Stem Cell Research 24 (2017) 188–194

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Convergent evolution of germ granule nucleators: A hypothesis

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ARTICLE INFO

Article history:
Received 28 February 2017
Received in revised form 18 June 2017
Accepted 15 July 2017
Available online 19 July 2017

Keywords:
Germ cells
Germ granules
Germ plasm
Nucleators
Convergent evolution

ABSTRACT

Germ cells have been considered "the ultimate stem cell" because they alone, during normal development of sexually reproducing organisms, are able to give rise to all organismal cell types. Morphological descriptions of a specialized cytoplasm termed 'germ plasm' and associated electron dense ribonucleoprotein (RNP) structures called 'germ granules' within germ cells date back as early as the 1800s. Both germ plasm and germ granules are implicated in germ line specification across metazoans. However, at a molecular level, little is currently understood about the molecular mechanisms that assemble these entities in germ cells. The discovery that in some animals, the gene products of a small number of lineage-specific genes initiate the assembly (also termed nucleation) of germ granules and/or germ plasm is the first step towards facilitating a better understanding of these complex biological processes. Here, we draw on research spanning over 100 years that supports the hypothesis that these nucleator genes may have evolved convergently, allowing them to perform analogous roles across animal lineages.

1. Introduction

Germ cells form an important subset of stem cells in metazoans, and are specified during embryogenesis either by the inheritance of a specialized cytoplasm, or via zygotic induction, i.e. extra-cellular signaling from neighboring somatic cells (Extavour and Akam, 2003). Germ cells are critical in maintaining the continuity of life and hence have been called "immortal" and "the ultimate stem cell" by some authors (Cinalli et al., 2008; Gao and Arkov, 2013; Nussbaum, 1880; Wilson, 1896). In the early 1890s, August Weismann pointed out that it was not that the germ cells per se remained continuous and immortal, but rather that the passage of certain "substances" from the parent germ cell to its progeny resulted in this "immortal" continuity (Weismann, 1892). He referred to these substances collectively as "germ plasm," thus providing, to our knowledge, the earliest recorded use of this term (*Das Keimplasma*: Weismann, 1892). Although Weismann had originally used the term germ plasm to mean nuclear genetic material (discussed by Lankenau, 2008), today germ plasm refers to a specialized cytoplasm, often morphologically and spatially distinct, that is contained within and confers fate upon the germ cell lineage (Eddy, 1975; Gao and Arkov, 2013; Guraya, 1979; Ikenishi, 1998; Voronina et al., 2011; Weismann, 1892). Contemporaneous with and following Weismann's discovery and description of germ plasm came independent observations that this cytoplasm contained granular material, termed "germ granules" (Hegner, 1911; Metschnikoff, 1866; Ritter, 1890). These granules were later found to contain specific ribonucleoprotein (RNP) complexes that often included apparent molecular determinants of germ line specification (see for example Illmensee and Mahowald, 1974, 1976; Strome and Wood, 1982). Germ line RNP complexes have been referred to using various terms in the literature over the years, oftentimes as a consequence of observed morphologies at various developmental stages in different organisms (Table 1). Throughout this review we will collectively refer to germ line RNP complexes as germ granules for simplicity and consistency.

Although germ granules were discovered more than a century ago, we are only recently beginning to understand the molecular-level biology behind their formation (also referred to as nucleation) and composition across metazoans (Gao and Arkov, 2013). In this review, we limit our discussion to the evolution of proteins that appear to cause or catalyze the nucleation of germ granules in non-mammalian species. Multiple pathways (reviewed by Voronina et al., 2011) that include the (inter)action of seed proteins known as "nucleators" (e.g. Oskar, Bucky ball, Xvelo1 and PGL), mitochondria (Huang et al., 2011; Watanabe et al., 2011), Tudor-domain containing proteins (Arkov et al., 2006), low-specificity protein-RNA interactions (Brangwynne et al., 2009) and small non-coding RNAs (Sengupta and Boag, 2012) are currently implicated in this process. Using a comparative approach that focuses on recent advances in our molecular understanding of the few germ granule nucleators listed above, we look at what is known about how novel nucleators arise, and ask whether the available literature can be used to address the hypothesis that these nucleators have evolved convergently to perform analogous roles.

