Post-transcriptional gene regulation instructs germline stem cell to oocyte transition during *Drosophila* oogenesis

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**Abstract**

During oogenesis, several developmental processes must be traversed to ensure effective completion of gametogenesis including, stem cell maintenance and asymmetric division, differentiation, mitosis and meiosis, and production of maternally contributed mRNAs, making the germ line a salient model for understanding how cell fate transitions are mediated. Due to silencing of the genome during meiotic divisions, there is little instructive transcription, barring a few examples, to mediate these critical transitions. In *Drosophila*, several layers of post-transcriptional regulation ensure that the mRNAs required for these processes are expressed in a timely manner and as needed during germline differentiation. These layers of regulation include alternative splicing, RNA modification, ribosome production, and translational repression. Many of the molecules and pathways involved in these regulatory activities are conserved from *Drosophila* to humans making the *Drosophila* germline an elegant model for studying the role of post-transcriptional regulation during stem cell differentiation and meiosis.

**Key words**

Splicing, Translation Control, RNA Modifications, Ribosome Biogenesis, Oogenesis, Drosophila, Germline Stem Cell, RNA regulation, Germline Differentiation, Gametogenesis, RNA Binding Proteins

1. **Introduction**

Gametogenesis gives rise to eggs or sperm in all sexually-reproducing organisms [@Cinalli2008d; @Ellis1994d; @Lesch2012b; @Seydoux2006]. Thus, understanding how gametogenesis is regulated is critical to comprehending this essential phenomenon that dictates fertility. Post-fertilization, the zygote gives rise to an entire organism, thus understanding how gametogenesis is regulated also has implications for the field of regeneration [@Lasko2012a; @Lee2014c; @Magnusdottir2014d; @Soldner2018d; @Tadros2009c; @Theunissen2017b]. *Drosophila melanogaster* has been one of the central organisms used to study heritability and gametogenesis for nearly a century due to its rapid generation time and genetic tractability [@Mattox1990; @Spradling1981b; @Spradling1993b; @Spradling2011f; @Spradling1997e; @Xie2007a]. These traits have facilitated the establishment of an extensive collection of informative and useful mutant and transgenic flies [@Hales2015a]. In addition, many of the gametogenic regulatory factors described in the *Drosophila* germ line are conserved to mammals and also play critical roles in other tissues, such as neurons [@Goldstrohm2018c; @Lin1997b; @Reichardt2018d; @Vessey2010b; @Zamore1999b; @Zhang2015c]. While both male and female *Drosophila* undergo meiosis to give rise to gametes, here we focus on the female germline as regulation of gametogenesis in males has been reviewed elsewhere [@Barreau2008d; @Fuller1998c; @Spradling2011f; @Yamashita2005d; @Zhao2002d].

The spatiotemporal stages of *Drosophila* oogenesis are discrete and can be easily identified by their morphology and molecular markers [@Gaspar2017b; @Jia2016b; @Spradling2011f]. At the anterior end of the ovary, germline stem cells (GSCs) reside in a structure known as the germarium and initiate differentiation to give rise to gametes [@Kai2005; @Twombly1996d; @Xie2000; @Xie1998d; @Xie2007a]. GSCs are maintained by signaling from the surrounding somatic niche. GSCs undergo asymmetric mitotic division, producing a stem cell daughter, or cystoblast (CB) which will begin the process of differentiation by expressing the essential differentiation factor *bag of marbles* (*bam*) [@Chen2003q; @McKearin1995b]. The differentiating CB then undergoes four incomplete mitotic divisions, giving rise to an interconnected 16-cell cyst [@McKearin1995b; @McKearin1990e]. In this cyst, one cell is designated to become the oocyte and the other 15 cells take on the role of nurse cells, which generate proteins and mRNAs that are provided to the developing oocyte [@Navarro2004b; @Spradling1997e]. The specified oocyte and its associated nurse cells are then encapsulated by somatic cells to form an egg chamber that buds off from the germarium (Figure 1B) [@Gilboa2004a; @Margolis1995a]. The nurse cells will enter into a unique state in which they undergo a modified version of the cell cycle without undergoing mitosis, creating polyploid nuclei capable of fulfilling the high transcriptional demand required to transcribe all of the mRNAs necessary for the egg [@Lilly2005d; @Royzman1998]. As this process ensues, the egg chambers and oocyte increase in size as the supply of mRNAs and proteins is created and deposited into the mature egg (Figure 1A) [@Lasko2012a; @Richter2011j].

Oocyte development entails multiple processes that ensure effective completion of gametogenesis and fertility. Among these are stem cell maintenance and asymmetric division, differentiation, mitosis and meiosis, and production of the maternal mRNA contribution, thus the germ line is a salient model for understanding how cells navigate fate transitions [@Chen2003q; @Fu2015h; @Harris2011i; @Lasko2012a]. During oogenesis, there is little instructive transcription, barring a few examples, to mediate these critical transitions [@Cinalli2008d; @Rangan2008]. Instead, the germline relies highly on post-transcriptional regulatory mechanisms to coordinate gametogenesis [@Slaidina2014h]. These include: alternative splicing, RNA modifications to modulate splicing, protein-RNA interactions, small RNA biology, and organization of the translation machinery to control the output of gene expression to mediate cell fate transitions. Here we focus on post-transcriptional processing of germline mRNAs and translational regulation both of which are required for successful oogenesis.

