactions between these molecules and their solvent (e.g., the cytosol) [7]. As a result, the molecules and the solvent demix into a condensed phase of a smaller volume and a dilute phase of larger volume [7]. The physical principles of phase separation and related theoretical and experimental concepts are beyond the scope of this review, but have been extensively reviewed elsewhere [8–11].

In biological systems, phase separation of proteins is mostly driven by weak interactions between these proteins. These multivalent interactions can be achieved with (i) multiple modular interaction domains, and (ii) large intrinsically disordered regions [7]. Intrinsically disordered regions lack a

Highlights

Many non-membrane-bound compartments in cells organize by phase separation. Recent research implicates phase separation in different steps during germline development.

Phase separation drives the formation and partitioning of germ granules that determine germ cell identity during early embryogenesis.

Phase separation promotes the interactions between ribonucleoprotein particles and membrane-bound organelles to form the Balbiani body in *Xenopus* and zebrafish oocytes.

Phase separation mediates pairing and synapsis of homologous chromosomes during meiotic recombination in various species.

Phase separation enriches microtubule regulatory proteins and promotes acentrosomal spindle assembly in mammalian oocytes.

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defined tertiary structure but contain low-complexity amino acid sequences that can engage in short-lived interactions with low affinity and no stereospecificity [7]. In addition, phase separation of proteins can be modulated by post-translational modifications and changes in the **physicochemical environment**, making it ideal for stress responses and signal transduction [12–14].

Phase separation plays fundamental roles in cell organization, and the number of subcellular compartments that form by phase separation is continuously expanding. It drives the assembly of RNA granules, the best-known **non-membrane-bound compartment**, in the cytosol and in the nucleus [15,16]. Moreover, phase separation plays pivotal roles in the formation of various biochemical interaction hubs. By locally concentrating factors such as enzymes, RNA, and DNA it can promote chemical reactions and activate the transduction of signals [17–26]. Most of these condensate-like hubs assemble via liquid–liquid phase separation, and have properties that resemble those of liquid droplets [8,27,28]. They are typically spherical and fuse into larger droplets upon contact. Their constituents are dynamic and exchange rapidly with the surrounding environment. Liquid-like condensates can transit into hydrogels or solids under some physiological or pathological conditions. During this process, the internal organization of the condensates changes from disordered to ordered, the mobility of their components drastically reduces, and the reversibility is compromised (Box 1) [29–32]. Such phase transitions can help to transform a condensate into a storage compartment, but may also lead to the formation of neurotoxic structures in neurological diseases. Accumulating evidence also reveals extensive interactions between non-membrane-bound and **membrane-bound compartments** [33]. Membranes provide platforms for the assembly of condensates and regulate their dynamics. In return, the condensates modulate the transport, clustering, or function of membrane-bound organelles.

Box 1. Condensates with Different Material Properties

Biomolecular condensates can have different material states, including the liquid, hydrogel, and amyloid states. How can these states be distinguished *in vitro* and *in vivo*?

- (i) Liquid-like condensates behave similarly to liquids. They are spherical when suspended in solution owing to surface tension. They can quickly fuse into larger droplets upon contact. Like liquids, they have the capacity to wet a surface. They can even flow and drip off the surface under shear stress [6]. These morphological features indicate that the constituents of a liquid-like condensate are mobile. If the liquid-like condensate is photobleached in one region, the signal recovers in seconds or dozens of seconds. While the signal recovers in the bleached region, the nonbleached region becomes dimmer owing to rapid internal rearrangement [6,98]. Another feature of liquid-like condensates is their reversibility. The droplet undergoes continuous condensation and dissolution. Post-translational modifications or environmental alterations can quickly dissolve the droplet [98]. P granules in *C. elegans* oocytes and the LISD domains in mammalian oocytes are two typical examples of liquid-like condensates [6,98].
- (ii) In contrast to liquid-like condensates, the constituents of hydrogels are immobile. Signals only recover slightly after photobleaching. A remarkable feature of a hydrogel is its elasticity. The hydrogel can resist distorting forces and return to its original shape when the force is removed. Whereas liquid droplets can wet a surface and fuse with each other, hydrogels largely retain their shape when they meet a surface or another hydrogel. Liquid and hydrogel are both reversible under physiological conditions, although a hydrogel is more stable than a liquid. A well-known case of a reversible hydrogel is the FG-repeat domains of nucleoporins that form the permeability barrier of nuclear pores [112]. It is noteworthy that many liquid-like condensates undergo liquid-to-gel transitions over time. Biological condensates are thus often in an intermediate state between a liquid and a gel, but not a pure gel. This is reflected by a gradual reduction of the recovery rates after photobleaching at sequential timepoints upon condensate formation [113].
- (iii) Amyloid-like fibers/assemblies can be formed physiologically or pathologically. It is generally thought that physiological amyloids are reversible, whereas pathological amyloids are not. Their constituents are typically immobile, and show almost no recovery after photobleaching. Amyloid-like fibers/assemblies can be formed through liquid-to-solid transitions [29–32]. It is possible that multivalent, transient, weak interactions among proteins drive quick liquid–liquid phase separation first. Concentrated proteins in a liquid-like condensate have a higher chance of interacting and can hence form stable amyloid-like interactions over time. The gradual increase of stable interactions converts the condensate into a hydrogel or amyloid.

Glossary

Autophagy: a process in which cells digest their own components or invading microorganisms with the aid of lysosomes. The breakdown products can be recycled for essential cellular processes.

Amyloid: an insoluble polymer formed by stacking of β -sheets. Amyloids have either physiological or pathological functions.

Blastomere: after fertilization, the zygote undergoes multiple rounds of cleavage. The cells of the cleavage stage embryo are termed blastomeres.

Centrioles: the core component of canonical centrosomes, consisting of singlet, doublet, or triplet microtubules arranged in ninefold symmetry; centrioles organize pericentriolar material.

Coiled coils: a left-handed superhelical array formed by winding two or more α -helices around each other.

Critical concentration: the protein concentration above which proteins self-aggregate into a biomolecular condensate.

1,6-Hexanediol: an aliphatic alcohol that can disrupts weak hydrophobic interactions. It is commonly used to probe the nature of interactions within liquid-like condensates.

Homologous chromosomes

(homologs): each chromosome is present in two copies, which are referred to as homologous chromosomes.

Membrane-bound compartment: a specialized unit within the cell that is surrounded by a single or double lipid bilayer membrane. The membrane separates the interior of the compartment from the rest of the cytoplasm.