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http://dx.doi.org/10.1016/j.scr.2017.07.018

Table 1
Germ granule nomenclature simplified by developmental stage and/or cell type.

Developmental stage	Referred to as	Select species examples	Select references
Immature and undifferentiated/ developing germ cells	Nuage, perinuclear granules	*Caenorhabditis elegans*	(Strome and Wood, 1982)
		Drosophila melanogaster	(Mahowald, 1968)
		Xenopus laevis	(Ikenishi et al., 1996)
		Danio rerio	(Knaut et al., 2000)
		Mus musculus	(Chuma et al., 2009)
Mature gametes such as oocytes and sperm	(Oocytes) Sponge bodies, Balbiani body, mitochondrial cloud, germ plasm	*Drosophila melanogaster*	(Cox and Spradling, 2003; Hurd et al., 2016; Snee and Macdonald, 2004)
		Xenopus laevis	(Kloc et al., 2004) (Bilinski et al., 2004)
		Danio rerio	(Bontems et al., 2009; Marlow and Mullins, 2008)
		Mus musculus	(Pepling et al., 2007; Spiegelman and Bennett, 1973)
	(Sperm) Chromatoid bodies, inter-mitochondrial cement	*Mus musculus*	(Chuma et al., 2009; Spiegelman and Bennett, 1973)
Embryos	P-granules	*Caenorhabditis elegans*	(Strome and Wood, 1982)
	Germ plasm, polar granules, Balbiani body	*Drosophila melanogaster*	(Illmensee and Mahowald, 1974, 1976)
		Xenopus laevis	(Kloc et al., 2004)
		Danio rerio	(Bontems et al., 2009; Marlow and Mullins, 2008)

2. Germ granules are characteristic of germ cells but may not always confer germ cell identity

Here we use the term "germ granules" to describe a class of cytoplasmic RNP complexes with differing morphologies and localization patterns during development (Table 1), unique to and characteristic of germ cells (Arkov and Ramos, 2010; Eddy, 1975; Gao and Arkov, 2013; Ikenishi, 1998; Schisa, 2012; Voronina et al., 2011). These complexes have been previously described as motile, electron dense, compact, highly dynamic, fibrillar or granular in appearance and lacking a membrane (Arkov and Ramos, 2010; Eddy, 1975; Gao and Arkov, 2013; Ikenishi, 1998; Schisa, 2012; Voronina et al., 2011). Although non-membrane bound, germ granules are organized in their architecture. Recently, it has been shown that some germ granules are divided into subdomains of specific protein and/or RNA composition (see for example Little et al., 2015; Schisa, 2012). Germ granules are required for germ cell function in all organisms, even though many organisms do not depend on them to specify germ cell fate (reviewed by Voronina et al., 2011). Thus, these granules can be formed *de novo* in primordial germ cells upon induction (e.g. *Mus musculus*) or inherited as part of the maternal germ plasm (e.g. *Drosophila melanogaster*, *Danio rerio* and *Xenopus laevis*) (see Table 1 for details and references). Recent advances in our understanding of animals that inherit germ granules suggest that germ plasm and germ granules are not equivalent, and may in fact represent distinct functional entities (reviewed by Marlow, 2015).

Germ cells can maintain other RNP complexes that are distinct entities from germ granules, including processing bodies (P-bodies) and stress granules. The latter two types of RNP complexes are also found in somatic cells (Balagopal and Parker, 2009; Nover et al., 1989). However, growing evidence suggests that all of these complexes share multiple components in common (Fig.1) and may therefore be related (Gallo et al., 2008; reviewed by Voronina et al., 2011). While the RNA within all of these RNP granules consists of both coding and non-coding components, three consistent protein classes are characteristic of all granules: RNA helicases (e.g. Vasa), Tudor-domain proteins (e.g. Tudor), and Piwi family proteins (e.g. Piwi) (Gao and Arkov, 2013). This may explain why the expression and function of genes such as *vasa* and *piwi*, which are often considered germ cell markers, are not restricted to the germ line, but are also integral to the maintenance and differentiation of somatic cells (see for example Alié et al., 2011; Ewen-Campen et al., 2010; Schwager et al., 2015; Yajima and Wessel, 2011).