1. **Alternative splicing ensures accurate production of critical germline mRNAs to regulate sex determination and differentiation**

Splicing decisions are crucial during the generation of mature mRNAs post-transcriptionally and significantly contribute to germline development. Splicing is mediated by a large ribonucleoprotein catalytic complex called the spliceosome, the core of which is made up of five small nuclear RNAs (snRNA), U1, U2, U4, U5 and U6, that work with spliceosomal proteins to form a small nuclear ribonucleoprotein complex (snRNP) [@Madhani1990; @Wahl2009; @Will2001; @Will2011d]. This complex removes introns from newly synthesized pre-mRNAs and links exonic sequences together [@Wahl2009]. Initially, U1 snRNP recognizes the donor site, which is located at the 5’ end of the intron, and U2 snRNP binds the branch site located at the 3’ end, leading to structural rearrangements of the complex and its associated substrate pre-mRNA [@Matera2014]. Catalytic actions of pre-mRNA splicing occur in two main steps. Cleavage at the 5’ splice site forms a lariat-like structure such that a 2’-5’ phosphodiester bond is created between the first nucleotide of the donor site and a conserved adenosine residue at the branch site [@Rymond1985]. Next, a second cleavage event occurs at the 3’ splice site and is followed by ligation of flanking exons to complete splicing [@Umen1995; @Wahl2009].

Alternative splicing is a process by which a single locus can give rise to many unique mRNA isoforms and their resulting protein variants [@Black2000]. The selection of the splice sites is exquisitely regulated to determine which exons will be included in the resulting alternatively spliced transcripts [@Wang2015a]. Alternative splicing is highly regulated and is critical to germline development [@Hager1997; @Kalsotra2011]. There are a myriad of RNA targets that must be differentially spliced, and a complex web of interacting proteins orchestrate production of their splice variants [@Lee2015]. One of the first described instances of alternative splicing in *Drosophila* females is the splicing of the sex determination gene *sex-lethal* (*sxl*) [@Bell1988]. *sxl* is alternatively spliced to generate isoforms that control sex determination in somatic tissues [@Chang2011]. In females, an autoregulatory loop forms between Sxl protein, U2AF splicing factor and U1 snRNP [@Nagengast2003]. In *Drosophila*, the protein component of the U1 and U2 snRNPs are encoded by a gene called *sans fille* (*snf*) [@Cline1999]. Loss of *snf* results in a sterility phenotype in females that specifically affects germline *sxl* splicing and leads to a tumor comprised of undifferentiated cells [@Johnson2010; @Nagengast2003]. When correctly spliced, the resulting Sxl protein recognizes its own pre-mRNAs by binding both upstream and downstream of Exon 3 [@Penalva2003]. In addition, Sxl protein interacts with the U2AF and U1 snRNP to block the recognition of splice sites at Exon 3 [@Nagengast2003]. As a result, exon 3 is spliced out of the pre-mRNA in the final transcript that is capable of being translated into a fully functional protein [@Penalva2003]. In contrast, males include exon three in the final *sxl* transcript. Exon 3 contains a premature stop codon within the *sxl* transcript that results in a truncated protein that lacks the activity of the female-specific variant [@Inoue1990]. Thus, *sxl* is differentially expressed in the male and the female gonad due to alternative splicing events.

In addition to control of *sxl* via alternative splicing, *sxl* expression is controlled at the level of transcription by several transcription factors, such as Ovo [@Salles2002]. Ovo is a zinc finger DNA binding protein that is required in the germline for proper gametogenesis[@Andrews2000a]. *ovo* is also alternatively spliced and each of its isoforms have different implications for *sxl* expression. Ovo-A and Ovo-B where the first splice variants of *ovo* shown to be expressed in the female germline during oogenesis [@Salles2002]. In addition to differences due to alternative exon usage, Ovo-A, unlike Ovo-B, contains a 381 amino acid N-terminal extension which arises due to alternative transcription start sites [@Andrews2000a]. Use of these promoters generates distinct Ova isoforms with unique temporal requirements during oocyte development; Ovo-B was found to be necessary and sufficient during early oogenesis and Ovo-A is critical in the later stages of egg development for a fully functional egg. The *ovo-B* gene has two characterized isoforms, Ovo+2B and Ovo-2B, which were discovered through a transposon insertion that disrupts exon splicing of *ovo-B*. This transposition event prevents inclusion of the exon 2b extension, producing a nonfunctional protein that accumulates during oogenesis. In the absence of retrotransposon insertion, the 178-amino acid extension encoded by exon 2b is included forming a fully functional Ovo protein, known as Ovo-+2B [@Salles2002]. Interestingly, Ovo-B promotes transcription of *ovarian tumor* (*otu*), which enhances *sxl* expression (Figure 2) [@Lu2001]. The mechanism by which Otu regulates sxl expression is unknown but various mutations in *otu* lead to a myriad of phenotypes such as loss of germ cell proliferation, and inability to complete the differentiation process. The *otu* gene produces two cytoplasmic protein isoforms, a 104-kDA isoform (Otu-104) and a 98-kDA isoform (Otu-98) [@Tirronen1995]. Strikingly, only Otu-104 is capable of rescuing all the *otu* mutant phenotypes, indicating its requirement during oogenesis, while Otu-98 is dispensable during this process [@Tirronen1995]. Despite the lack of insight into how the *otu* splice forms regulate GSC development, its alternative splicing is critical for oogenesis [@Sass1995]. Thus, a cascade of alternative splicing events regulate production of Sxl in the female germline to promote oogenesis (Figure 2).