Non-membrane-bound

compartment: a specialized unit within the cell formed by spontaneous phase separation of proteins and/or nucleic acids. A lipid bilayer membrane is not necessary to separate the compartment interior from the rest of the cytoplasm. Some compartments (i.e., the Balbiani body) are formed by phase separation, but contain membrane-bound compartments (i.e., mitochondria). It might hence be misleading if they are referred to as 'membraneless compartments'. Because these compartments are not surrounded by membranes, it is still appropriate to refer to them as 'non-membrane-bound compartments'.

Phase Separation during Germ Granule Assembly

Germ granules are prominent examples of phase-separated structures in the germline. In some species, such as *C. elegans*, *Drosophila*, zebrafish, and *Xenopus*, germ granules are already deposited in the oocyte. Upon fertilization, they determine which regions of the cytoplasm will eventually become the germ cells. In others, including all mammals, the germ cells only become determined during early embryo development by signals from the surrounding somatic cells. These germ cells also assemble germ granules, albeit *de novo* [5].

The most fascinating property of germ granules is their dynamic reorganization throughout oogenesis and embryogenesis, which can be explained by principles of phase separation [1–3,5]. In general, germ granules are first formed in close vicinity of the nucleus in **oogonia**, early oocytes, or **nurse cells** [1–5]. Germ granules then increase in size and number, and redistribute during oocyte growth. Some of them form contacts with mitochondria and other membrane-bound organelles [3–5]. In organisms such as *Xenopus*, germ granules and associated membrane-bound organelles coalesce to form the Balbiani body. The Balbiani body is fragmented as it translocates to the **vegetal pole**, and reassembles into multiple large assemblies upon fertilization [4,34]. In other organisms such as *C. elegans*, there is no Balbiani body, but the P granules still undergo continuous dissolution and condensation [1,6]. In this review we use P granules of *C. elegans* and polar granules of *Drosophila* as two examples to illustrate how phase separation contributes to the assembly, dynamics, and functions of germ granules.

Phase Separation Controls P Granule Organization and Function in *C. elegans* Embryos

P granules of *C. elegans* interact with the nuclear envelope in growing oocytes, and detach from the nucleus before fertilization [2]. They are initially evenly distributed in the newly fertilized one-cell zygote, and then become enriched in the posterior half of the cell upon symmetry breaking as the embryo becomes polarized (Figure 1A) [1,2,6]. Subsequently, the zygote divides into a P granule-free anterior cell (AB) and a P granule-containing posterior cell (P1). The posterior cell eventually gives rise to the primordial germ cells (Z2 and Z3) after four rounds of division (Figure 1A). P granules are maintained in the germline **blastomeres** (P2, P3, P4) throughout the divisions and reattach to the nucleus at the 4–8 cell stage.

Multiple lines of evidence indicate that P granules are assembled via liquid–liquid phase separation [6]. P granules are spherical in the cytosol, but become nonspherical when they attach to the nuclear membrane, resembling liquid droplets wetting a surface. Moreover, P granules occasionally fuse with each other – another characteristic feature of liquid droplets. **Photobleaching** showed that components of P granules undergo rapid internal rearrangement and exchange with soluble components in the cytoplasm, both hallmarks of structures that form by phase separation (Box 2). Notably, multiple P granule proteins phase separate into P granule-like droplets and behave like a liquid or hydrogel *in vitro* [35–37]. Among them, the RGG motif-containing proteins PGL-1 and PGL-3 form a liquid-like core, whereas MEG-3 and MEG-4 form a hydrogel-like dynamic shell at the periphery that may stabilize the liquid-like PGL phase (Figure 1B) [38]. DEAD-box RNA helicases, including LAF-1 and GLH-1, may regulate P granule assembly and disassembly through a conserved ATP-dependent mechanism [35,39,40].

Phase separation controls not only the formation of P granules but also their distribution within the *C. elegans* embryo. The asymmetric enrichment of P granules in the posterior half of the embryo was initially thought to be driven by cytoplasmic flows. However, particle-tracking experiments revealed that similar amounts of P granules moved in anterior and posterior directions, excluding flow as the driver of P granule redistribution [6]. Spatiotemporal monitoring of P granule size

Nurse cells: these cells transfer their cytoplasm (RNAs and proteins) into the oocyte to support oocyte development in the *Drosophila* egg chamber.

Oocyte: a female germ cell that becomes a fertilizable egg after meiosis.

Oogonia: the plural of oogonium. Oogonia are formed by mitosis of primordial germ cells, and are precursor cells of primary oocytes.

Pericentriolar material: a mixture of hundreds of proteins which help to nucleate and anchor microtubules.

Photobleaching: permanent loss of the ability of a fluorophore to fluoresce after excitation with high-intensity illumination.