Central to the idea of germ granule assembly was the discovery of a handful of proteins that function as germ granule inducers, assemblers or nucleators. These proteins help initiate the assembly of germ granules (and/or germ plasm) by recruiting several similar downstream components. Some of these components are highly conserved, such as Vasa and Piwi family members, and others may be more species-specific (Hanazawa et al., 2011; Hay et al., 1988a, 1988b; Lasko and Ashburner, 1988; Raz, 2000). Examples of apparent germ granule nucleators include Oskar (*osk*) from *D. melanogaster* (Lehmann and Nüsslein-Volhard, 1986), PGL proteins (*pgl-1* and *pgl-3*) from *C. elegans* (Hanazawa et al., 2011), and the vertebrate-specific Bucky ball (*buc*) from *D. rerio* (Bontems et al., 2009; Marlow and Mullins, 2008) along with its *X. laevis* homolog, Vegetally localized 1 (*xvelo1*) (Nijjar and Woodland, 2013). *osk*, *pgl* and *buc* share low sequence similarity with each other and are not orthologous, suggesting that they are novel lineage-specific genes that arose independently. *osk*, being the best understood of these genes, is discussed in a comparative context below.

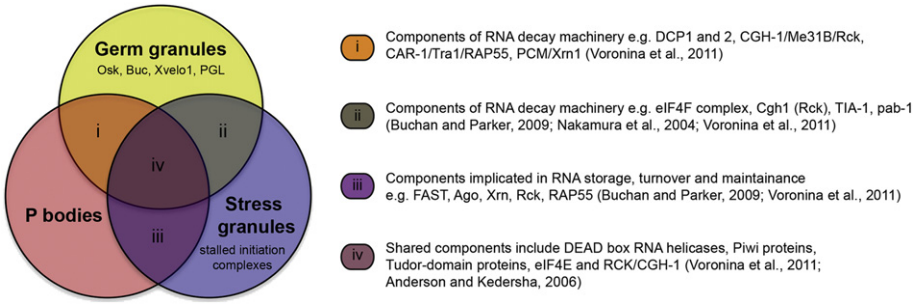

Fig. 1. Shared and distinct components of RNP granules in germ cells and somatic cells. A venn diagram (left) showing some examples of shared components (detailed at right) between germ granules, P-bodies and stress granules (Anderson and Kedersha, 2006; Buchan and Parker, 2009; Nakamura et al., 2004; Voronina et al., 2011). Components currently thought to be unique to each class of RNP complexes are also listed. It should be noted that a complete list of components for any RNP granule-type is currently lacking, limiting our understanding of shared and unique components.

3. oskar (osk) as a germ granule nucleator in insects

Some of our best understanding of germ plasm biology comes from studies on *D. melanogaster*, where *osk* has been shown to be indispensable for the localization of germ plasm (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991; Lehmann and Nüsslein-Volhard, 1986). Translation of localized *osk* mRNA at the oocyte posterior yields two protein isoforms: Long Osk, which localizes to endocytic membranes, and Short Osk, which is integral to polar granules (Tanaka and Nakamura, 2008; Vanzo et al., 2007). Despite these isoforms being almost identical save for the N-terminal 138 amino acids present only in Long Osk, there is evidence that they perform distinct developmental roles within the oocyte (Breitwieser et al., 1996; Jeske et al., 2015; Markussen et al., 1995; Vanzo et al., 2007; Vanzo and Ephrussi, 2002). Osk is a partially disordered protein, but recently, crystal structures were solved for two well-ordered domains that are common to both isoforms (Jeske et al., 2015; Yang et al., 2015). These structures and their possible implications are briefly discussed below.