Sxl expression in the female gonad regulates both sex determination as well as differentiation [@Chau2012]. One critical task of Sxl is to represses Tudor domain containing protein 5-like (*tdrd5l*) [@Primus2019]. Tdrd5l is present in the cytoplasm of the male germline, localizing to granules associated with RNA regulation, to promote male identity and differentiation. Sxl expression the female gonad represses translation of Tdrd5l to promote female identity [@Primus2019]. In addition, female Sxl has been found to regulate transcription of *PHD finger protein 7* (*phf7*), a key regulator of male identity [@Yang2012]. Sxl was found to recruit SETDB1, a chromatin writer, to deposit trimethylated H3K9 (H3K9me3) repressing transcription of *phf7* [@Smolko2018]. Thus, alternative splicing of *sxl* results in different sexes helps promote proper sex determination in the germline (Figure 2). Sxl also fulfills additional functions outside of sex determination. Sxl is required in the female germline for germline stem cell GSC differentiation. Loss of Sxl protein causes an accumulation of single cells and two cell cysts [@Chau2009]. It is thought that Sxl binds *nanos* (*nos*) mRNA, an RNA binding protein that is necessary for GSC self-renewal, using a canonical Sxl binding sequence in the 3’ UTR [@Chau2012]. Loss of Sxl leads to an accumulation of excess of Nanos protein, which is thought to limit? GSC differentiation [@Boerner2016; @Chau2012; @Li2013h]. While regulation by Sxl is beginning to be deciphered, several aspects remain to be discovered. For example, Sxl, a splicing factor, is predominantly cytoplasmic in undifferentiated cells but becomes nuclear as differentiation proceeds [@Chau2009], yet, how it works as translational regulator while in the cytoplasm and how it is transported to the nucleus to function as splicing factor during differentiation are not known.

Polypyrimidine tract binding proteins (PTBs) promote splicing by binding polypyrimidine tracts that are ~10nt long and bring splice sites together by means of protein dimerization to promote alternative splicing [@Polydorides2000; @Romanelli2013]. A PTB, *half pint* (*hfp*),a homolog of human PUF60, is important for oogenesis [@Maniatis2002]. Loss of *hfp* results in missplicing of the *otu* transcripts described above [@VanBuskirk2002]. In addition, *hfp* also regulates alternative splicing of *eukaryotic initiation factor 4E* (*eIF4E*) during development through 3’ splice site selection [@Reyes2008]. Hfp is required to increase the relative abundance of the longer *eIF4E* transcript [@VanBuskirk2002]. Lastly, *hfp* also regulates splicing of *gurken*, a critical regulator of dorsal-ventral patterning [@Kalifa2009]. Thus, sex determination, differentiation and production of the determinants of embryonic patterning for the next generation are all regulated by mechanisms involving alternative splicing in the female germline.

1. **RNA modifications direct splicing of sex determinants and translation of differentiation promoting genes in the germline**

Post transcriptional RNA modifications are abundant and conserved in all branches of life [@Yi2011]. There have been over 100 described RNA modifications that can alter stability, function and splicing of RNAs [@Licht2016; @Roundtree2017]. A well-known example of an mRNA modification is the 5’ methylguanosine cap that is added to all mRNAs to promote their stability and aid in translation initiation [@Mitchell2010; @Mukherjee2012]. A variety of RNA modifications have been linked to developmental transitions, such as those affecting GSC fate [@Batista2014; @Roundtree2017]. Specifically during oogenesis, N6A-methyladenosine (m6A) has been shown to be important for differentiation of germline stem cell daughter cells in females by ensuring proper female-specific splicing of *sxl* [@Haussmann2016]. Additionally, the H/ACA box complex, an RNP complex responsible for depositing pseudouridine on rRNA, has been suggested to be regulated by Sxl during the germline stem cell to daughter cell transition and is required for proper cyst differentiation [@Kiss2010; @Morita2018].

m6A is prevalent on mRNA and is mediated by a methyltransferase complex that deposits a methyl-group at the sixth nitrogen on adenosine [@Yang2018]. In *Drosophila,* m6A is placed by a m6A writer complex consisting of Xio, Virilizer (Vir), Spenito (Nito), female lethal d (fl(2)d), Methyltransferase like 3 (Mettl3) and Methyltransferase like 14 (Mettl14) [@Yan2015]. Some described roles of m6A involve modulating RNA-structure, facilitating mRNA degradation, promoting translation initiation and mediating alternative splicing [@Roundtree2017]. Interestingly, the m6A writer complex has been linked to *sxl* splicing during *Drosophila* oogenesis [@Kan2017]. miCLIP data revealed that m6A must be placed at intergenic regions of the *sxl* mRNA in order to produce the female-specific isoform [@Kan2017]. Accordingly. loss of m6A complex members such as *spenito* result in expression of the male specific isoform of *sxl*, and tumors of undifferentiated cells, similar to loss of *sxl* [@Mattox1990]; [@Kan2017]. This suggests that m6A enables proper splicing of female-specific *sxl*, which allows for proper differentiation of germline stem cells into cystoblast daughter cells (Figure 2).