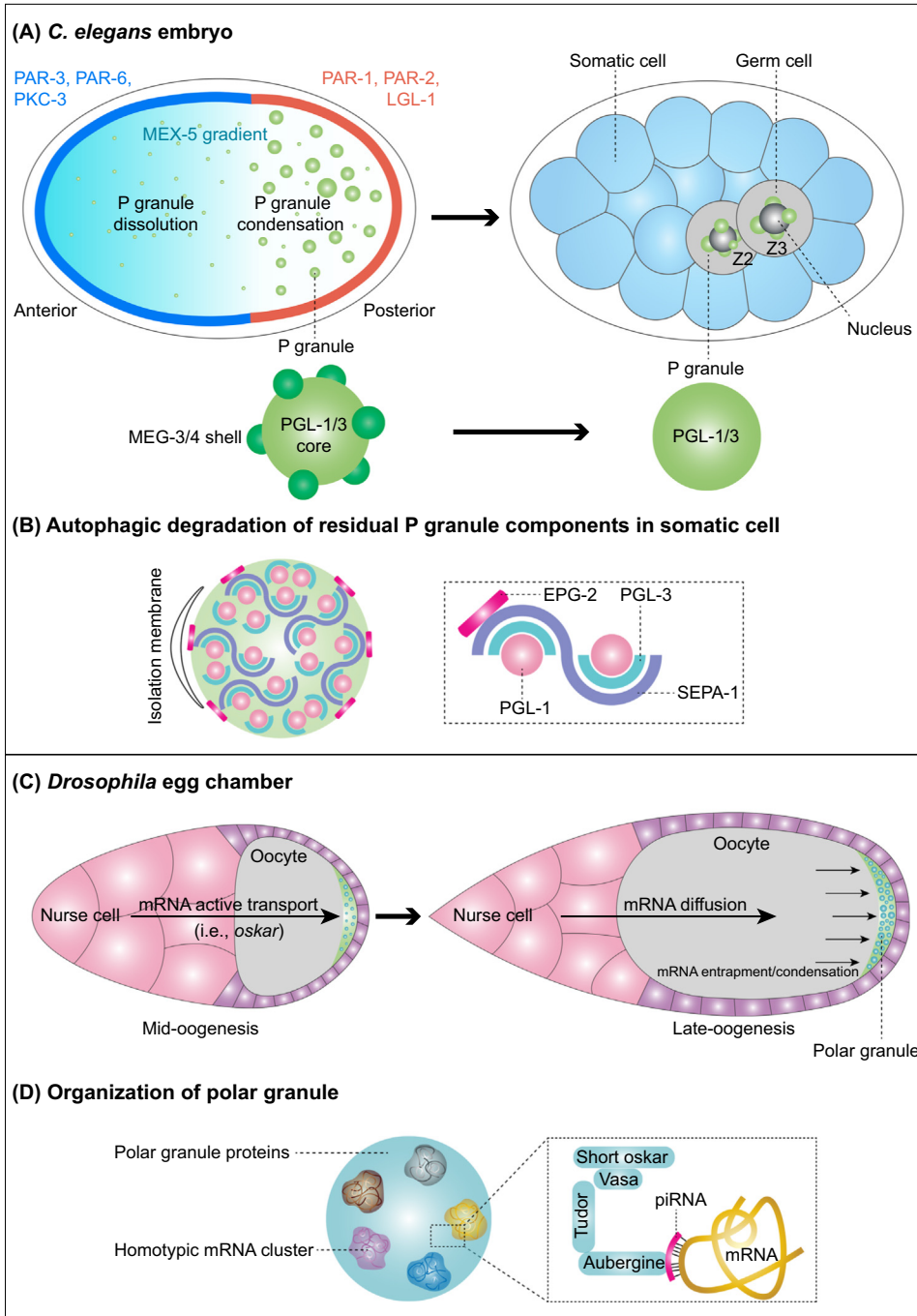
Photoconversion: change in excitation and emission spectra of a fluorophore after excitation at a specific wavelength (usually 405 nm).

Physicochemical environment: the condition jointly created by different physical and chemical parameters, such as temperature, pH, and ionic strength.

RanGTP pathway: the microtubule nucleation pathway mediated by a chromosome-centered gradient of GTP-loaded Ran GTPase.

Synapsis: describes the state in which the homologs are fully paired by the synaptonemal complex.

Vegetal pole: in *Xenopus* and zebrafish, the animal–vegetal axis appears in the primary oocyte. The animal hemisphere contains the nucleus and the vegetal hemisphere contains the Balbiani body.



Trends in Cell Biology

Figure 1. Phase Separation Regulates Germ Granule Assembly and Segregation. (A) The *Caenorhabditis elegans* one-cell embryo is polarized through asymmetric partitioning of PAR-3/PAR-6/PKC-3 and PAR-1/PAR-2/LGL-1, which leads to a cytosolic gradient of the RNA-binding protein MEX-5. MEX-5 competes with PGL-3 and MEG-3 for mRNA binding, and thus impairs their phase separation and induces a P granule gradient in the opposite direction to the MEX-5 gradient. PGL-1 and PGL-3 form a liquid-like core of the P granule, whereas MEG-3 and MEG-4 form a hydrogel-like shell. P granules are attached to the nuclear membrane in the primordial germ cells, Z2 and Z3, which are generated at

(Figure legend continued at the bottom of the next page.)

Box 2. Studying Liquid–Liquid Phase Separation *in Vivo*

There are multiple ways to test whether a spherical, non-membrane-bound structure is a liquid-like condensate in a cell. Other than filming fusion events using time-lapse microscopy, one can measure the contact angle when the structure attaches to other surfaces such as the nuclear surface [6]. If the structure displays wetting behavior, it will become nonspherical and spread on the surface, reducing the contact angle. Another way is to perform fluorescence recovery after photobleaching (FRAP). Because proteins within liquid-like condensates are dynamic, FRAP can be used to show the exchange of proteins between the structure and the cytosol [28]. If the structure is large enough, one can further perform a 'half-FRAP'. By bleaching half of the structure, internal rearrangement can be demonstrated by the redistribution of fluorescence from the unbleached into the bleached area [28]. For smaller structures, photoconversion or photoactivation might be better suited for selectively labeling half of the structure [87,98] because photobleaching is typically less precise. Once the liquid-like properties are demonstrated by the aforementioned methods, one can further probe the nature of interactions within the condensate with 1,6-hexanediol. 1,6-Hexanediol is a cell-permeable, aliphatic alcohol that lowers aqueous surface tension and disrupts the weak hydrophobic interactions that help to assemble and/or maintain many liquid-like condensates [114,115]. Whereas many liquid-like condensates can be dissolved by a low concentration of 1,6-hexanediol, solid-like condensates are resistant to 1,6-hexanediol [116]. However, extended exposure to 1,6-hexanediol is cytotoxic and could cause side effects owing to the nonspecific disruption of other cellular structures [117]. Therefore, phenotypes associated with the use of 1,6-hexanediol should be interpreted with caution.

Probing the material properties of liquid-like condensates is more challenging in cells. To determine the permeability of condensates, one can microinject dextran of different sizes into cells [118]. Exclusion of dextrans above a particular size reflects the pore size of the condensate. To extract information on protein diffusion within condensates, one can use fluorescence correlation spectroscopy [119–121]. By measuring fluctuations in fluorescence intensity caused by movement of labeled proteins within small regions, one can determine their diffusion coefficient. Determining the viscosity and surface tension of condensates is, however, not trivial in cells. Nevertheless, one can calculate the inverse capillary velocity, the ratio of the viscosity to surface tension, using the time required for two condensates to fully fuse [28]. In this way, one can compare the properties of different condensates even if the absolute viscosities are not known.

revealed that P granules tend to dissolve in the anterior region, whereas they enlarge in the posterior region after the onset of symmetry breaking (Figure 1A). The components released by the dissolving anterior P granules may become absorbed by P granules in the posterior region, ultimately leading to the asymmetric partitioning of P granules [6].

One crucial question is why P granule assembly and disassembly occurs differently in the anterior and posterior regions of the embryo. It is well known that the establishment of the anterior–posterior polarity of the one-cell embryo depends on anterior partitioning of the polarity proteins PAR-3/PAR-6/PKC-3, and on posterior partitioning of PAR-1/PAR-2/LGL-1 at or near the cortex region [41]. This in turn leads to a cytosolic gradient of the RNA-binding protein MEX-5 (Figure 1A) [42]. P granules are condensates of proteins and RNAs [43]. The P granule proteins PGL-3 and MEG-3 phase separate with RNA. RNA binding stimulates their phase separation at lower concentration [36,37]. MEX-5 competes with PGL-3 and MEG-3 for mRNA. It thereby impairs their phase separation and induces a P granule gradient in the opposite direction to the MEX-5 gradient (Figure 1A) [36,37].

