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I want to thank a few people.

Preface

This is an example of a thesis setup to use the reed thesis document class (for LaTeX) and the R bookdown package, in general.

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Abstract

All dynamic biological processes require control over transcription, translation, or post-translational products. My work has focused on characterizing this control using RNA sequencing techniques, primarily on transcription and translation, to better understand how stem cell differentiation occurs. Stem cells are cells with the unique ability to develop into more specialized cell types in a process called differentiation. Some stem cell, including those focused on in my work, also have the ability to "self-renew", a process that allows one stem cell to copy itself giving rise to two stem cells. These processes must be carefully balanced as excess self-renewal will result in cells that do not give rise to differentiated cells necessary for further development or biological function. However, excess differentiation will result in the lack of an available pool of stem cells, preventing future differentiation and development. The decision of a stem cell to either self renew or differentiate is controlled by specific cellular pathways that can act at the level of transcription, translation, or post-translation. To study the regulation of these pathways in-vivo I have used the female *Drosophila* germline as a model system. The female *Drosophila* germline is contained within two pairs of ovaries. Ovaries consist of two main types of tissue, soma and germline. Each ovary is made up of strands called ovarioles. Ovarioles represent an assembly line of successive development. At the anterior tip of each ovariole a structure called a germarium is present. At the anterior of the germarium two to three stem cells are housed in a somatic niche. These germline stem cells (GSCs) can self-renew, or differentiate giving rise to a daughter cell called a cystoblast (CB). The CB turns on a differentiation factor called bag of marbles (bam). This CB then undergoes four incomplete cellular divisions, resulting in interconnected cysts consisting of two, four, eight, and finally sixteen cells. One of these cells is designated as the oocyte while the rest of the cells will become nurse cells. The sixteen cell cyst is then encapsulated by somatic cells, forming egg chambers. Egg chambers successively grow in size in fourteen stages. During this time the nurse cells produce mRNAs and proteins that are transported to the oocyte. The oocyte continues to grow, while the nurse cells eventually die, dumping their contents into the oocyte. Once the oocyte reaches the final, 14th stage it is known as an egg. Each of the steps from GSC to egg require changes in cellular pathways. These changes can occur at the level of transcription, translation, or post-translation. Decades of research has elucidated many of the changes that occur during oogenesis, however, many players in this process still remain mysterious. My work has helped to identify and characterize novel developmental mechanisms that are required for the successive developmental transitions that take place during oogenesis. I have leveraged RNAseq and polysome-seq to probe the global transcriptional and translational landscape over development.

I have also used the power of *Drosophila* genetics in concert with these sequencing techniques to identify and characterize misregulated pathways.

Dedication

You can have a dedication here if you wish.

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

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0.1.1 Contents

0.1.2 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 (Cinalli, Rangan, & Lehmann, 2008; Ellis & Kimble, 1994; Lesch & Page, 2012; Seydoux & Braun, 2006). 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 (Lasko, 2012; Lee & Lee, 2014; Magnúsdóttir & Surani, 2014; Soldner & Jaenisch, 2018; Tadros & Lipshitz, 2009; Theunissen & Jaenisch, 2017). *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 (Mattox, Palmer, & Baker, 1990; Spradling, 1993; Spradling et al., 1997; Spradling & Rubin, 1981; Spradling, Fuller, Braun, & Yoshida, 2011; Xie & Li, 2007). These traits have facilitated the establishment of an extensive collection of informative and useful mutant and transgenic flies (Hales, Korey, Larracuente, & Roberts, 2015). 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 (Goldstrohm, Hall, & McKenney, 2018; Lin & Spradling, 1997; Reichardt et al., 2018; Vessey et al., 2010; Zamore, Bartel, Lehmann, & Williamson, 1999; Zhang & Smith, 2015). 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 (Barreau, Benson, Gudmannsdottir, Newton, & White-Cooper, 2008; Fuller, 1998; Spradling et al., 2011; Yamashita & Fuller, 2005; Zhao & Garbers, 2002).