Osk has two structured domains, separated by a highly-disordered region (Jeske et al., 2015; Yang et al., 2015). Towards the N-terminus is the LOTUS or OST-HTH domain (Anantharaman et al., 2010), which is a winged helix-turn-helix globular domain also present in other germ plasm/germ granule components, such as some Tudor family members (Callebaut and Mornon, 2010; Jeske et al., 2015). At the C-terminus is the OSK domain (Jeske et al., 2015), a lipase-related domain with sequence similarity to SGNH hydrolases (Ewen-Campen et al., 2012; Jeske et al., 2015; Juhn et al., 2008; Lynch et al., 2011). The OSK domain is predicted to be enzymatically inactive, but important in binding mRNAs including nanos (*nos*), polar granule component (*pgc*) and germ cell-less (*gcl*) (Ewen-Campen et al., 2012; Jeske et al., 2015; Juhn et al., 2008; Lynch et al., 2011; Yang et al., 2015). Based on current data it appears likely that Long Osk controls mitochondrial inheritance in primordial germ cells (Hurd et al., 2016) and helps anchor Short Osk at the posterior pole of the embryo (Vanzo et al., 2007). Osk dimerization and interaction with Vasa were recently shown to occur via the LOTUS domain (Jeske et al., 2015; Vanzo et al., 2007). Whether the Osk isoforms form homodimers or heterodimers *in vivo* is currently unknown.

Interestingly, the Long Osk domain (i.e. the 138 amino acid N-terminal extension) seems specific to the Drosophilid lineage. BLAST searches of non-Drosophilid insect sequence databases (e.g. the wasp *Nasonia vitripennis*, the ant *Messor pergandei*, the mosquito *Aedes aegypti*, the beetle *Acanthoscelides obtectus*), and the cricket *Gryllus bimaculatus* recover only the short isoform, suggesting that Long Osk may be a recent evolutionary innovation in insects (Lynch et al., 2011). Nevertheless, as shown by studies in *N. vitripennis* (Lynch et al., 2011; Ewen-Campen et al., 2012) at least some of these *oskar* orthologues that lack the Long Osk domain code for *oskar* gene products that are localized to functional germ plasm at the posterior pole of the egg. This indicates that although *D. melanogaster* may rely on the Long Osk domain for posteriorly localized germ plasm assembly (Vanzo et al., 2007) and mitochondrial accumulation (Hurd et al., 2016), other insects have Long Osk-independent mechanisms for accomplishing these tasks.

4. Examples of germ granule nucleators in other animals

In this section, we discuss some key similarities between Osk from *D. melanogaster* and germ plasm nucleators from other species. In *C. elegans*, the P-granule abnormality (PGL) proteins (*pgl-1* and *pgl-3*) are proteins that are dynamically associated with and integral to the formation of P-granules, the germ granules of nematodes, but are not phylogenetically related to *osk* (Aoki et al., 2016; Updike et al., 2011). In addition to having RNA binding motifs (the RGG domain), PGL-1 and 3 both have dimerization domains (DD) and form homodimers (Aoki et al., 2016). Based on these results, Aoki et al. (2016) have suggested that these homodimers may comprise fundamental building blocks of P-granules. The observation that Osk also forms dimers *in vitro* (Jeske et al., 2015; Yang et al., 2015) raises the possibility that fruit fly germ plasm may be assembled using analogous molecular mechanisms to those posited for *C. elegans*. The PGL-1 DD encodes a novel guanosine-specific, single-stranded RNA endonuclease (RNase T1-like), whose structure assumes a novel fold and contains no recognizable classic RNase active site (Aoki et al., 2016). While the exact molecular mechanisms remain unknown, this RNase is implicated in translational inhibition (Aoki et al., 2016). Novel enzymatic domains (and enzymatic roles novel to germ granule nucleators, such as that of the endonuclease described above) currently remain unidentified in Osk and other germ granule nucleators.