Given the physical properties of phase separation, it is impossible to segregate all P granule components into the germline. Some P granule components will end up in somatic cells, where

around the 100-cell stage of the embryo. The MEG-3/4 phase of P granule disappears in Z2, Z3, and later developmental stages. (B) In somatic blastomeres, the remaining PGL-1 and PGL-3 proteins reassemble into phase-separated compartments under the control of SEPA-1 and EPG-2, and are subsequently cleared by autophagy. (C) Polar granules of *Drosophila* are formed in the germlasm that is localized in the posterior region of the oocyte. Polar granule formation initiates from active mRNA transport (i.e., *oskar*) from nurse cells to the posterior pole of the oocyte at mid-oogenesis. Polar granules assemble through phase separation of core proteins and associated mRNAs at the posterior pole of the oocyte, where they recruit further mRNAs. (D) In polar granules, mRNAs self-assemble into spatially distinct homotypic clusters in a homogenous protein phase composed of the short isoform of Oskar, Vasa, Tudor, and the Piwi protein Aubergine. Abbreviation: piRNA, PIWI-interacting RNA.

they are likely soluble because their concentration in somatic cells is below the **critical concentration** required for phase separation, or because high MEX-5 concentration leads to their dissolution. What is the fate of the remaining P granule components in somatic blastomeres? Some of the components, such as PGL-1 and PGL-3, reassemble phase-separated compartments under the control of SEPA-1, EPG-2, and post-translational modifications, and are subsequently cleared by **autophagy** (Figure 1B) [44,45]. This indicates that the critical concentration for phase separation is changed by physicochemical alterations during development.

P granules change their material state as the embryo develops, and this has been linked to changes in P granule composition and function during germline development. Although PGL-1, PGL-3, and GLH-1 are present in P granules throughout germline development, MEG-3 and MEG-4 are only expressed in early embryos (1–100 cell stage) [46]. Therefore, the material state of P granules changes from liquid-like core/gel-like shell in early embryos to liquid-like for the rest of germline development (Figure 1A). This transition in material state coincides with a change in P granule function. It is possible that P granules in the zygote do not yet play an active role in germ cell fate specification but instead act as passive passengers. They must be robustly built to ensure the transmission of maternal mRNAs and epigenetic factors to the germline founder cells [43]. Their gel-like state might stabilize and protect the P granules during this time-period. MEG-3 and MEG-4 fade when primordial germ cells (Z2 and Z3) are generated at around the 100-cell stage. During the later developmental stages, P granules attach to the cytoplasmic side of the nucleus, forming a complex with nuclear pores [47]. The function of perinuclear P granules is to survey newly transcribed mRNAs and to silence unwanted transcripts before they are released into the cytosol to maintain germ cell identity [48–50]. The material state transition from gel-like to liquid-like upon MEG-3 and MEG-4 dissociation might allow the P granules to attach better to the pores.

In summary, phase separation drives the assembly of P granules, the selective stabilization of P granules in the posterior half of the embryo, and the removal of unwanted P granule components from somatic cell. Phase separation also coordinates P granule composition, organization, and localization with P granule function during germline development.

Phase Separation Controls Polar Granules in *Drosophila* Embryos

Drosophila oocytes assemble so-called polar granules via phase separation. The polar granules form in the germplasm that is localized in the posterior region of the oocyte, and are later segregated into primordial germ cells during early embryogenesis [3]. Polar granules are enriched in maternal mRNAs that are required for germ cell fate specification and maintenance. Polar granule formation starts with the active transport of *oskar* mRNA from nurse cells to the posterior pole of the oocyte where Oskar protein is synthesized (Figure 1C). The long isoform of Oskar is required for polar granule anchoring at the posterior pole, whereas the short isoform is directly involved in polar granule formation, likely through direct interaction with the DEAD-box RNA helicase Vasa (Figure 1D) [3]. DDX4, the homolog of Vasa, has the capacity to phase-separate *in vitro* [51]. Vasa may regulate polar granule assembly in a similar way to other DEAD-box ATPases [40]. The multi-Tudor domain-containing protein, Tudor, acts as scaffold by interacting with both Vasa and the Piwi protein Aubergine (Aub) via the Tudor domain dimethylated arginine module (Figure 1D) [3]. Polar granules display both liquid-like and hydrogel-like properties, with slower recovery of Oskar after photobleaching than PGL-1 in P granules, probably due to the presence of high-affinity interaction/crosslinking between the core proteins [52].

Around 200 different mRNAs accumulate in the germplasm, including *oskar*. Most mRNAs are targeted to the posterior pole via a diffusion and entrapment mechanism (Figure 1C) [53]. Partial base-pairing between Aubergine-loaded PIWI-interacting RNAs (piRNAs) and targeted mRNAs

contributes to mRNA trapping (Figure 1D) [54]. The entrapment mechanism can be explained by phase separation. *oskar* mRNAs become initially enriched at the posterior pole by active transport, where they are translated. The synthesized short Oskar proteins phase separate with other core proteins (Tudor, Vasa, and Aub) and associated RNAs (piRNAs and mRNAs) upon reaching a critical concentration. The condensates enlarge as more proteins are synthesized and incorporated. The enlarged condensates recruit and accommodate more mRNAs, thereby establishing the polar granules (Figure 1D).