Pseudouridine is one of the most abundant RNA modifications [@Zhao2015]. Although most commonly found on tRNAs, pseudouridine is also found on mRNAs as well as rRNA [@Penzo2018]. Unlike the canonical nucleoside uridine which is attached to the sugar via a nitrogen-carbon bond, pseudouridine is a uridine isomer attached through a carbon-carbon bond [@Cohn1960]. Pseudouridine can be placed by two different classes of enzymes; either by a sequence specific pseudouridine synthase or a small RNA guided complex called the box H/ACA ribonucleoprotein [@DeZoysa2017]. Depletion of the H/ACA box complex member Nucleolar Protein Family A Member 2 (NHP2) in the germline leads to an accumulation of 4- and 8- cell cysts that do not transition to the 16-cell cyst stage [@Morita2018]. Interestingly, the accumulation of single cells due to loss of *sxl* is partially rescued by loss of *NHP2* indicating that this *sxl* phenotype is due to excess NHP2[@Morita2018]. Consistent with this notion, Sxl interacts with *nhp2* mRNA suggesting that Sxl may impose a regulatory function, in this case likely repression of *nhp2* to allow initiation of the differentiation program (Figure 2) [@Morita2018]. Thus, although it is clear that RNA modifications help to ensure proper splicing of sex determination factors, but the pathway, mechanism, and direct targets remain unresolved.

1. **Production of ribosomes is finely tuned to facilitate differentiation**

While splicing mediates proper mRNA production, access of the mature mRNAs to ribosomes controls their translation. Once mRNAs are gated for translation, proper ribosome levels control protein production. The levels of ribosomes during early oogenesis are strictly regulated and shockingly dynamic. Ribosome biogenesis is the process of transcribing and processing the ribosomal RNA (rRNA) components, as well as transcribing and translating the protein constituents of the ribosome [@Granneman2004a; @Nazar2004a; @Teng2013; @Yelick2015a]. This process is exquisitely regulated as ribosome biogenesis is one of the most energy intensive tasks of maintaining cell homeostasis and is even more crucial in proliferative cells [@Phipps2011a]. In addition to the high energy requirement of ribosome biogenesis, all of the components of the ribosome must be coordinated in their production. The process of ribosome biogenesis involves a series of coordinated steps of processing and assembly that involve dozens of non-coding RNAs and proteins and the molecular details of this process have been thoroughly covered in detail in several recent reviews [@Granneman2004a; @Yelick2015a; @You2015]. Briefly, ribosomal DNA (rDNA) is present in multicopy stretches within the genome; these areas of DNA are localized to a subnuclear organelle called the nucleolus [@Karpen1988a; @Ritossa1965a; @Schwarzacher1993]. rDNA is transcribed into rRNA in the nucleolus and processing steps begin cotranscriptionally [@Kos2010] to remove internal and external spacers found in immature rRNA [@Granneman2011; @Granneman2004a; @Schafer2003a; @Tafforeau2013a]. As these processing steps occur, the rRNA is covalently modified and ribosomal proteins begin to interact with the partially processed rRNA [@Agalarov2000; @Deshmukh1993a; @Gumienny2017c; @Jady2001c; @Kiss2004c]. When the rRNA is mostly mature it is exported from the nucleus to the cytoplasm where the small and large subunits of the ribosome fully mature and assemble [@Lo2010a; @Schafer2003a; @Sloan2017e; @Tschochner2003a; @Zemp2007]. Errors at any of these steps can result in ribosome biogenesis defects which in humans result in disease states known as ribosomopathies [@Armistead2014a; @Barlow2010a; @Brooks2014b; @Higa-Nakamine2012o; @Mills2017c; @Sloan2017e].

Curiously, despite the presence of ribosomes across cell types and sharing similar molecular origins, ribosomopathies manifest as tissue specific defects rather than pleiotropic phenotypes [@Brooks2014b; @Higa-Nakamine2012o; @Mills2017c; @Pereboom2011a; @Yelick2015a]. The reasons behind the unique, tissue-specific manifestations are still being investigated but in several cases it seems that stem cells may be particularly sensitive to perturbations in ribosome biogenesis [@Brooks2014b; @Morgado-Palacin2012a; @Pereboom2011a; @WatanabeSusaki2014a]. Indeed, a growing body of evidence is beginning to suggest that *Drosophila* GSCs not only have a specific requirement for ribosome biogenesis, but also that ribosome biogenesis, as well as global translation, vary greatly over the course of GSC differentiation and are uncoupled during early oogenesis [@Sanchez2016h; @Zhang2014d]. These attributes make *Drosophila* oogenesis an excellent system to address how perturbations of ribosome levels affects stem cell differentiation.

In order to maintain stem cell fate, GSCs asymmetrically partition factors required for ribosome biogenesis by retaining more of this machinery than they pass on to daughter cells [@Fichelson2009a; @Zhang2014d]. In particular, Underdeveloped (Udd), an rRNA transcription factor segregates asymmetrically to the GSC during mitosis and seems to promote a high rate of rRNA synthesis within the GSC [@Zhang2014d]. Furthermore, Wicked (Wcd), a U3 snoRNP complex member required for rRNA maturation, is also asymmetrically partitioned to GSCs and associates with the original spectrosome, an ER rich organelle found in GSCs and CBs [@Spradling1997e], of the dividing GSC. How GSCs carry out this specialized cellular division requires further investigation, however, asymmetric stem cell division is crucial for proper differentiation [@Chen2003q; @Chen2003o; @Lin1997b].Consistent with this loss of *wcd* results in premature differentiation of GSCs [@Fichelson2009a]. Nascent rRNA production, measured by BrUTP incorporation, and presumably ribosomes, are produced at high levels in GSCs but this production drops in CBs and in subsequent stages (Figure 3) [@Zhang2014d]. Additionally, it has been observed that certain ribosome biogenesis components are expressed at high levels specifically in the germline [@Kai2005]. In particular, RNA exonuclease 5 (Rexo5) is an RNA exonuclease that facilitates ribosome biogenesis by trimming snoRNAs as well as rRNAs [@Gerstberger2017]. Depletion of *rexo5* in the germline results in an accumulation of egg chambers that bud off from the germarium, but do not grow in size, and causes defects in GSC proliferation [@Gerstberger2017]. These observations suggest that the machinery for ribosome biogenesis is not only critical for germline development but is also dynamically regulated.

