Chapter 1

Germ Cell Specification: The Evolution of a Recipe to Make Germ Cells

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71557

Abstract

Multicellular species use gametes for their propagation. Gametes are formed from primordial germ cells (PGCs), which develop during embryogenesis. In some species, PGCs are specified by the inheritance of a RNA granule known as germ plasm. During germ cell specification, the germ plasm conveys a unique set of properties, e.g. the germ cell specific meiotic cell cycle to the PGCs. Germ plasm assembly is controlled by independently evolving organizer proteins like Oskar in *Drosophila* or Bucky ball in zebrafish. These organizers are intrinsically disordered proteins, which rapidly changed their amino acid sequence during evolution. A common recipe has emerged by studies on organizer proteins for animals that use germ plasm to specify their germline. Investigating the nature of these organizers might therefore provide a clue to germ cell specification in other species, which are less accessible to molecular-genetic and embryological approaches. Moreover, we might understand how the first metazoans modified their existing cellular structures from unicellular eukaryotes to ensure their reproduction.

Keywords: zebrafish, germ plasm, primordial germ cell, Bucky ball, Oskar, intrinsically disordered protein, stem cells

1. Introduction

Germ cells are precursors to animal gametes. After fusion, gametes have the impressive capacity to develop into a new organism. As all cells of this organism are descendants of PGCs, they are considered totipotent. Interestingly, gametes are also formed in every subsequent generation from the same germ cell. These features identify germ cells as a truly immortal cell line, whereas somatic cells die at the end of life. These are the same characteristics seen in stem cells, thus making germ cells the superior stem cell.

Germline development has to be tightly regulated and controlled to ensure the development of a fertile adult organism. Any misregulation in the pathway would affect fertility and might lead to no offspring. Eventually, sterility might therefore result in the end of that lineage and ultimately in the extinction of the species. Hence, any errors in the germ cell program could have disastrous consequences for a species compared to mistakes in a somatic cell program like forming an organ.

Compared to somatic tissue, very little is known about the critical period of PGC specification. Understanding the biochemical activity of all germ plasm components could help us to grasp, how germ cells get specified. Furthermore, it could identify how "stemness" is achieved at the molecular level. This knowledge might help to treat many degenerative Wof new drug targets for therapy.

2. Mechanisms of germ cell specification

Two different modes of germ cell specification have been described.

2.1. Inductive mode

Germ cell specification by induction is often described as the ancestral or more prevalent mode (**Figure 1A**) [2]. In the induction mode, germ cell fate is specified through external signals from developing embryonic cells. Induction was described in some invertebrates and in some vertebrates like mammals [3, 4]. The most studied example is the mouse [5–7]. One of the signals inducing germ cells is BMP4 [8]. However, it is currently not clear how conserved this signal is during germ cell specification in other species of the animal kingdom.

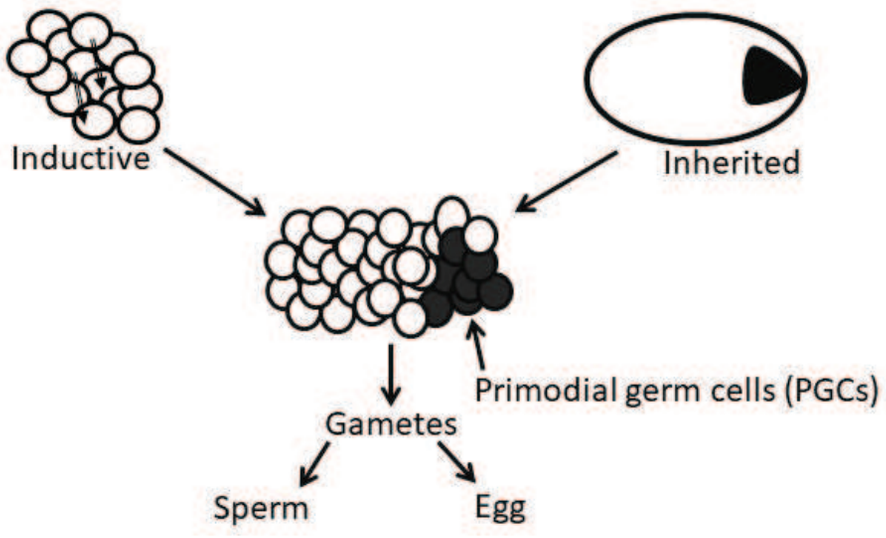


Figure 1. Inherited vs. inductive mode. (A) Inductive mode. Somatic cells induce germ cells (white arrows) within the blastula to express germline factors and differentiate into PGCs (red). (B) Inherited mode. Maternal RNP granules or germ plasm (red) are asymmetrically localized in the oocyte and are inherited by a subset of blastomeres, which specifies PGCs [1].

Regardless whether PGCs are specified by induction or inheritance, they show several commonalities at the molecular level. In most species, numerous proteins and mRNAs like Vasa, Piwi, and Nanos are conserved [9, 10]. In spite of two different modes of specification, they activate common downstream components. We will address the evolutionary conservation of germ plasm again at the end of this chapter, when we describe a potential origin of germ plasm in unicellular organisms.