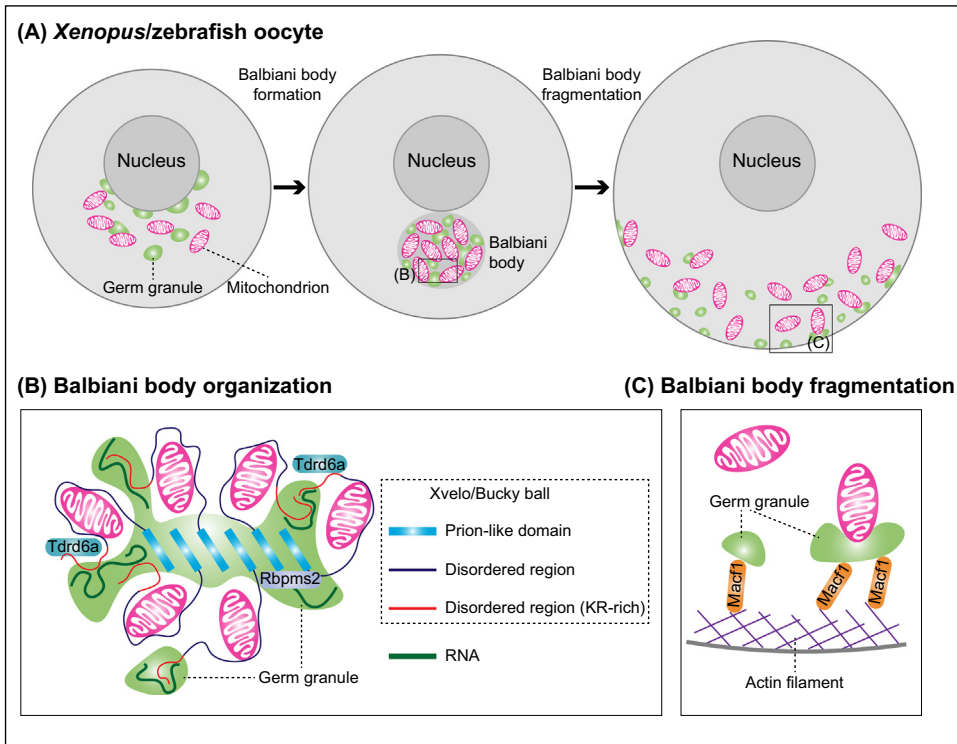
In *C. elegans*, P granules of the early embryo contain two distinct protein phases, the liquid-like PGL phase and the gel-like MEG phase. By contrast, in *Drosophila* polar granules, the proteins are homogeneously distributed, whereas mRNAs are organized into spatially distinct homotypic clusters within the condensate (Figure 1D) [55–57]. A recent study revealed that the homotypic cluster is formed via self-assembly of mRNAs derived from the same gene [58]. RNA self-assembly is driven by nonspecific disordered RNA–RNA interactions, but not by sequence specific interactions [58]. It is possible that proteins and mRNAs condensate into a homogenous phase first, and then mRNAs derived from the same gene assemble and form the second phase when more proteins and mRNAs are recruited [57]. Nevertheless, it remains unclear why mRNAs derived from different genes form distinct domains and do not intermix. It seems that the miscible properties of mRNAs are determined by the sum of many factors, including primary sequences, modifications, structures, and bound proteins [58]. When the miscible properties are altered to some degree, no matter whether it is by a change of primary sequence or by other means, the mRNAs will be sorted into distinct clusters.

The homotypic clustering of mRNAs seems to be a conserved mechanism because it is also observed in the germline of zebrafish [59]. Moreover, RNA self-assembly contributes to stress granule assembly and the formation of abnormal nuclear foci in some neurological diseases [60,61]. Taking all the evidence together, RNA–RNA interactions also play a pivotal role in phase separation and the formation of RNA-containing non-membrane-bound compartments, in addition to protein–protein and protein–RNA interactions.

Phase Separation during Balbiani Body Formation

Phase separation is not only involved in the formation of non-membrane-bound compartments but also clusters membrane-bound organelles to form 'super-organelles' such as the synaptic vesicle cluster at the synapse and the Balbiani body in oocytes [62,63]. The Balbiani body is a remarkably large non-membrane-bound compartment composed of membrane-bound organelles (mitochondria, endoplasmic reticulum, and Golgi) and germline that is essential for primordial germ cell formation (Figure 2A) [5]. It has long remained a mystery how membrane-bound organelles are assembled into such a giant structure – until the principle of phase separation emerged. Studies in *Xenopus* demonstrated that physiological **amyloids** are involved in Balbiani body formation [63]. To identify the key protein that mediates Balbiani body formation, the Balbiani bodies were isolated and analyzed by mass spectrometry. Xvelo was one of the most enriched proteins. Xvelo–GFP intermingled with mitochondria in the Balbiani body (Figure 2B). Photobleaching indicated that Xvelo forms a stable matrix that could entrap mitochondria and other organelles. In line with this observation, recombinant Xvelo–GFP proteins self-assembled into amyloid-like fibers that interact with mitochondria and RNA *in vitro*. The prion-like domain at the N-terminus of Xvelo is responsible and sufficient for the formation of the amyloid-like fibers, whereas other parts of the protein are involved in the recruitment of mitochondria and RNA (Figure 2B) [63].

In zebrafish, the protein Bucky ball (Buc) is required for Balbiani body formation [64,65]. Buc is also a prion-like domain-containing protein, and resembles Xvelo in terms of localization and



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Figure 2. Amyloid-like Assembly of the Balbiani Body in *Xenopus* and Zebrafish Oocytes. (A) The Balbiani body is assembled in the vicinity of the nucleus in the vegetal hemisphere of the early oocyte, and is later fragmented into many small islands that are docked to the vegetal cortex during oocyte growth. (B) Amyloid-like self-assembly of the prion-like domain in Xvelo or Bucky ball (Buc) drives Balbiani body formation. The disordered regions of Xvelo and Buc may directly recruit mitochondria and mRNAs. Buc may also recruit mRNA indirectly by interacting with the RNA-binding protein Rbpms2. The multi-Tudor domain-containing protein Tdrd6a interacts with Buc, and modulates the mobility and aggregation properties of Buc in the Balbiani body. (C) The microtubule–actin crosslinking factor 1 (Macf1) and actin filaments mediate Balbiani body fragmentation. Abbreviation: KR, lysine/arginine.

dynamics, implying that the self-assembly of prion-like domains is a conserved mechanism of Balbiani body formation [34,63]. Xvelo or Buc may interact with mRNA directly via their K/R rich regions, or indirectly through other RNA-binding proteins such as Rbpms2 (Figure 2B) [66]. The multi-Tudor domain-containing protein Tdrd6a interacts with Buc via the dimethylated tri-RG motif, and modulates the mobility and assembly properties of Buc in the Balbiani body (Figure 2B) [34].

Amyloids are often associated with pathologic processes such as amyotrophic lateral sclerosis (ALS), Parkinson disease (PD), and Alzheimer disease (AD). Pathological amyloids are irreversible, whereas physiological amyloids are reversible probably through post-translational modifications [67]. The Balbiani body is formed in the early oocyte, and is fragmented into many small islands that are docked to the vegetal cortex during oocyte growth (Figure 2A). Temporally controlled phosphorylation of Xvelo and Buc could be a possible mechanism for Balbiani body disassembly [63,68]. Given the stability of amyloid-like fibers and the huge size of the Balbiani body, it is reasonable to investigate whether the cytoskeleton is involved in the rupture of this compartment. Studies in zebrafish reveal that the microtubule–actin crosslinking factor 1 (Macf1) and actin filaments are crucial for the fragmentation of the Balbiani body (Figure 2C) [68]. A possible model is that the Balbiani body is anchored to the actin network by Macf1 at multiple points,

and that these anchor points are physically pulled apart during oocyte growth, leading to disruption of the Balbiani body [5]. Alternatively, the Balbiani body may be broken up directly by actin filaments and unidentified motor proteins.