Sanchez et al. demonstrated that the dynamic nature of rRNA transcription during germline development is not simply a consequence of the differentiation process. Instead, lowering ribosome biogenesis is required for timely differentiation, but severe loss of ribosome biogenesis causes formation of stem-cysts, a product of perturbed cytokinesis of GSC daughters [@Mathieu2013d; @Matias2015g; @Sanchez2016h] . Somewhat surprisingly, despite their increased retention of ribosome biogenesis components, GSCs exhibit a lower rate of translation compared to daughter cells and cyst stages (Figure 3). This finding invokes the hypothesis that despite the GSCs elevated capacity for ribosome biogenesis, GSCs do not intrinsically require higher ribosome levels for translation. Instead, the data is suggestive of the possibility that GSCs produce high levels of ribosomes in order to pass them on to and facilitate differentiation of their daughter cells. We thus hypothesize that a ribosome biogenesis checkpoint could couple ribosome production to cell cycle progression to ensure a sufficient ribosome concentration is passed from the GSC to the daughter CB. Conversely, increasing ribosome biogenesis via overexpression of TIF-IA, an RNA Pol I transcription initiation factor that is required for rRNA synthesis [@Grewal2007c], results in a failure of germ cells to differentiate, causing a marked overproliferation of undifferentiated GSC daughters [@Zhang2014d]. This overproliferation may be caused by bypassing or rapid progression through the proposed ribosome biogenesis checkpoint such that the cell cycle is hastened in response to elevated ribosome biogenesis. The overproliferation of undifferentiated germ cells when ribosome levels are elevated is consistent with observations that high ribosome levels lead to rapidly growing cancers [@Belin2009a; @Deisenroth2010e; @Vlachos2010a].

Although reducing ribosome biogenesis tends to result in the formation of a stem-cyst as previously described, some factors that play a role in ribosome biogenesis have a less severe phenotypes. For example, some mutants of the ribosomal protein S2 (rps2) gene have a repeating egg-chamber mid-oogenesis defect, wherein ovarian development halts at stage 5 and successive egg chambers do not grow in size and eventually die, resulting in sterility [@Cramton1994a]. This phenotype may be the consequence of incomplete loss of function as the allele that results in the repeating egg chamber phenotype reduces mRNA expression of *rps2*, incompletely, by 60-70%, while other allelic combinations result in embryonic lethality [@Cramton1994a]. Incomplete loss of function alleles for another ribosomal protein, *ribosomal protein S3*, result in a similar repeating egg chamber phenotype [@Sæbøe-Larssen1998]. These observations suggest that partial loss of ribosome biogenesis during oogenesis may be tolerated during differentiation but results in phenotypes at a later phase of egg production, consistent with the model that high levels of biogenesis in early stages supply the ribosomes for subsequent differentiation and development.

Not only do ribosome levels vary but a class of ribosomal protein paralogs are enriched specifically in early germ cells [@Xue2012]. Several variant ribosomal proteins such as *ribosomal proteins S5b* (*rps5b*), *s10a*, *s19b*, and *l22*-like are enriched in the germline and others are enriched during early oogenesis [@Kai2005]. The role of these ribosomal proteins has not been thoroughly explored, but their presence indicates either a role for specialized ribosomes early during germline development or as a way to further increase the availability of ribosomal proteins to facilitate the high level of ribosome production in GSCs. One of these ribosomal protein paralogs, RpS5b, has recently been characterized [@Kong2019]. *rps5b* is most highly expressed in ovaries in contrast to its paralog, *ribosomal protein S5a* (*rps5a*), which is expressed at high levels ubiquitously [@Kong2019]. Loss of *rps5a* in the germline does not cause a germline phenotype, however, loss of *rps5b* results in a mid-oogenesis defect that is further exacerbated when *rps5a* is depleted in a *rps5b* mutant background [@Kong2019]. This could suggest that RpS5a and RpS5b are functionally similar and that the RpS5b phenotype results from lowering the overall amount of RpS5 available during oogenesis. However, RpS5b was also found to interact preferentially with mRNAs that encode proteins involved in mitochondrial electron transport, in contrast to RpS5a which binds mRNAs from a broad spectrum of gene categories [@Kong2019]. In accordance with the binding data, *rps5b* depleted ovaries expressed lower levels of proteins involved in oxidative phosphorylation and mitochondrial respiration [@Kong2019]. This evidence suggests that the expression of ribosomal protein paralogs may be a part of specialized ribosomes that translate specific groups of mRNAs; however, these ribosomal protein paralogs must be carefully analyzed to determine if they make up bonafide special ribosomes or instead have ribosome independent functions [@Dinman2016a].