PGCs adopt different lineages, if transplanted to different parts of the embryo. In the mouse, which uses the induction mode, transplanted PGCs later on colocalize with neural plate and surface ectoderm cells [6]. In *Xenopus*, which uses the inherited mode, transplanted PGCs generate lineages of the three germ layers [11]. These results suggest that despite different specification modes, both types of PGCs still require signaling from extrinsic sources to maintain their fate as fully determined PGCs [12]. Hence, even though the two mechanisms seem starkly different, there may be a common underlying signaling mechanism which is universal.

The key to understanding the specification of PGCs is to separate species-specific adaptations from a core program of germ cell formation. As information about the initial phase of germ cell specification is still quite fragmentary in different organisms, the core program of germ cell specification is unclear. For instance, the molecule that acts as a master or "kick starter" for the germ plasm or PGC program appears to be different in each organism. Therefore, in the rest of this chapter, we will concentrate on the inherited mechanism of germ cell specification.

Publication	Finding/Hypothesis
Weismann (1893)	Inheritance depends on germ cells. Postulates that germ plasm localizes to the nucleus.
Hegner (1911), Boveri (1910)	Germline determinants (germ plasm) localize to the cytoplasm. Germ plasm is necessary (Hegner) and sufficient (Boveri) for germline development.
Bounoure (1934)	Germ plasm for the first time visualized in a vertebrate egg.
Smith (1966)	UV-irradiation of *Drosophila* eggs reduces the number of PGCs. The UV-wavelength suggest that nucleic acids are critical for germline development.
Illmensee and Mahowald (1977)	Ectopic germ plasm is sufficient for PGC formation.
Heasman (1984)	The Balbiani body of *Xenopus* contains the germ plasm.
Ephrussi and Lehman (1992)	Ectopic expression of a single protein termed Oskar gives rise to functional PGCs in *Drosophila*.
Hashimoto (2004)	Ablation of germ plasm in zebrafish reduces PGCs.
Bontems (2009)	Ectopic Expression of a single protein termed Bucky ball induces PGCs in zebrafish.
Brangwynne (2009)	Biophysical studies on embryonic germ plasm reveal a liquid-like hydrogel in *C. elegans*.
Tada (2012)	Germ plasm transplantation in Xenopus induces ectopic germ cells.
Boke (2016)	The *Xenopus* Balbiani body forms amyloid aggregates.

Table 1. Listing selected discoveries that paved the way for the current model of the inherited strategy of germ cell specification.

2.2. Inherited mode

Inheritance of cytoplasmic determinants represents the second mode, by which germ cells are specified (**Figure 1B**). This mechanism of germ cell specification is described amongst others in dipteran insects (e.g. *Drosophila*), nematodes (e.g. *C. elegans*) anuran amphibians (e.g. *Xenopus*), zebrafish, and birds [2, 13]. The molecular mechanisms of germ cell specification are probably better understood at the molecular-genetic and biochemical level than induction, because forward genetics identified most of the known key factors [14, 15]. The best studied examples are probably *Drosophila* and *C. elegans* [16, 17]. **Table 1** summarizes some historical highlights in the context of germ plasm research.

3. Germ plasm

3.1. Composition

Germ plasm is a collection of maternally provided RNAs, proteins, and organelles like mitochondria and endoplasmic reticulum [ER]. The entire assembly forms a cytoplasmic structure in the oocyte named Balbiani body [18]. Sometimes it is also referred to as the mitochondrial cloud in *Xenopus* [19]. The Balbiani body [Bb] was discovered in spiders and it seems to be omnipresent in oocytes of most species of invertebrates (e.g. spiders, insects, and molluscs) and vertebrates (e.g. frogs, birds, teleosts, and mammals) [20–22].

Studies in *Xenopus* and *Drosophila* suggest that the Bb accumulates a subset of mitochondria. These mitochondria are designated to be delivered to the germ plasm and ultimately to the next generation *via* primordial germ cells [23–25]. Interesting experiments in *Drosophila* proposed that germ plasm selects a healthy set of mitochondria by their level of ATP production [23, 24]. The mitochondria in oocytes show high levels of mitochondrial inner membrane potential [26, 27]. Perhaps this mechanism provides germ cells and by extension gametes with the fittest organelles. This ensures that the healthiest mitochondria and its descendants are passed on to the next generation.

3.2. Function

Loss of germ plasm leads to a decrease or no germ cells, whereas in gain of function experiments more germ plasm leads to more germ cells [28] (**Table 1**). Germ plasm components are believed to act in stem cells to convey longevity and totipotency, similar to the magic substances *ambrosia/amrit* in Greek or Hindu mythology, which kept the gods immortal. Many components of germ plasm, like Vasa, are also present in multipotent stem cells flatworms [29]. Nanos is present in stem cells involved in regeneration in planarians [30]. Finally, Piwi also functions in maintaining both germline and somatic stem cells in *Drosophila* [31].

As several germ plasm components have a role in stem cells, it should have a much greater effect in maintaining "stemness" and increased longevity than their somatic stem cell counterparts. As germ plasm conveys a high degree of longevity to germ cells, it would be of stupendous importance to further dissect the germ plasm and study this network of protein and RNA to get further insights into these stemness features.