The function of the Balbiani body is not yet fully understood. In *Xenopus* and zebrafish, the Balbiani body is localized in the vegetal hemisphere, and is an early marker of animal–vegetal polarity. The Balbiani body contains germplasm that determines primordial germ cell formation at the vegetal pole. Oogenesis in *Xenopus* and zebrafish takes much longer than in *C. elegans* and *Drosophila*. Amyloids are more stable than liquid droplets or hydrogels and display minimal exchange with the cytosol. The amyloid nature of the Balbiani body may thus help to prevent scattering of the germplasm during oogenesis [4].

Phase Separation during Meiotic Recombination

Apart from the organization of cytoplasmic organelles and RNA granules during oogenesis, phase separation plays essential roles in meiotic chromosome segregation, which is a crucial step in the development of both eggs and sperm. To reduce the number of chromosomes in germ cells by half, **homologous chromosomes** (homologs) first need to be paired before segregation. This is achieved by different mechanisms in different species. Although the precise pairing mechanisms differ, they do have in common that they use phase separation in various ways.

In most organisms, including budding yeast, mammals, and plants, pairing requires meiotic recombination, a process that involves the exchange of DNA strands (crossover) between homologs [69]. This exchange relies on the formation of double-strand breaks, whose distribution is controlled by phase separation. During leptotene, an early stage of meiotic recombination, double-strand breaks are introduced by the topoisomerase-like protein SPO11 [69,70]. Subsequently, a stretch of single-stranded DNA is generated and searches for the corresponding DNA sequence on the homolog, facilitating contacts between homologs [69,71,72]. Because the number of double-strand breaks is directly related to the extent of pairing, SPO11 activity must be spatiotemporally regulated. How the meiotic double-strand break machinery is assembled and regulated is largely unclear. In budding yeast, the Spo11 accessory proteins Rec114, Mei4, and Mer2 (RMM) bind stably to the chromosome axis as foci [73–77]. In the presence of DNA, RMM can phase-separate *in vitro*, and the resulting condensates can recruit Spo11 [78]. RMM mutants with reduced DNA-binding activity form fewer foci and fail to form double-strand breaks *in vivo* [78]. Together, these data have led to the model that phase separation drives the self-assembly of hyperstoichiometric RMM complexes on chromosome axes and thereafter the recruitment of multiple Spo11 proteins (Figure 3A). This ensures localized activation of Spo11 which could otherwise create excessive double-strand breaks and compromise genome integrity. Because Rec114, Mei4, and Mer2 are conserved in mice (REC114, MEI4, and IHO1) and *Arabidopsis* (PHS1, PRD2, and PRD3/PAIR1) [70,78], it is likely that this self-assembly mechanism is also conserved in mammals and plants.

In other organisms such as *C. elegans*, *Drosophila*, and fission yeast, recombination-independent pairing mechanisms exist [69]. In fission yeast for instance, long noncoding RNAs (lncRNAs) mediate pairing. Pairing here also involves phase separation (Figure 3B). In particular, fission yeast expresses *sme2*, a meiosis-specific lncRNA that accumulates at its gene locus and mediates robust pairing [79]. In a recent study, several *sme2* RNA-associated proteins (Smp) were identified and were found to be similarly required for robust pairing [80]. Acute addition of **1,6-hexanediol** dissolves Smp foci and unpairs the *sme2* RNA-decorated loci (Box 2). In addition, it unpairs another Smp protein-decorated loci, A55 [80]. Washout of 1,6-hexanediol