bucky ball (buc) was the first gene shown to be necessary and sufficient for germ plasm organization in a vertebrate. In *D. rerio*, *buc* controls the formation of the Balbiani body (Marlow and Mullins, 2008), a perinuclear structure found in many animal oocytes (Guraya, 1979; Kloc et al., 2004), and specifies germ line identity (Bontems et al., 2009). Even though unrelated by sequence, *buc* and *osk* are both rapidly evolving proteins, have similar localization patterns, and exist as different isoforms (reviewed by Marlow, 2015). Buc is predicted to be a disordered protein with no recognizable characterized domains that could help explain its biochemical function. Nevertheless, Buc contains two conserved motifs (suggested by alignment of 15 related vertebrate Buc proteins) with no known homology to other protein domains. The first of these is the N-terminal 100 amino acids, termed the BUVE (Buc-Velo) motif, and containing a highly conserved 15 amino acid sequence (Bontems et al., 2009). Using prion detection algorithms (PLAAP and PAPA), Boke and colleagues (2016) found evidence for a second type of conserved domain: prion-like domains (PLDs) (Boke et al., 2016). These domains, found in both Buc and Osk, are defined as protein regions with sequence similarity to yeast prions (Alberti et al., 2009; Si et al., 2003), and are low complexity, intrinsically disordered regions that cause amyloid-like self-assemblies (Courchaine et al., 2016; Kato et al., 2012). Research on yeast has indicated that translational control mediated via proteins that form amyloid-like aggregates is required for gametogenesis in this single-celled eukaryote (Berchowitz et al., 2015). These results have led to the speculation that the diverse processes of spore formation in yeast and sperm formation in animals are fundamentally related, and may rely on an ancient mechanism that involves amyloid-like aggregates (Berchowitz et al., 2015). Nevertheless, the exact mechanism of germ plasm assembly in zebrafish remains unknown (Bontems et al., 2009).

xvelo1, the *X. laevis* homolog of *buc*, exists as two splice variants, with the longer variant being an abundant constituent of the Balbiani body (Claussen and Pieler, 2004; Nijjar and Woodland, 2013; Skugor et al., 2016). Xvelo1 is predicted to be a disordered protein, with no recognizable functional protein domains, although it does possess the BUVE motif and the PLD described above for Buc. For Xvelo1, the N-terminal PLD is necessary and sufficient for Balbiani body localization, and is implicated in forming amyloid-like networks *in vitro*, by binding RNA and sequestering mitochondria, a function similar to that of Long Osk (Boke et al., 2016; Hurd et al., 2016). An interesting observation is that when expressed in *X. laevis* oocytes, the PLD of Buc causes its co-localization to the Balbiani body as well, suggesting that PLDs are required for Balbiani body association. It has been suggested that amyloid-like assemblies of disordered proteins such as Xvelo1 and Buc may be an evolutionarily conserved mechanism for Balbiani body formation (Boke et al., 2016). The inter-relationship between the PLD and the Buc/Xvelo1 BUVE motif (or the conserved 15 amino acid peptide within the BUVE), however, remains unexplored.

To explore the idea that PLDs might be important for nucleation of RNP granules in general, we searched the literature for their presence in non-germ granule nucleators, and found that a known nucleator of stress granules (somatic granules), the RNA binding protein TIA-1, has been reported to have a C-terminal PLD which promotes stress body assembly in environmentally stressed cells (Gilks et al., 2004). This

commonality of possessing a PLD among RNP granule nucleators might therefore have evolutionary implications and warrants further detailed study.

In contrast to all examples discussed above, mammalian germ cells lack germ plasm and are specified via induction later at the post-implantation epiblast stage of embryogenesis. Nonetheless, once specified, mammalian germ cells have been reported to contain germ granules (Eddy, 1975; Spiegelman and Bennett, 1973). These germ granules are newly assembled once the primordial germ cells have synthesized germ granule components *de novo*, which is well past the induction of PGC fate. In mouse PGCs, germ granules are reported approximately two to three days after PGC induction (thought to occur at E7.5) (Spiegelman and Bennett, 1973). The granules persist until later stages of germ cell differentiation, including spermatogenesis and oogenesis (Chuma et al., 2009). To our knowledge, a mammalian germ granule nucleator, or an alternative mechanism that regulates germ granule formation in mammalian PGCs, has not been identified. Given the presence of germ granules at E9.5–E10.5 in mice, analysis of PGCs at this midgestation period, rather than at the time of PGC specification (E7.5), would perhaps aid in the identification of such a nucleator or other assembly mechanism. The current lack of any known germ granule nucleators from mammals thus limits our comparison of nucleators to that of non-mammalian species alone.