3.3. Assembly

In the section below, we will concentrate on the two organizer proteins Oskar in invertebrates and Bucky ball in vertebrates that are involved in germ plasm assembly. Both molecules specify germ cells indicating that their biochemistry and mode of action is similar.

4. Oskar in invertebrates

Oskar protein acts as a master regulator of germ plasm assembly [32]. In *Drosophila*, germ plasm is localized to the posterior pole during late oogenesis and hence, also known as pole plasm (**Figure 3**). *Oskar* was isolated in mutagenesis screens for maternal-effect genes required for embryonic patterning [33]. Oskar mutants showed posterior patterning defects and no germ cells [34]. *osk* RNA localizes to the posterior pole, where the protein gets translated and starts the assembly of germ plasm [34, 35]. Mutations in *oskar* affect the enrichment of other RNAs and proteins at the posterior pole, which are present in the germ plasm. This shows that Oskar indeed is essential to initiate germ plasm formation and by extension germ cells.

Mislocalization of Oskar protein at the anterior end of the embryo leads to ectopic germ cells and a second abdomen [32]. Oskar was the first protein, which is both necessary and sufficient to assemble germ plasm. Increasing the amount of Oskar protein in the fly embryo causes an increase in activity of the Nos protein. Thus, the amount of Osk protein and the level of Nos protein accumulation are related. Possibly the heightened expression of Nos represses the somatic cell fate pushing it to a germ cell lineage [36, 37]. Such an activity supports the role of Oskar as a master regulator of PGC specification in invertebrates.

osk mRNA is translated into two protein isoforms by alternative translation initiation [42, 43]. Long Osk (lOsk) is translated at the first start codon and encodes a protein of 606 amino acids. LOsk mainly anchors germ plasm at the posterior end. Long Oskar also traps and accumulates mitochondria at the site of PGC formation. Mutating specifically this long oskar form strongly decreases the number of mtDNA molecules inherited by PGCs [44]. Short Oskar (sOsk) starts at Methionine 139 and encodes a protein of 467 amino acids [42, 43]. sOsk assembles germ plasm and thereby plays a critical role to specify PGCs (**Table 2**).

Long Oskar	Short Oskar
606 amino acids long	467 amino acids long
Anchoring germ plasm	Assembling germ plasm
Associated with endosomes	Associated with RNA granules
Interacts with Lasp to be tethered to posterior pole	Interacts with Lasp to be tethered to posterior pole
Not essential for patterning and germ cell formation	Necessary for germ cell formation and posterior patterning

Table 2. Differences between long and short Oskar.

5. Germ cell specification by Oskar

Fascinating insight into sOsk function was recently gathered by crystallizing two of its domains. These were a domain at the N-terminus of sOsk [139–240aa], which was termed LOTUS domain and previously predicted to be involved in RNA-binding. The second structure described the C-terminal "OSK" domain, which resembles a SGNH hydrolase [40, 41] (**Figure 2**). However, looking carefully at the biochemical interactions and crystallizing sOsk with these binding partners revealed some unexpected information.

sOsk directly interacts with Vasa [45], which is an ATP-dependent helicase [41, 46]. Interesting biochemical and biophysical studies show that the eLOTUS domain of Oskar does not interact with RNA, but in fact binds to the RNA helicase Vasa, which is an important component of germ plasm. Surprisingly, the extension of the LOTUS domain (eLOTUS) encodes an intrinsically disordered motif, which forms a structured domain upon Vasa binding. This stretch of 18 amino acids outside of the LOTUS domain is essential for the Vasa interaction. Moreover, binding the eLOTUS domain increases the ATPase activity of Vasa. This is the first time an instructive role was assigned to Oskar, which was previously regarded as a scaffold protein aggregating germ plasm components within the *Drosophila* oocyte [46].

The OSK domain shows a lot of similarity to a SGNH hydrolase, but lacks three of the four residues of the SGNH motif, as well as the serine triad to be an active hydrolase [41]. The C-terminal OSK-domain forms a globular structure, which carries several basic, positively charged residues at its surface suggesting it could interact with nucleic acids. Indeed, this domain binds in *in vitro* experiments mRNAs like the *osk* and *nos* 3'UTRs [40]. When the basic residues of the OSK domain are mutated, binding to RNA is disrupted [40]. *In vivo* pull-down experiments after UV-crosslinking suggest that Osk interacts with *nos*, *pgc*, and *gcl* mRNA *in vivo* [41]. All three RNAs are known to be localized to the germ plasm. Again, these exciting discoveries identify sOsk as a novel RNA-binding protein and suggest a more instructive role of in germline development than previously anticipated.

Taken the interaction data of sOsk together, a modified picture of germ cell specification emerges. sOsk initiates the assembly of germ plasm by binding to Vasa and mRNA. This interaction activates Vasa and might sterically bring it in proximity with specific RNA(s). This could regulate translation or stability of the RNA(s) involved in specifying PGCs [37]. Hence, Vasa and Osk seem to act in a co-operative manner to specify germ cells.