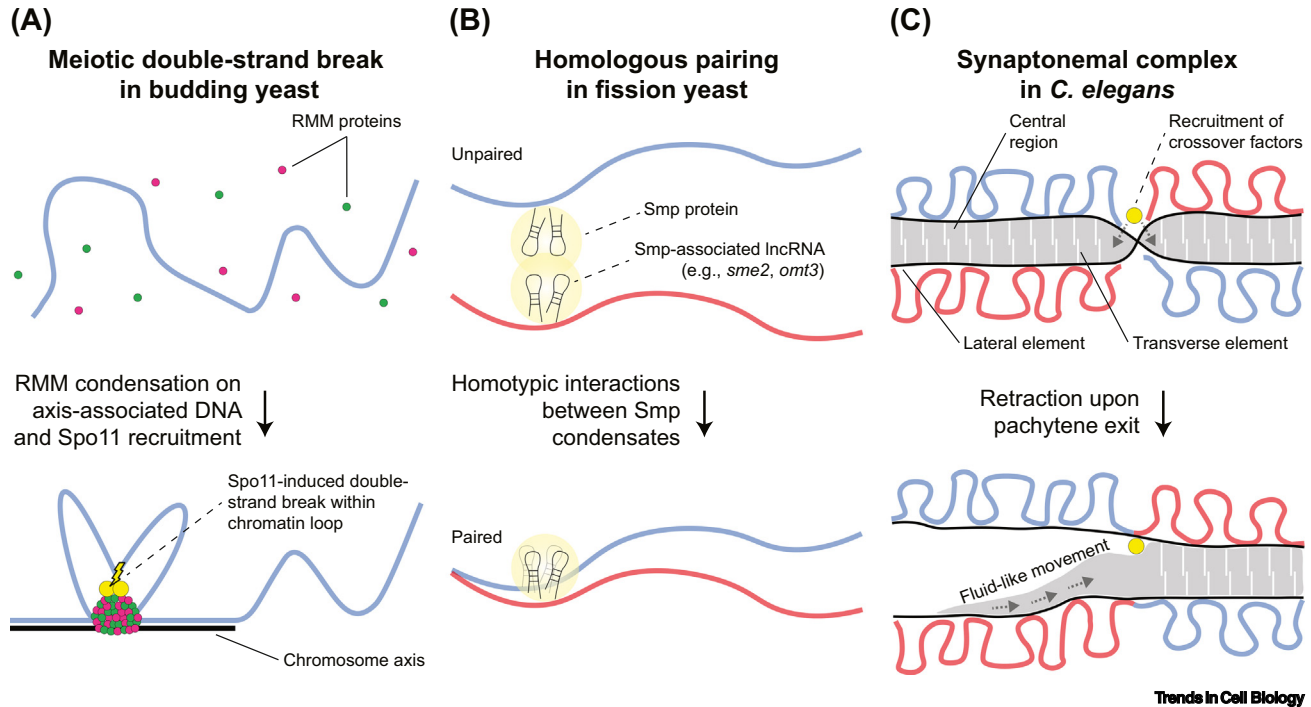


Figure 3. Phase Separation Facilitates Pairing and Synapsis during Meiotic Recombination in Various Species. (A) In budding yeast in which recombination-dependent pairing occurs, the Spo11 accessory proteins Rec114, Mei4, and Mer2 (RMM; in green and magenta) phase-separate in the presence of DNA. This drives the self-assembly of hyperstoichiometric RMM complexes on chromosome axes and thereafter the recruitment of multiple Spo11 proteins (in yellow), ensuring local activation of Spo11 and thus induction of double-strand breaks within chromatin loops. (B) In fission yeast where recombination-independent pairing occurs, distinct long noncoding RNAs (lncRNAs) such as *sme2* and *omt3* are transcribed from different loci and associate with Smp protein (in yellow) to form distinct Smp–lncRNA condensates. Homotypic fusion of distinct Smp–lncRNA condensates ensures specific pairing at each locus. (C) In *Caenorhabditis elegans*, the central region of the synaptonemal complex is a liquid-like compartment (in grey). Crossover factors such as ZHP-3 (in yellow) are selectively recruited to this compartment, and probably regulate local recombination. Upon pachytene exit, the fluid-like properties of the synaptonemal complex may also be required for its retraction towards one end of the chromosome, a behavior resembling the flow of a liquid.

reverses both effects, confirming that the formation of Smp–lncRNA complexes is reversible [80]. Substitution of the *omt3* RNA transcribed from an A55 locus with *sme2* RNA disrupts pairing of the A55 loci, but not when both A55 loci are substituted with *sme2* RNA [80], highlighting the importance of homotypic interactions between lncRNAs in Smp-mediated pairing. Thus, by transcribing different lncRNAs from each locus, homotypic fusion of distinct Smp–lncRNA condensates ensures specific pairing at each locus.

Recent work in several species revealed that a further key structure involved in meiotic recombination – the synaptonemal complex – is formed by phase separation. After the initial pairing step, the synaptonemal complex is nucleated at the homolog contact sites, and subsequently spreads along the entire length of the homologs to promote their tight association [69]. The synaptonemal complex consists of a transversely striated central region that is symmetrically flanked by two parallel lateral elements running in the direction of the chromosome axis [69]. Initially, the synaptonemal complex was thought to be a stable, zipper-like structure formed by **coiled-coil** interactions [69]. This was because many of the synaptonemal complex proteins that form the transverse elements were predicted to contain coiled coils. Later work in different model organisms revealed that synaptonemal complex proteins turn over, and that the synaptonemal complex can incorporate newly translated proteins and undergo reorganization after assembly [81–86], suggesting that it is a dynamic structure. Indeed, recent studies in

C. elegans suggest that synaptonemal complex proteins behave like a liquid [87,88]. Their overexpression results in droplet-like foci in mammalian cells [88]. When **synapsis** is perturbed in the absence of axis components, synaptonemal complex proteins self-assemble into large bodies called polycomplexes outside of the chromosomes [87]. Polycomplexes are non-membrane-bound structures that can undergo fusion and are susceptible to 1,6-hexanediol [87], suggesting the presence of internal rearrangement and their dependence on weak hydrophobic interactions. Consistently, **photoconversion** revealed that proteins within the central region, but not axis components can undergo internal rearrangement (Box 2) [87]. In addition, central region proteins, but not axis components, can be reversibly dispersed by 1,6-hexanediol [87]. A similar behavior was observed for central region proteins and axis components in *Drosophila* and budding yeast [87]. By contrast, in fission yeast, where the synaptonemal complex is absent but lateral element-like linear elements are present, linear element proteins have a slow turnover and are resistant to 1,6-hexanediol [89]. Altogether, these data suggest that the central region of the synaptonemal complex is a liquid-like compartment (Figure 3C). Such a compartment may play a role in locally regulating recombination via co-partitioning synaptonemal complex proteins with crossover factors such as ZHP-3, which is also susceptible to 1,6-hexanediol within the synaptonemal complex and can be concentrated in polycomplexes [87]. In addition, when crossovers emerge upon pachytene exit, fluid-like properties of the synaptonemal complex may be required for its gradual retraction towards one end of the chromosome [88], a behavior resembling the flow of liquid.

Phase Separation during Meiotic Spindle Assembly

Upon completion of meiotic recombination, the homologs are linked and are ready for segregation. However, in females, meiosis is arrested in prophase and oocytes become stored in the ovaries. Only after puberty, oocytes that reach their full size can resume meiosis and segregate their chromosomes. Recent work shows that phase separation is also involved in this final step of meiosis by promoting the assembly of a specialized microtubule spindle in oocytes. The spindle is the cellular machinery that aligns and segregates the chromosomes. In mitotic cells and male germ cells, the spindle is assembled by two centrosomes (Figure 4A) which consist of a pair of

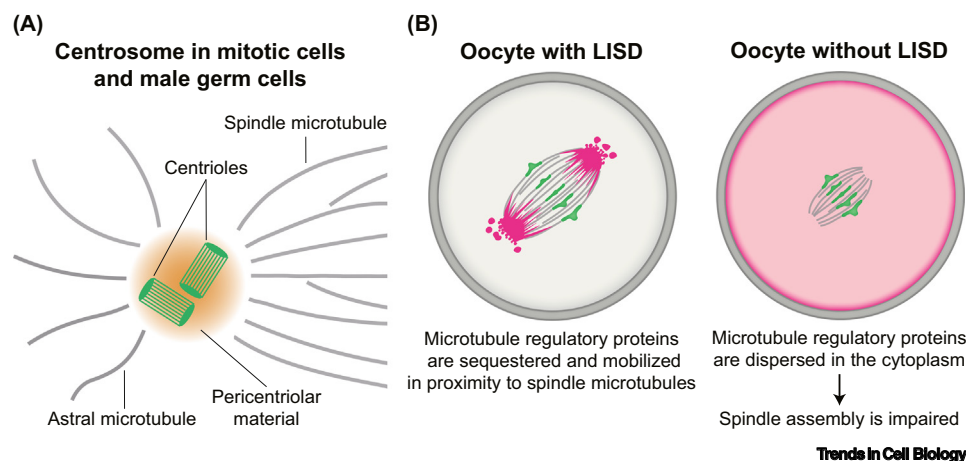


Figure 4. Phase Separation of Microtubule Regulatory Proteins Promotes Acentrosomal Spindle Assembly in Mammalian Oocytes. (A) In mitotic cells and male germ cells, centrosomes serve as the dominant microtubule-organizing centers during spindle assembly. Centrosomes consist of a pair of centrioles (in green) surrounded by pericentriolar material (in orange) which efficiently nucleates spindle microtubules. (B) In oocytes, centrioles degenerate during early oogenesis, and spindles (in gray) are assembled in the absence of centrosomes. Mammalian oocytes sequester microtubule regulatory factors in the liquid-like spindle domain (LISD; in magenta) via phase separation, and thereby enrich and mobilize them in proximity to spindle microtubules. In the absence of the LISD, microtubule regulatory factors are dispersed in the large cytoplasmic volume, thus impairing spindle assembly.