What regulates ribosome biogenesis to allow for it to be dynamic during early *Drosophila* germline development? The best understood regulator of ribosome biogenesis is the Target of Rapamycin (TOR) pathway [@Chymkowitch2017a; @Magnuson2012c; @Wei2009a; @Yerlikaya2016a] TOR is a kinase that is part of two distinct subcomplexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2) [@Wullschleger2006b]. These complexes have distinct biological roles. TORC2 has been shown to function as an important regulator of the cytoskeleton [@Wullschleger2006b]. Whereas, TORC1 receives and integrates several different signals including nutritional and growth factors and its activity promotes pro-proliferative activities such as global translation, ribosomal protein translation, and cell cycle progression [@Kim2008b; @Magnuson2012c; @Texada2019]. TORC1 activity also helps to coordinate the transcription and translation of the components required for ribosome biogenesis [@Grewal2007c; @Magnuson2012c; @Martin2006a]. In *Drosophila,* TORC1 activity is high in GSCs through the 4-cell cyst, but TORC1 activity dips in 8 and 16 cell cysts and subsequently increases after the cyst stages [@Wei2018a]. Interestingly, the landscape of TORC1 activity resembles the landscape of ribosome biogenesis, but not global translation (Figure 3) [@Sanchez2016h; @Zhang2014d]. However, loss of TORC1 components does not phenocopy perturbation of ribosome biogenesis [@Sanchez2016h]. This is possibly because TORC1 plays a broader role in early oogenesis given the myriad of regulatory functions TORC1 is known to play in other systems [@Kim2008b; @Li2009n; @Moreno-Torres2015; @Noda2017; @Wei2009a]. A downstream effector of mTORC1, La related protein 1 (Larp1) is known to silence ribosomal protein translation in mammals through binding to terminal oligopyrimidine tracts in the 5’UTR of its targets [@Fonseca2015a; @Hong2017a; @Lahr2017b; [CSL STYLE ERROR: reference with no printed form.]]; however, the same has yet to be demonstrated for the *Drosophila* ortholog, La related protein (Larp). Tantalizingly, Larp is required for male and female fertility in *Drosophila*, but details of Larp’s precise role in the female and oogenesis are lacking [@Blagden2009f; @Ichihara2007a]. In contrast, in males Larp is required for proper spindle pole formation as well as proper cytokinesis [@Blagden2009f]. Given the regulatory role Larp plays in ribosome biogenesis in mammals and the data from *Drosophila* spermatogenesis, Larp could facilitate the dynamic nature of ribosome biogenesis during GSC differentiation and meiosis. However, further study is required to understand the role of Larp during GSC differentiation and oogenesis to determine its function in this context.

The process of differentiation requires major cellular reprogramming. Surprisingly, despite being required for cell viability ribosome biogenesis and global translation are two key programs that are modulated to shape GSC differentiation[@Sanchez2016h; @Zhang2014d]. When ribosome production is improperly modulated during GSC differentiation it results in characteristic phenotypes, accumulation of single cells if biogenesis components are overexpressed and formation of a stem-like cyst if ribosome biogenesis components are knocked down in the germline [@Sanchez2016h; @Zhang2014d]. Additionally, several ribosomal protein variants are highly enriched in ovaries and they may perform special functions, however, these variants are just beginning to be studied. Additionally, based on what we know of the mechanisms and networks that control ribosome biogenesis in *Drosophila* oocytes, the dynamic nature of ribosome biogenesis seems likely to be conserved; however, further investigation is required to determine and compare the basis of ribosome biogenesis control.

1. **Hand off mechanisms facilitated by combinatorial RNA binding proteins dynamically shape the translational landscape during oogenesis**

While some mRNAs are translated post-transcriptionally, other critical mRNAs are translationally regulated. For efficient translation of mRNAs, it is thought that the mRNAs must be circularized - bringing their 5’ cap and 3’ poly A tail in close proximity to each other [@Fukao2009c; @Martineau2008c; @Preiss1998g]. This interaction is mediated by cap binding proteins such as eukaryotic initiation factor 4E (eIF4E) and the poly-A binding protein (PABP)[@Eichhorn2016n; @Kronja2014; @Subtelny2014a; @TarunJr1997l]. A longer poly-A tail and uninhibited access to the 5’ cap for eIF4E is believed to promote efficient translation [@Jalkanen2014h]. A major mode of translational regulation is that RNA binding proteins (RBPs) recognize cognate sequences in the 3’ UTRs of their target mRNAs [@Harvey2018f]. The binding of the RBP prevents circularization of the mRNA and inhibits efficient translation initiation, leading to reduced translation [@Mazumder2001k]. RBP binding to the 3’ UTR can mediate translation inhibition by recruiting cofactors to inhibit circularization [@Szostak2013l].This inhibition of circularization can be achieved by RBP binding to the cap and competing with eIF4E, removal of the cap by the decapping machinery, or recruitment of factors such as the CCR4-Not complex to shorten poly-A tail length [@Rissland2017k]. In some cases, RBPs can both block initiation as well as mediate shortening of the poly-A tail [@Neve2017i].