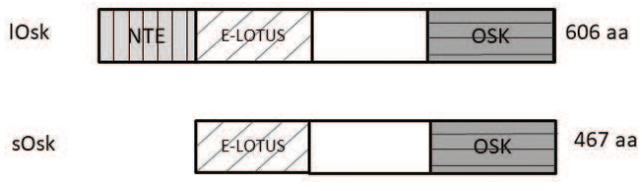


Figure 2. Comparison of long (lOsk) and short (sOsk) Oskar proteins. The NTE domain in lOsk inhibits Vasa-interaction and RNA-binding [16, 38, 39]. The eLOTUS (extended LOTUS) domain consists of the minimal LOTUS domain along with a short disordered region of 18 aa, which together are essential to bind Vasa. The OSK domain binds to RNA [40, 41].

Vasa is also involved in piRNA processing. The amount of Vasa in the germ plasm, therefore, prevents the degradation of the germ cell genome by transposon activity, but piRNAs could also play an undiscovered early role in germ cells [47]. Aubergine, a well-known component of the piRNA pathway, is needed for Osk translation, which also needs Vasa to localize. This could indicate a feedback mechanism ensuring all the downstream germ plasm members are expressed [48]. Figuring out the biochemical process, which is initiated by sOsk/Vasa, is probably the key to understand the molecular mechanism of the germ cell specification program.

6. Zebrafish as a model organism to study germ cell specification in vertebrates

Compared to invertebrates such as *Drosophila* and *C. elegans*, much less is known about the molecular processes occurring in the germ plasm of vertebrates. In *Xenopus*, germ plasm research is mostly focused on processes during oogenesis [49–51]. However, among vertebrates that specify their germ cells through inheritance of germ plasm, there are a numerous studies in the zebrafish. Zebrafish combines a number of features helpful for early developmental studies. Embryos and oocytes are easily accessible and available in high numbers. Moreover, its transparent embryos enable tracing of fluorescently tagged proteins *in vivo* and allow detection of endogenous proteins by immunostaining. The genome is completely sequenced, and genomic manipulations via CRISPR/Cas9 are easy. Therefore, zebrafish as a vertebrate model is very well suited for the analysis of germ cell specification [52].

7. Bucky ball in zebrafish

To identify maternal factors controlling early vertebrate development, a maternal-effect mutant screen was carried out in zebrafish [15]. Among the mutants with a defect prior to midblastula transition (MBT), one line produced embryos with radial segregation of cytoplasm instead of animal pole aggregation. In addition, the fertilized embryo from the mutant mother does not show cellular cleavages and hence does not develop beyond the 1-cell stage. As the mutant embryo lacks polarity similar to Buckminsterfullerenes, it was referred to as *bucky ball* (*buc*) [15].

In the oocyte, Buc mutants fail to assemble germ plasm into a Balbiani body (Bb) (**Figure 3A**). Instead, germ plasm components like *nanos* and *vasa* mRNA are no longer localized to the Balbiani body, but rather distributed ubiquitously in the ooplasm [54]. This result described the first gene in vertebrates required for the formation of the Balbiani body and the localization of germ plasm components in the oocyte. Moreover, if the cDNA of Buc is ectopically expressed from a transgene, ectopic Bbs are seen (**Figure 3D**) [55]. This leads to the conclusion that Buc, similar to sOsk in *Drosophila*, is necessary and sufficient for germ plasm assembly.

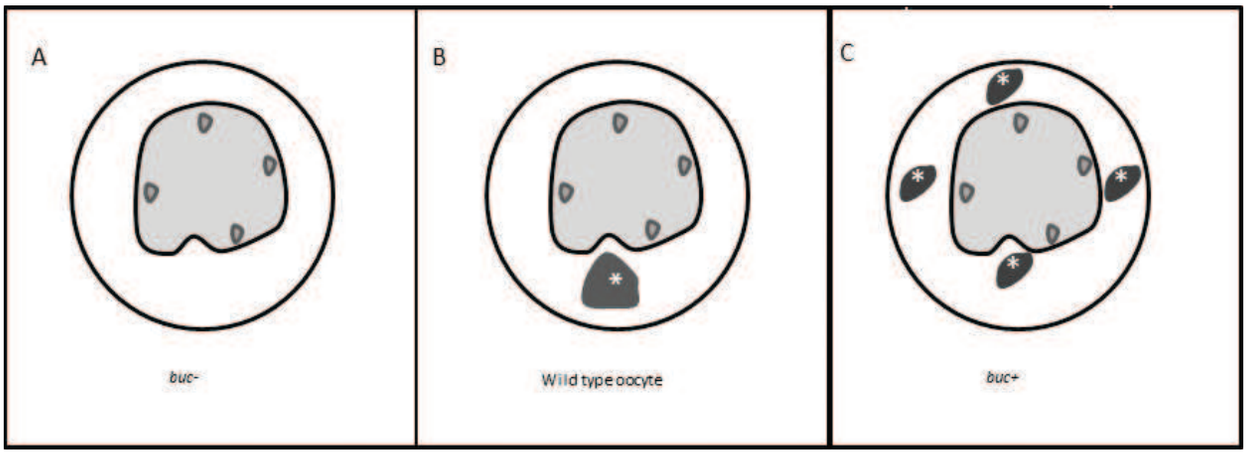


Figure 3. Scheme summarizing the role of Buc for germ plasm assembly during zebrafish oogenesis. (A) In *buc* mutant oocytes (*buc*-), germ plasm assembly is disrupted, and Balbiani body components are ubiquitously distributed in the oocyte (red haze) [54]. (B) Wild-type stage I zebrafish oocyte, the central nucleus (germinal vesicle; gray), germ plasm/Balbiani body (red). (C) A transgene with the Buc cDNA is over-expressed, which leads to the ectopic formation of multiple Bbs (red) [54, 55].