5. Germ granule nucleators as examples of convergent evolution?

Multiple lines of evidence support the idea that germ granule nucleators might have evolved convergently to perform analogous roles in nucleating germ plasm, and hence in germ line specification across animals (Fig. 2). Osk, Buc (Xvelo1) and PGL are all novel genes specific to distinct animal lineages and are not orthologs. Moreover, they are all either intrinsically disordered proteins or have disordered regions, in addition to having PLDs that are implicated in specifying germ line identity. In the context of their own animal lineages, Osk, Buc (Xvelo1) and PGL are all fast-evolving proteins (Ahuja and Extavour, 2014; Bezares-Calderon et al., 2010; Skugor et al., 2016). At the biochemical level, Osk, Buc (Xvelo1) and PGL are known to interact with similar conserved players such as Vasa and Piwi (or their homologs) and for the formation of self-assemblies. It has been previously proposed that to function as a germ plasm nucleator, rather than a specific gene sequence, what may be more important are the biochemical or physicochemical properties of their respective proteins (Extavour, 2011; Lynch et al., 2011). Nevertheless, none of these nucleators are enzymes with catalytic activities in a classical sense. To date, germ granule nucleator activity has only been observed in the orthologs of these genes found in animal lineages that have germ plasm. Given that germ plasm is thought to have evolved convergently in animals (Extavour and Akam, 2003; Johnson et al., 2011), it is plausible that such a convergently evolved cytoplasmic environment favors the evolution of nucleators with certain biochemical properties as well. Lastly, we note that these nucleators exhibit several differences, including varied spatial expression patterns during development, some non-overlapping interaction partners, and lack of a protein-independent RNA role for some (e.g. Buc) (reviewed by Marlow, 2015). These differences are consistent with the hypothesis that germ granule nucleators evolved independently to perform a similar function.

6. Do novel genes facilitate novel developmental processes?

osk is an excellent example of how novel genes can facilitate novel developmental processes. The surprising discovery of *osk* and its non-germ line function in the basally branching insect lineage of the Orthopteran cricket *Gryllus bimaculatus*, was key in showing that the germ line role of *osk* may in fact be a recent evolutionary innovation, specific to

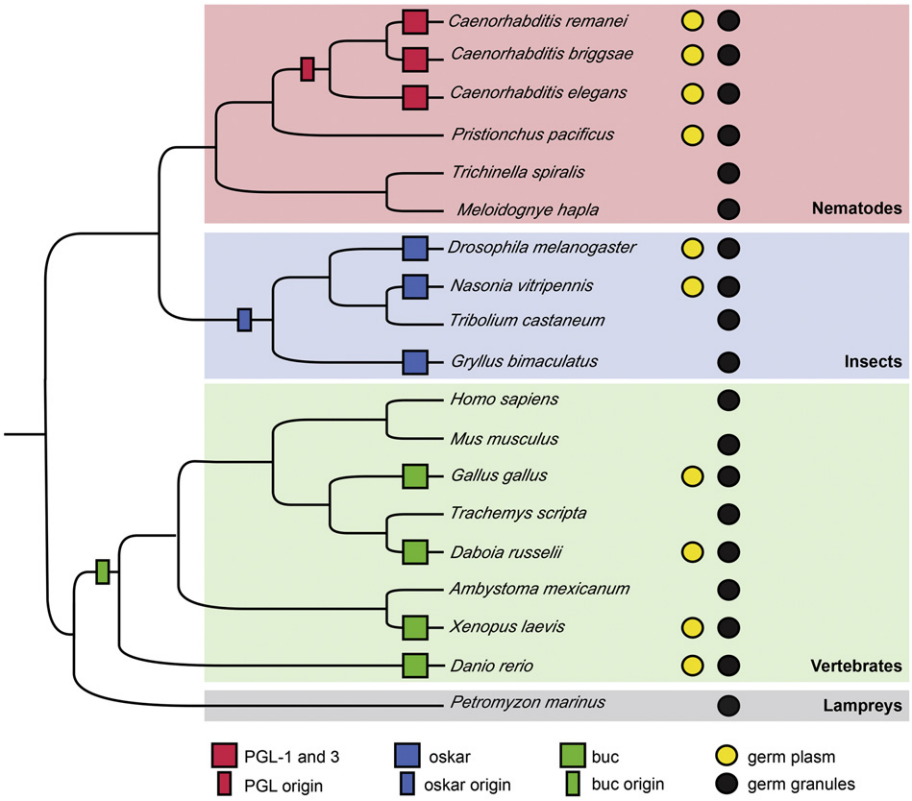

Fig. 2. Scenarios for the evolution of germ plasm nucleators. Based on current literature, the distribution of germ granule nucleators on this cladogram of different animal lineages (nematodes in red, insects in blue and vertebrates in green) suggests possible convergent evolution of these nucleators. Species that inherit germ plasm maternally are indicated in yellow.