As mentioned in the germline several developmental processes such as stem cell maintenance, differentiation, mitosis and meiosis are coordinated and successful transition through these diverse programs relies on precise translational control (Figure 4) [@Joshi2010l; @Slaidina2014h]. As factors that interfere with translation such as the decapping machinery and the poly-A tail shortening CCR4-Not complex are expressed continuously during oogenesis, and cannot support dynamic translational control on their own, a dynamic and diverse landscape of translational regulators has evolved to allow for fine-scale temporal control of mRNA translation [@Eichhorn2016n; @Flora2018k]. To add an additional layer of complexity, the expression or abundance of several RBPs that regulate translational control oscillate as oogenesis progresses (Figure 4) [@Flora2018k; @Rangan2009; @Richter2011j]. As the levels of RBPs decrease, their bound mRNA targets are licensed for translation [@Flora2018k; @Lasko2000; @Linder2006]. There are three major themes that work to control mRNA translation: 1. RBPs collaborate in a combinatorial manner to regulate mRNAs, 2. Target mRNAs are handed off from one RBP complex to another as levels oscillate during oogenesis to consistently repress or promote target mRNA translation, and 3. Multiple feedback mechanisms operate to mediate each transition (Figure 4) [@Flora2018k]. The feedback mechanism has been extensively reviewed elsewhere and is not the focus of this chapter [@Flora2018k; @Slaidina2014h]. Here, we outline how RBPs both collaborate as well hand off mRNAs during the transition from GSC to mature oocyte.

GSCs rely on several factors to maintain self-renewal, two of the main factors are Pumilio (Pum) and Nanos (Nos), which work in a combinatorial fashion to repress the translation of differentiation-promoting mRNAs (Figure 4) [@Forbes1998g; @Gilboa2004a; @Joly2013f; @Lin1997b]. Pum, a member of the conserved Pum- and Fem-3-binding factor (PUF) family of proteins, is present at high levels in the undifferentiated germline cells of the ovary, including GSCs, CBs, and early-differentiating cysts [@Forbes1998g; @Kai2005]. Independent of other factors, Pum can directly bind mRNA, but it requires the catalytic activity of other proteins to regulate translation of its targets in the *Drosophila* germline [@Sonoda1999a; @Tadauchi2001a]. Pum is known to have dynamic interactions with two critical regulators, Nos in GSCs, and Brain tumor (Brat) in CBs (Figure 4) [@Arvola2017n; @Goldstrohm2018c; @Harris2011i; @Reichardt2018d; @Sonoda2001d; @Sonoda1999a]. Nos, a well conserved RNA binding protein, has the ability to bind mRNA, albeit at low affinity and requires the presence of Pum to recognize its targets [@Arvola2017n]. Nanos directly interacts with Not1, a member of the CCR4-Not complex, recruiting it to target mRNAs, such as *meiotic P26* (*mei-p26*) and *brat*, to regulate their translation [@Bhandari2014h; @Raisch2016h; @Temme2014j]. While in some systems Pum can directly recruit the CCR4-Not complex, activity of *nos* is required for this interaction in the *Drosophila* germline [@Joly2013f; @Temme2014j]. Upon loss of Pum, Nanos or Twin, GSCs fail to maintain stem cell fate and differentiate into stem cell daughters, resulting in the inability to sustain oogenesis as outlined below.

An example of distinct, stage-specific translational control by Pum/Nos/CCR4-Not complex in the germline is the mechanism by which *polar granule component* (*pgc*), a germline-specific transcriptional repressor, is controlled (Figure 4) [@Flora2018k]. Pgc interacts with the Positive Transcription Elongation Factor (P-TEFb) complex and inhibits the phosphorylation of the Serine-2 residue that is critical for transcriptional elongation, resulting in global transcriptional silencing [@Hanyu-Nakamura2008g]. A single pulse of expression of Pgc protein in the CB allows for epigenetic and transcriptomic reprogramming during differentiation [@Flora2018l]. While *pgc* mRNA is expressed highly and ubiquitously throughout oogenesis, translation of *pgc* mRNA is tightly regulated to mitigate the effects of its potent transcriptional silencing activity. The *pgc* 3’ UTR contains a conserved consensus sequence that is transiently and sequentially bound by multiple distinct, developmentally regulated RBPs [@Flora2018k]. This 3’ UTR sequence is required for post-transcriptional control of *pgc* as Pgc protein expression is restricted to the CB*.* In the GSCs, Pum and Nos bind the *pgc* 3’ UTR and recruit Twin a component of the CCR4-Not complex to deadenylate *pgc* mRNA and inhibit its translation (Figure 4) [@Flora2018k]. In addition to *pgc*, Pum/Nos and Twin also regulate Brain tumor (Brat) [@Joly2013f]. Brat is a TRIM-NHL domain protein expressed in the germline that represses translation by engaging with d4EHP and competing with the cap-binding protein eIF4E to prevent translation initiation (Figure 4) [@Arvola2017n; @Harris2011i; @Sonoda2001d]. While *brat* mRNA is expressed in the GSC, it is specifically repressed by Nos and Pum . In addition to these targets, several differentiation promoting mRNAs such as *meiP26* are also repressed [@Joly2013f]. Thus, in the GSCs, a combination of Pum, Nos and CCR4-Not complex are required for repressing translation of several critical differentiation promoting mRNAs [@Flora2018k; @Lasko2012a; @Lasko2000; @Slaidina2014n].