7.1. The conservation of Buc across the vertebrate kingdom

Buc is present in vertebrates; however, across its homologs in the vertebrate phylum, the sequence changes quite rapidly [54]. Zebrafish has two paralogs of Buc in its genome, whereas the salmon has three [56]. Currently, the function of the other paralogs is not clear. The *Xenopus* Buc homolog Xvelo exists in two splice forms, long Xvelo and short Xvelo. Both seem to play redudant roles in maintaining germ plasm assembly [51]. In humans, two genetic loci show homology to Buc protein (Gene ID EU128483, EU128484) [54], but the sequence is interrupted by STOP-codons and hence, does not encode an open reading frame. Human ovaries show RNA expression from these loci, but their function is not known (Lyautey et al., unpublished). BUC might act as a noncoding RNA or encode a short peptide [54, 57]. Whether the homologs from other mammals have an open reading frame, like Velo in *Xenopus* or Buc in zebrafish and can in fact induce germ cells, would open an exciting new avenue for stem cell research as well as regenerative medicine.

8. Similarities between Oskar and Buc

Buc and sOsk show a striking homology at the genetic level regarding germ plasm formation. Both mutants show a defect in polarity and a failure of germ plasm aggregation [54, 58]. Remarkably, ectopic overexpression of sOsk and Buc induces the formation of additional germ cells [32, 54]. To this end, no other proteins have been described, which can induce PGC formation in an organism.

Fascinatingly, ectopic expression of *Drosophila* sOsk in zebrafish induces the formation of primordial germ cells similarly to Buc (**Figure 4**) (Krishnakumar et al., unpublished). At the

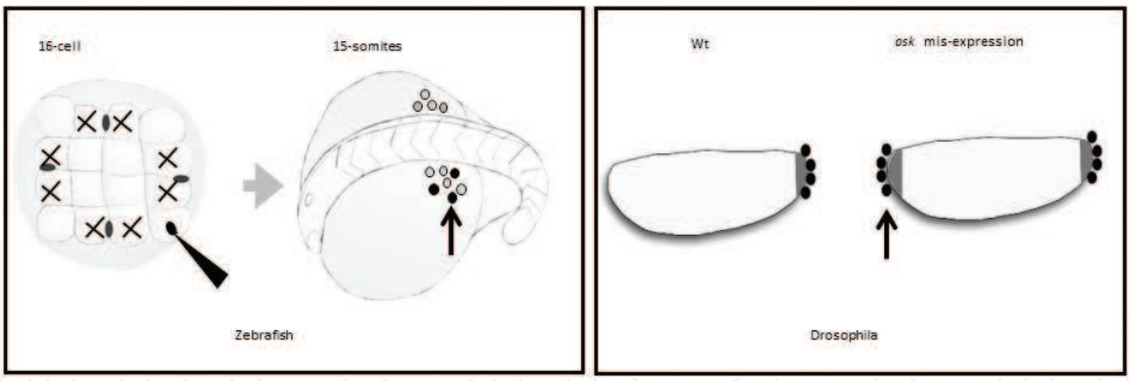
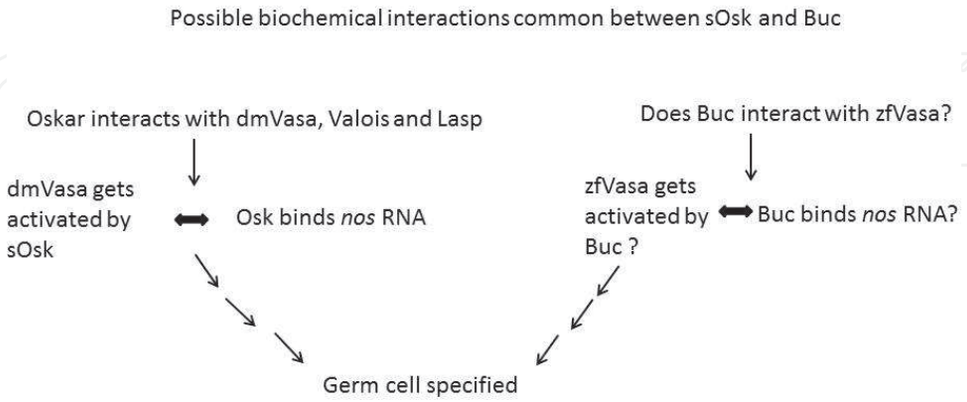