the Holometabola (Ewen-Campen et al., 2012). Ewen-Campen and colleagues (2012) showed that in crickets, *Gb-osk* is essential for the development of the nervous system but not the germ line. Additionally, *Gb-osk* is co-expressed with *Gb-vasa* in neuroblasts, suggesting the possibility that a functional link between *osk* and germ line genes may have predated the evolution of germ plasm. If future results provide evidence that the upstream and downstream interactors of *osk* are conserved in *Gryllus* and *Drosophila*, then this could support their hypothesis that *osk* arose early in insect evolution with a (predominant) function in the nervous system and was then secondarily co-opted for a germ line role in the Holometabola (Ewen-Campen et al., 2012).

Within the Holometabola, there is support for the hypothesis that *osk* has intercalated between two evolutionarily ancient pre-existing regulatory networks, each with conserved roles in the germ line across Metazoans, and that this intercalation might have required relatively few evolutionary steps (Lynch et al., 2011; Quan and Lynch, 2016). Even though *osk's* germ line role is conserved across Holometabolan insects, there is ample evidence for Osk's divergence in function in assembling germ plasm in this clade. For example, knockdowns of *osk* in *D. melanogaster* and *N. vitripennis* show defects in somatic patterning, suggesting that *osk* plays additional roles outside of the germ line in these species (Lynch et al., 2011). Within the Drosophilids, germ granule morphology and size is dictated by *osk* (Jones and Macdonald, 2007), and is highly variable across the genus (Mahowald, 1968). In addition, the divergence of Osk's germ line function among Dipterans is now well documented. This is illustrated by the observation that *osk* from *D. virilis* cannot induce functional germ plasm formation in *D. melanogaster* (Webster et al., 1994), while *osk* from the equally distant *D. immigrans* can (Jones and Macdonald, 2007). Notably, however, *D. virilis osk* is capable of posterior patterning activity in *D. melanogaster* (Webster et al., 1994). These variations in the function of Drosophila *osk* orthologs within the genus, and its capabilities in a trans species context, were hypothesized to result from differences in the properties of their respective Osk proteins (Jones and Macdonald, 2007; Webster et al., 1994). In recent years, the divergence of Osk protein function in *D. virilis* has been linked to some parts of the gene evolving under positive selection in Drosophila (Ahuja and Extavour, 2014). In light of current information, future experiments with *osk* could help pinpoint which region(s) are important for these different biological functions across Drosophilids, and more broadly across insects. Finally, in some insect lineages, a secondary loss of *osk* coincides with a change in germ line determination strategies (Lynch et al., 2011).

7. How do novel nucleators arise?

How new genes arise has been a long-standing question in biology. Some events leading to the formation of new coding sequences include gene duplications, retro-transposition, domain swapping among genes, horizontal gene transfer and *de novo* formation from previous non-coding DNA (Ponce et al., 2012). Despite our current understanding of these mechanisms, *osk*, *buc*, *xvelo1* and *pgl* have unclear evolutionary origins, as they are novel proteins not obviously known to belong to any gene family. Phylogenetic analysis of *osk* indicates an origin ~-400 million years ago (mya) in the last common insect ancestor (Ewen-Campen et al., 2012; Misof et al., 2014). Based on BLAST search results, Osk's N-terminal domain is speculated to have arisen from a duplication and subsequent divergence of the highly conserved Tudor-domain-containing gene locus *tdrd7*. While further assessment is warranted, the sequence of the C-terminal OSK domain appears somewhat similar to that of bacteria, which may suggest that it was acquired through horizontal gene transfer (Ewen-Campen et al., 2012; Lynch et al., 2011). Thus, Lynch et al. (2011) have raised the possibility of a single origin for *osk* in the common Holometabolan ancestor, though the possible fusion of a *tdrd7* paralog to a gene possessing a hydrolase domain.