centrioles surrounded by the **pericentriolar material** [90]. The centrosome is the dominant microtubule-organizing center (MTOC) in these cell types [90], but is absent in mammalian oocytes. Centrioles degenerate during early oogenesis, and the pericentriolar material is lost in most species (except for rodents) [91]. Whereas rodent oocytes nucleate spindle microtubules using both acentriolar MTOCs (aMTOCs) and the **RanGTP pathway** [92–94], non-rodent oocytes seem to require the RanGTP pathway only [95,96]. Apart from nucleation factors, spindle assembly involves motor proteins as well as proteins that regulate the growth and stability of microtubules. Given the large size of oocytes, controlling the concentration of these proteins is likely more challenging than in mitotic cells and male germ cells. Recent work has revealed that oocytes also use phase separation to achieve this task.

Despite the absence of centrosomes, many centrosomal proteins could be detected in mammalian oocytes [97]. Systematic mapping of the localization of centrosomal and spindle-related proteins in mouse oocytes identified a previously undescribed spindle domain in which many microtubule regulatory factors are stored [98]. This domain, which is also conserved in oocytes from other mammalian species, infiltrates the spindle poles and forms prominent protrusions that extend beyond the poles [98]. Because these protrusions are spherical and can undergo fusion, the domain was named the liquid-like meiotic spindle domain (LISD) [98]. The LISD is formed by liquid–liquid phase separation: it is not enclosed by membrane, its proteins turn over and undergo internal rearrangement, and it can be dissolved by 1,6-hexanediol [98]. When spindle assembly is disrupted by nocodazole, a microtubule-depolymerizing agent, LISD proteins reversibly self-assemble into large condensates with liquid-like properties [98].

LISD assembly *in vivo* is driven by the regulatory kinase AURA [98], which is found at aMTOCs and on spindle microtubules [98–100]. In the presence of AURA kinase activity, transforming acidic coiled-coil-containing protein TACC3 and clathrin heavy chain CHC17 nucleate the LISD on spindle microtubules and recruit other LISD proteins [98]. Unlike TACC3, CHC17 is not required for the assembly of condensates induced by nocodazole, implying that CHC17 is not essential for phase separation in the absence of spindle microtubules. Indeed, TACC3 alone can phase-separate *in vitro* via its intrinsically disordered N-terminus [98], which is not required for microtubule binding [101,102]. Disruption of the LISD by AURA inhibition, or by TACC3 or CHC17 depletion, similarly disperses other LISD proteins throughout the cytoplasm and results in substantial microtubule loss [98]. Expression of the N-terminal deletion mutant of TACC3 in TACC3-depleted oocytes restores the recruitment of CHC17, but not that of other LISD proteins, and fails to rescue the microtubule loss [98]. Together, these data suggest a model whereby the LISD sequesters and mobilizes microtubule regulatory factors in proximity to spindle microtubules, thus stabilizing microtubules within the meiotic spindle (Figure 4B).

Endogenous TACC3 does not appear to phase-separate in mitotic cells, regardless of the presence or absence of centrosomes [98,103,104]. The LISD is thus likely a meiosis-specific mechanism for enriching microtubule regulatory factors within the large cytoplasmic volume of oocytes. In the absence of the LISD, the low cytoplasmic concentration of microtubule regulatory factors is unlikely to be sufficient for the assembly of spindle microtubules, especially in the absence of additional mechanisms (such as the centrosomes) to locally enrich tubulin dimers [105].

Concluding Remarks

Phase separation recurs as an important organizing principle throughout germline development – from fate determination in germ cells to the meiotic divisions of oocytes. It is directly involved in the physical assembly of germ granules and the Balbiani body, and represents a smart strategy for segregating germplasm into germ cells. Germ granules and the Balbiani body are the hubs for

Outstanding Questions

How are germ granule and Balbiani body constituents and dynamics spatiotemporally controlled during oogenesis and early embryogenesis?

Is it possible to isolate or reconstitute germ granules to investigate their behavior and functions *in vitro*?

Do membrane-bound organelles only act as platforms for germ granule assembly, or do germ granules also regulate the fate or function of membrane-bound organelles at the oocyte-to-embryo transition?

What is the function of the Balbiani body in mammalian oocytes?

Is the formation and function of phase-separated compartments discovered in different model organisms conserved during human germline development?

Why is phase separation used as the principle for targeting Spo11 to DNA?

Which proteins are responsible for phase separation in the central region of the synaptonemal complex?

Is the LISD present during early embryogenesis?

Could defects in phase separation be linked to infertility?

post-translational gene regulation. The different material states of these condensates could help to meet the requirements for maternal mRNA processing, storage, and translational control. During meiotic recombination, phase separation helps to assemble diverse machineries that promote chromosome pairing and synapsis. Given the divergence in pairing and synapsis mechanisms in various species, it is not unlikely that more examples of phase separation will be discovered. During oocyte maturation, phase separation helps to prevent the dispersal of microtubule regulatory factors during spindle assembly. After fertilization, the embryo reacquires centrosomes from the sperm or via *de novo* assembly [106]. Whether the LISD is required for spindle assembly during early embryogenesis remains to be investigated. Other work implied phase separation in the generation of universal intracellular structures such as the nuclear pore complexes, nucleoli, and centrosomes [105,107,108], and in various other processes such as signal transduction, gene expression control, and membrane trafficking [17–26,33]. It is clear that additional universal and germ cell-specific phase-separation mechanisms must be at work during germline development. With emerging tools that now allow spatiotemporal control over phase separation *in vivo* [109–111], exciting times for studying the assembly and function of phase-separated compartments during germline development are coming up (see Outstanding Questions).

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