Subsequent differentiation of the GSC daughters relies on several factors to repress expression of *nos* mRNA [@Lasko2012a; @Lasko2000]. Differentiation is initiated upon Bam expression in the CB, where Bam and its binding partner benign gonial cell neoplasm (Bgcn) act through a sequence in the *nos* 3’ UTR to its inhibit translation (Figure 4) [@Li2009h; @McCarthy2018h]. This repression mechanism includes deadenylation activity by Twin, which works in conjunction with Bam and Bgcn [@Fu2015h]. As Nos protein levels decrease in the CB, *pgc* and *brat* mRNAs are translated [@Flora2018k]. The expressed Brat protein now partners with Pum to repress translation of GSC self-renewal genes (Figure 4) [@Harris2011i]. In addition, expression of Mei-P26 increases initiating interactions with Bam, Bgcn and Sxl. Mei-P26 then promotes translational repression of GSC fate promoting genes such as *nos*, allowing for further differentiation by cooperating with Bam and Bgcn [@Li2013h; @Reichardt2018d]. As the CB differentiates into 2-, 4-, 8- and 16- cell cysts, levels of Nanos protein rebound. However, in spite of the presence of Nos, Pum partners with Brat to suppress *pgc* translation in the 4- to 16-cell cyst stages (Figure 4) [@Flora2018k]. Thus, in CBs, absence of Nos allows for Pum to complex with a different subset of proteins as well as license expression of new translational regulators to promote differentiation.

After cyst differentiation, Pum protein levels decrease and expression of another translational repressor, Bruno (Bru), increases [@Kim-Ha1995i; [CSL STYLE ERROR: reference with no printed form.]; @Schupbach1991f; @Webster1997a]. Downregulation of Pum expression is critical for the transition from GSC to an oocyte [@Carreira-Rosario2016e; @Forbes1998g]. Rbfox1, an RBP whose cytoplasmic isoform regulates the translation of specific mRNAs in the germline is responsible for repressing Pum translation through binding of a consensus sequence in the *pum* 3’ UTR (Figure 4) [@Carreira-Rosario2016e]. Loss of Rbfox1 leads to an expansion of Pum protein expression and a disruption of differentiation [@Carreira-Rosario2016e]. Repression of Pum levels by Rbfox1 allows for Bru expression [@Carreira-Rosario2016e]. Surprisingly, Bru can bind to a sequence in the 3’ UTR that is very similar to Pum binding sequence (Figure 4)[@Reveal2011j]. Bruno blocks translation initiation by interacting with Cup, a conserved eIF4E binding protein [@Kim2015m; @Nakamura2004g]. In fact, Bru binds the same sequence in the *pgc* 3’ UTR as Nos/Pum to prevent *pgc* translation [@Flora2018k]. This mode of translation repression is not restricted to *pgc*, rather a cohort of maternal mRNAs are co-regulated by Pum and Bru representing a hand-off mechanism for repression of maternal mRNAs [@Flora2018k].

1. **Summary**

Decades of work using elegant genetics has revealed several paradigms in which splicing machinery, RNA modifying enzymes, ribosome levels, and translational regulation mediates the transition from GSC to oocyte fate. However, several critical details such as the direct targets and mechanisms still need to be deciphered.  Together the advent of cost-effective sequencing technologies combined with the increasing ability to easily create mutants in previously uncharacterized genes will allow us to further elucidate the regulatory logic (underlying or of) this critical transition.

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**Figure 1.** (A) Schematic of *Drosophila* an ovariole. *Drosophila* females have two ovaries consisting of 16–20 ovarioles, which are assembly lines for producing mature eggs. The germarium, the structure that houses the germline stem cell (GSC), is present at anterior tip of the ovariole. The germline stem cell asymmetrically divides, giving rise to another GSC and a GSC daughter. The daughter cell then will undergo four incomplete rounds of mitosis, giving rise to a 16-cell cyst. Of the 16 cells one will be specified as the egg while the others serve as polyploid nurse cells that support oocyte and egg development. The surrounding somatic cells encapsulate the 16-cell cyst creating egg chambers. As development proceeds, the nurse cells provide mRNAs and proteins allowing the oocyte to grow in size and to eventually become a mature egg. (B) Inset of a germarium showing the developing germline, with the GSC located at the most anterior tip. Upon differentiation, the CB will undergo 4 incomplete mitotic divisions giving rise to a 16-cell cyst. Only one cell of the sixteen cells completes meiosis and is destined to become the oocyte.

**Figure 2.** Schematic of the pathway that promotes alternative splicing of *sxl* to generate the female sex determining variant in the germline. Ovo-B promotes the transcription of *otu,* which enhances splicing of *sxl*. The female-specific splice form of *sxl* is further enhanced by RNA modification by the m6A writer. Formation of the female-specific form generates a functional Sxl protein. Sxl represses Tdrd5l, a protein that promotes male identify. Additionally, Sxl post-transcriptionally represses *nhp2* to promote cyst formation during differentiation.

**Figure 3.** Schematic representing the germarium and plots representing relative changes in global translation rate, rRNA transcription rate, and mTorc1 activity during development at the developmental stages indicated. As germline stem cell differentiation occurs rRNA production decreases, while global translation initially increases as differentiation occurs then falls off post differentiation. A global regulator of both translation and rRNA production, mTorc1 activity decreases during differentiation and increases post differentiation.

**Figure 4.** Schematic of combinatorial and dynamic translation regulation in the *Drosophila*germarium. In the GSCs Nos, Pum and Twin form a complex to inhibit the translation of differentiation mRNAs such as *pgc*, which increases throughout oogenesis. Expression of Bam in the CB initiates differentiation by interacting with its partner Bgcn and Mei-P26 to repress the translation of GSC-expressed mRNAs, specifically *nos*. As Nos protein levels decrease in the CB, Pum is available to partner with Brat to repress the translation of self-renewal genes and *pgc*. In cyst stages, Rbfox1 binds the *pum* 3’ UTR to inhibit its translation. Throughout oogenesis Bru and Cup continuously block translation of *pgc*.