Preliminary data on the evolution of other nucleators is slowly emerging. *Buc* is estimated to have arisen more than 400 mya with a proposed ancestral role in the germ line of vertebrates (Skugor et al., 2016). Evidence for tandem duplications of *buc* among different vertebrates (with further gains and/or losses of *buc* paralogs) suggests additional roles of Buc outside of the germ line and warrants further study of Buc proteins. The PGL family of proteins, which appears specific to the nematode genus Caenorhabditis, is estimated to have evolved anywhere between 280 and 770 mya, depending on whether *C. elegans* divergence time is compared with *Pristionchus pacificus* or *Trichinella spiralis* respectively (Bezares-Calderon et al., 2010; Dieterich et al., 2008; Mitreva and Jasmer, 2006). Phylogenetic analysis suggests that PGL-1 and 3 are paralogs that formed after *C. elegans* diverged from other Caenorhabditis species including *C. remanei*, *C. briggsae* and *C. brenneri* (Bezares-Calderon et al., 2010). Only one homolog of PGL-1/-3 is present in a non-*elegans* Caenorhabditis species. However synteny in the region of this gene is not conserved with *C. elegans*, precluding use of local genome structure as a test for orthology (Bezares-Calderon et al., 2010).

8. Conclusions

Novel germ plasm nucleator genes such as *osk* display rapid evolutionary rates (Ahuja and Extavour, 2014). Advances in genome sequencing coupled with transcriptomics now facilitate the discovery of such genes and their homologs from distantly related species, thus allowing a fundamental understanding into putative instances of neofunctionalization and evolution of novel protein function at the biochemical level. The biophysical properties of nucleator proteins appear to be of more consequence in assembling RNP granules than their primary amino acid sequences (Extavour, 2011; Lehmann, 2016; Lynch et al., 2011). Given that multiple nucleators, unrelated by their sequence and derived from distinct animal lineages, share multiple biophysical properties, it appears likely that these germ granule nucleators evolved independently, and thus acquired these properties convergently. As more data accumulates, we anticipate that evidence for convergent evolution of germ plasm components may be more prevalent than we have previously recognized. This might help explain why proteins as distinct as Maelstrom (a *Drosophila* germ granule component recently identified as a novel guanosine RNase) and the structurally unrelated PGL proteins of nematodes, have predicted similarities in enzymatic functions (Aoki et al., 2016; Matsumoto et al., 2015).

With an increased understanding of their biochemistry, will come an insight into the macro-level stability and the micro-level dynamics so beautifully embodied by germ granules (Sheth et al., 2010; Snee and Macdonald, 2004). The intriguing possibility that prion-like templating proteins give germ granules not only their unique structural characteristics but also the ability to propogate from germ cell to germ cell, and from generation to generation in animals with germ plasm, has been previously highlighted (Boke et al., 2016; Voronina et al., 2011). If these PLDs are indeed important, then the differences separating the behavior of a germ granule nucleator's PLD from the documented promiscuous behaviors of protein PLDs in disease causing amyloids, remain to be determined. In addition, the role of nucleating factors in regulating the behaviors of organelles such as mitochondria, which often accompany germ granules, also needs further assessment (Huang et al., 2011; Watanabe et al., 2011).

Of late, the uncovering of several similarities in RNA processing and key components between germ granules and P-bodies or stress granules of somatic cells (Strome and Lehmann, 2007; Voronina et al., 2011) points towards a primary role of all of these granules in the post-transcriptional regulation of RNA (Seydoux and Braun, 2006; Strome and Lehmann, 2007). Whether germ granules represent specialized P-bodies or P-body/stress granule hybrids, however, remains to be ascertained (Voronina et al., 2011). Taken together, these findings emphasize that we have much to learn about the fascinating roles germ granules play within a cell.

Acknowledgements

We thank John R. Srouji, Tarun Kumar and Sam Church for discussions, and members of the Extavour lab for helpful comments on the manuscript. This work was supported by Harvard University.

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