Figure 4. Ectopic PGC induction by germ plasm organizer overexpression. (A) Scheme showing a zebrafish 16-cell embryo in animal view. The middle blastomeres (red) contain endogenous germ plasm and hence, contribute to the PGCs of the embryo. The yellow blastomeres will not participate in germline development and form somatic structures e.g. neurons, muscle, etc. Buc overexpression (green) in a somatic blastomere is sufficient to reprogram germ cells formation. 24hpf stage embryo in a lateral oblique view, anterior to the left. Red cells highlight the endogenous germ cells. Overexpression of Buc in a somatic blastomere leads to the formation of ectopic germ cells (green). (B) Scheme showing *Drosophila* embryos at stage 5, anterior to the left, dorsal to the top. In wt embryos, sOsk is localized to the posterior pole (red), where it induces the formation of ectopic germ cells. Right embryo: germ plasm transplantation or anterior *oskar* localization (red) is sufficient for the specification of ectopic germ cells. Blue arrows point at extra germ cells.

molecular level, *buc* as well as *osk* mRNA localize with other germ cell specific molecules to the germ plasm during oogenesis. This result suggests that Osk and Buc have an overlap in their biochemical network, which they use to form germ plasm and specify germ cells.

sOsk was shown to interact with Vasa, Valois, and Lasp [45, 53, 59]. For example, if Buc also binds to zebrafish Vasa, it could mean that Buc uses a similar set of germ cell core factors like Osk to specify germ cells. *Vice versa*, it would also suggest that Oskar might use zebrafish Vasa to induce germ cells. Taken together, identifying the Buc-interactome might identify conserved factors, which were already core components of the germ cell specification pathway in the first multicellular animals (**Scheme 1**).



Scheme 1. Osk and Buc could have an overlap in their biochemical network, which they use to form germ plasm and specify germ cells.

8.1. Conservation between Oskar and Buc

According to the sequence-structure-function paradigm, proteins with a conserved activity contain homologous sequence motifs to interact with similar binding partners. Conserved sequences were previously not identified between sOsk and Buc [41, 54, 60]. Buc does not have a visible LOTUS domain, which is required for multimerization and takes part in the interaction with Vasa [46]. Moreover, Buc has no motif with homology to any known RNA binding domain. However, the OSK RNA-binding domain was also not described previously in other proteins and many RNA binding motifs do not show conserved domains [61]. Presently, none of the published bioinformatic analysis detected sequence similarities between the two germ plasm organizers Osk and Buc. Hence, their conserved activity remains a mystery. Overall, this would suggest that the structure or biophysical nature of both proteins might be similar in order to accomplish the same activity by which both would give rise to the "core" RNA-protein complex. sOsk and Buc might, therefore, represent the first protein pair of a frequently postulated phenomenon: Two proteins with similar function without sequence similarity [62].

9. Vasa: the ubiquitous germ cell marker

Vasa seems to be the most widely used molecular marker to identify germ cells [63–67]. Vasa is well conserved during evolution and required for germline development. Vasa is a member of the DEAD-box protein family of RNA helicase suggesting that it resolves duplex RNA or RNA-protein hybrids. Mutations in Vasa show defects in posterior patterning and in germ cell specification in the *Drosophila* embryo [63] . Vasa mutant zebrafish do not form gametes and develop as sterile males [68]. Vasa-null male mice are infertile because their germ cell do not proliferate and differentiate [69]. The VASA-like gene *DBY* in humans also appears to be required for male fertility [70]. In gain of function experiments, ectopic Vasa expression in chicken embryonic stem cells induces expression of specific germline and meiotic genes [71]. When these cells are transplanted into chick embryos, they migrate to the gonad anlagen and differentiate into gametes. Overall these results support the theory that Vasa has a central role in establishing germ line identity and function, however the exact function is still not known.

Vasa RNA or protein expression is frequently used to label PGCs in animals. As at least one homolog seems to be present in all metazoans, Vasa is also an easily accessible marker across the animal kingdom [72]. However, the restriction of Vasa at the blastula stage to the germ plasm and prospective PGCs varies across species. In some species like the zebrafish, Vasa protein is ubiquitous at early stages and later gets restricted into PGCs [73], which raised concerns about the role of Vasa during germ cell specification.

Exciting results from *Drosophila* provided a novel perspective on Vasa and germ cell specification [46]. Vasa has been shown to be activated by sOsk. This would mean that not the localization of Vasa protein or RNA labels the region of the early embryo, where germ cells are specified, but it only matters, where Vasa is active. So far, the activity of Vasa was only determined biochemically by the hydrolysis of ATP, but we still do not know what the activity of Vasa *in vivo* is. It would, therefore, be interesting to differentiate between inactive and active Vasa in the developing embryo and whether the active form labels specified germ cells.

In conclusion, regulatory proteins of Vasa activity like sOsk seem to be a much more reliable marker for germline specification.

10. Low complexity proteins

Low complexity (LC) proteins are of two types, amyloid and intrinsically disordered proteins (IDPs) [74–76]. **Table 3** compares the differences between the two types of LC-proteins.

Both Buc and sOsk have been suggested to have low complexity regions [41, 75, 77]. Indeed, it was shown that sOsk contains an intrinsically disordered region critical for Vasa binding. In Buc and Velo1, it was shown that parts of the conserved BUVE-motif form prions or amyloid-like aggregates. IDPs frequently evolve faster than structured proteins [74, 82]. This feature might hide conserved motifs in both proteins, which are critical to interact with the same biochemical network.

IDPs are also known to act as hubs for supra-molecular complexes and are also more prevalent in RNA-binding proteins. As sOsk fits this profile, it would be interesting to know whether Buc binds RNA to explain their conserved activities. Moreover, IDPs form liquid-liquid phase separations such as RNA-granules, which were also described for the germ plasm in *C. elegans* [79]. Some evidence was provided by *in vivo* imaging of germ plasm in zebrafish [83] and *Drosophila* [84] that germ plasm is liquid. Nonetheless, the level of intrinsic disorder of germ plasm organizers and the liquid properties of germ plasm in fly and fish are still not clear. It is presently unknown how the protein components like Oskar, Vasa, assemble into a germ granule aggregate. RNA-binding proteins have been shown to undergo phase transitions from a soluble to viscous state [85–87]. Thus, RNAs may be trapped by germ plasm aggregates, which become a granule and thereby facilitate more RNA-RNA and RNA-protein interactions. Oskar has been suggested to contain disordered regions, which connect the domains that were crystallized. These regions could push for the propensity to form aggregates as well.

Interestingly, Buc has been discussed to have both amyloid and IDP regions. In *Xenopus*, the Buc homolog Velo1 aggregates into an amyloid like assembly forming the Balbiani body [77].

Properties	Amyloid	IDP
Structure	Low complexity regions form beta sheets.	Very low complexity with FG or FXXG repeats, in most cases with no secondary structure formation.
Chemical	Aggregates are resistant to SDS and high salt concentrations.	Aggregates are dissolved by SDS or high salt concentrations.
Aggregation	Aggregates are resistant to 1,6 hexanediol.	1,6-hexanediol dissolves hydrogels formed by IDPs.
Staining	Stain positively with Thioflavin S and T.	No accumulation of Thioflavin.
	Examples: Amyloid plaques, Balbiani body Xvelo protein in *Xenopus* (Boke et al. [77]).	**Examples***:* Nuclear pores [Nucleoporins (Frey et al. [81])], germ plasm in *C. elegans*.

References [77–81].

Table 3. The differences between the two classes of low complexity proteins, amyloid and IDP.

By contrast, BucGFP molecules showed hydrogel or liquid droplet-like behavior in the early zebrafish similar to the P-granules in *C. elegans* [79, 83]. This suggests a controlled transition from an amyloid plaque to a soluble hydrogel at the end of oogenesis. Understanding, how the same protein can generate different aggregates and how these transitions are regulated *in vivo* will be quite exciting. Finding the molecular mechanism, by which the oocyte dissolves amyloids, might also provide a therapeutic strategy to dissolve protein aggregates during neurodegenerative diseases like Alzheimer's.

Overall the aggregation of IDPs emerge as a central theme in germ cell specification. Just like Vasa, which is also intrinsically disordered region [88] and like the polymerizing substrates of P-granules which are the MEG1 and MEG 3 proteins in *C. elegans* [89], Buc and Osk self-aggregate and assemble germ plasm *via* phase transition.

11. A common recipe to make germ cells

If Osk and Buc have diverged from a common ancestor, their precursor would have been an ancient protein of low complexity, which induces germ cell formation. Both proteins probably have unrelated sequences as consequence of their role as intrinsically disordered scaffolds. This structural role releases the constraints to maintain a defined protein structure as described for other IDPs [90]. This divergence probably hides conserved motifs, which bind to a similar interactome such as Vasa, Valois, and probably other common mRNA binding partners (**Figure 5**). Finding interaction partners and mapping the interaction motifs like for the sOsk-Vasa interaction will determine, to which level interaction motifs are conserved between sOsk and Buc.

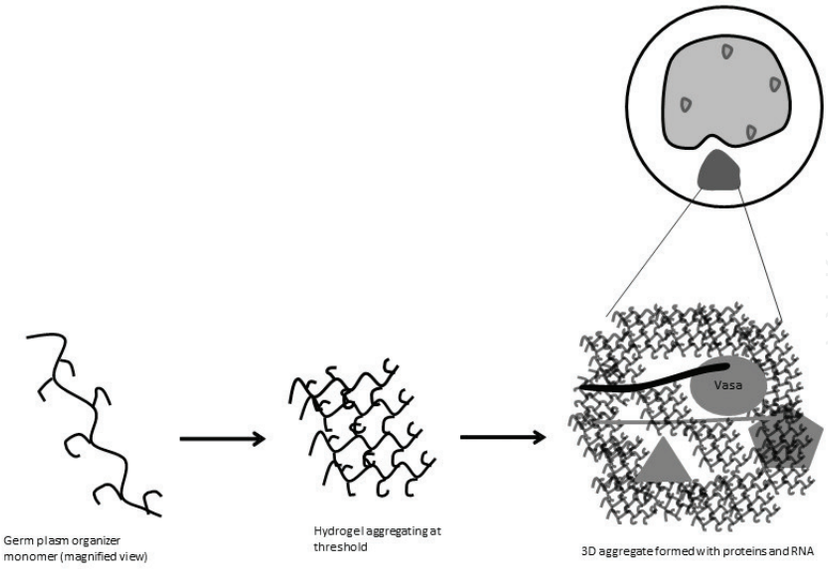


Figure 5. Model for germ plasm formation. Single monomer molecules of germ plasm organizer (red) aggregate through weak interactions of their intrinsically disordered regions (hooks and loops), until a threshold concentration is reached. This leads to a liquid-liquid phase separation (red haze) to form hydrogel-like germ plasm. The aggregate then selectively recruits protein (geometric shapes) and mRNA (lines). This gets packed into germ plasm *e.g.* as shown above in the Balbiani body of the oocyte.

Describing the Balbiani body, a picture of the popular "bubble tea" comes to mind. In this picture, the organizer proteins form a scaffold probably via self-aggregation or upon binding with their interactors similar to the chewy alginate balls, which form during polymerization. During this process, germ plasm assembles and thereby integrates RNA and proteins into this 3D liquid lattice. The assembly also initiates Vasa's activity to start the downstream program, e.g. to protect RNAs and proteins from degradation [91]. The germ plasm also exchanges components with the cytoplasm similar to those spheres floating in the bubble tea. When inherited into a cell, the germ plasm probably releases some proteins whose translation and stability is tightly controlled. Once these factors are unleashed from the bubble spheres, they change the transcriptional program to specify the maturation of a PGC to a gamete.

12. Conclusion

Why should germ cell specification be conserved? Reproduction is a conserved feature of all biological systems and must have been, therefore, be present in the first metazoans before other cell types like neurons, muscle or a vascular system. Germ cell specification was, therefore, present before the formation of an eye or even a nervous system. Nonetheless, the conservation of the master regulator Pax6/Eyeless showed that light sensing organs were already present at the base of metazoan evolution [92]. Although this hallmark finding is currently accepted in the scientific literature, the insect compound eye and the vertebrate camera-eye were regarded as a paradigm for convergent adaptations. We, therefore, speculate that germ cell formation is the more ancient tissue compared to eyes, would use an even more conserved molecular regulation than Pax6/Eyeless.

When animals started to become multi-cellular, they could no longer continue to reproduce by simple cell cleavage. They needed to set the germline apart from the soma for their reproduction [93]. For this task, they had to evolve proteins, which served as master switches for germ cell specification. Any changes to the function of these proteins could have lasting consequences on the propagation of that species. However if these proteins were IDPs, they could still perform their function, despite of rapid (localized or random) changes. These changes could have roles in speciation or better coordinated control of specification. Whatever the case, if they still aggregated and setup the "core" complex, a germ cell would have still formed.

13. Future directions and recommendations: back to the future

Ciliates form a cytoplasmic aggregate called the conjusome [94]. This structure is present only during sexual reproduction. Similar to the Balbiani body in *Xenopus* and the P-granules in *C. elegans*, the conjusome is made up of fibrous, electron dense material [94]. It also contains a Piwi related protein TWI, which protects the integrity of the genome [95]. These commonalities with germ plasm are very striking and suggest that the conjusome might be the ancestral form of germ plasm. Hence, the organizer protein in Ciliates probably displays a very different amino acid sequence from Osk and Buc. However, the Ciliate

organizer might have similar characteristics like Osk and Buc, such as forming the protein-RNA core or even induce germ cells in zebrafish. If indeed a germ plasm like structure existed in unicellular organisms, germ cell specification by induction would have emerged after the transition to multicellularity, because signaling requires a multicellular environment. It will, therefore, be quite interesting to find out to which level germ plasm in metazoans and structures like the conjusome in unicellular organisms are conserved. Therefore, if these conjusomes could be chemically isolated, its proteins and RNA can be compared to the known components of germ plasm. This will show if there is an evolutionary clue between the conjusome in lower organisms and germ plasm in higher organisms thus providing the missing link.

Expanding on this hypothesis, protein phase transition might have been present before the first unicellular organisms. If the beginning of life was an RNA world [96] and formation of a cell was needed to protect the genetic material, it would have been easier to have a hydrogel aggregate of slime or protein lock the RNA into an RNA granule than to establish a lipid bilayer with an internal framework. Indeed if that was the case, this structure would have been more similar to the germ plasm that we see today than to a membrane-bound cell. Thus the origin of life would have been from a germ plasm ancestor similar to a drop of Amrit or Ambrosia spilled from the heavens.

Acknowledgements

Pritesh Krishnakumar was supported by the German Academic Exchange Service (DAAD). Research in the Dosch lab is supported by the Deutsche Forschungsgemeinschaft, the GGNB Junior Group Stipend and the “Forschungsförderungsprogramm” of the University Medical Center Goettingen.

Abbreviations

Buc	Bucky ball
Bb	Balbiani Body
C. elegans	*Caenorhabditis elegans*
IDPs	Intrinsically disordered proteins
Osk	Oskar
Pax6	Paired box protein 6
PGCs	Primordial germ cells
RNA	Ribonucleic acid
Xvelo-1	Xenopus Vegetal localized 1

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