The spatiotemporal stages of *Drosophila* oogenesis are discrete and can be easily identified by their morphology and molecular markers (Gáspár & Ephrussi, 2017; Jia, Xu, Xie, Mio, & Deng, 2016; Spradling et al., 2011). 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 (Kai, Williams, & Spradling, 2005; Twombly et al., 1996; Xie & Li, 2007; Xie & Spradling, 1998, 2000a). 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*) (D. Chen & McKearin, 2003b; McKearin & Ohlstein, 1995). The differentiating CB then undergoes four incomplete mitotic divisions, giving rise to an interconnected 16-cell cyst (McKearin & Spradling, 1990; McKearin & Ohlstein, 1995). 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 (Navarro, Puthalakath, Adams, Strasser, & Lehmann, 2004; Spradling et al., 1997). 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) (Gilboa & Lehmann, 2004; Margolis & Spradling, 1995). 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 transcrip-

tional demand required to transcribe all of the mRNAs necessary for the egg (Lilly & Duronio, 2005; Royzman & Orr-Weaver, 1998). 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) (Lasko, 2012; Richter & Lasko, 2011).

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 (D. Chen & McKearin, 2003b; Fu et al., 2015; Harris, Pargett, Sutcliffe, Umulis, & Ashe, 2011; Lasko, 2012). During oogenesis, there is little instructive transcription, barring a few examples, to mediate these critical transitions (Cinalli et al., 2008; Rangan, DeGennaro, & Lehmann, 2008). Instead, the germline relies highly on post-transcriptional regulatory mechanisms to coordinate gametogenesis (Slaidina & Lehmann, 2014a). 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.

2. 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) (Madhani, Bordonne, & Guthrie, 1990; Wahl, Will, & Lührmann, 2009; Will & Lührmann, 2001, 2011). This complex removes introns from newly synthesized pre-mRNAs and links exonic sequences together (Wahl et al., 2009). 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 (Matera & Wang, 2014). 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 (Rymond & Rosbash, 1985). Next, a second cleavage event occurs at the 3' splice site and is followed by ligation of flanking exons to complete splicing (Umen & Guthrie, 1995; Wahl et al., 2009).

Alternative splicing is a process by which a single locus can give rise to many unique mRNA isoforms and their resulting protein variants (Black, 2000). The selection of the splice sites is exquisitely regulated to determine which exons will be included in the resulting alternatively spliced transcripts (Wang et al., 2015). Alternative splicing is highly regulated and is critical to germline development (Hager & Cline, 1997; Kalsotra & Cooper, 2011). 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 (Lee & Rio, 2015). One of the first described instances of alternative splicing in *Drosophila* females is the splicing of the sex determination gene *sex-lethal* (*sxl*) (Bell, Maine, Schedl, & Cline, 1988). *sxl* is alternatively spliced to generate isoforms that control sex determination in somatic tissues (Chang, Dunham, Nuzhdin, & Arbeitman, 2011). In females, an autoregulatory loop forms between Sxl protein, U2AF splicing factor and U1 snRNP (Nagengast, Stitzinger, Tseng, Mount, & Salz, 2003). In *Drosophila*, the protein component of the U1 and U2 snRNPs are encoded by a gene called *sans fille* (*snf*) (Cline, Rudner, Barbash, Bell, & Vutien, 1999). 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 (Johnson, Nagengast, & Salz, 2010; Nagengast et al., 2003). When correctly spliced, the resulting Sxl protein recognizes its own pre-mRNAs by binding both upstream and downstream of Exon 3 (Penalva & Sánchez, 2003). In addition, Sxl protein interacts with the U2AF and U1 snRNP to block the recognition of splice sites at Exon 3 (Nagengast et al., 2003). 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 (Penalva & Sánchez, 2003). 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 (Inoue, Hoshijima, Sakamoto, & Shimura, 1990). 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 (Salles, Mével-Ninio, Vincent, & Payre, 2002). Ovo is a zinc finger DNA binding protein that is required in the germline for proper gametogenesis(Andrews et al., 2000). *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 (Salles et al., 2002). 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 (Andrews et al., 2000). 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 (Salles et al., 2002). Interestingly, Ovo-B promotes transcription of *ovarian tumor* (*otu*), which enhances *sxl* expression (Figure 2) (Lu & Oliver, 2001). 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) (Tirronen, Lahti, Heino, & Roos, 1995). 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 (Tirronen et al., 1995). Despite the lack of insight into how the *otu* splice forms regulate GSC development, its alternative splicing is critical for oogenesis (Sass, Comer, & Searles, 1995). 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 (Chau, Kulnane, & Salz, 2012). One critical task of Sxl is to represses Tudor domain containing protein 5-like (*tdrd5l*) (Primus, Pozmanter, Baxter, & Van Doren, 2019). 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 (Primus et al., 2019). In addition, female Sxl has been found to regulate transcription of *PHD finger protein 7 (phf7)*, a key regulator of male identity (Yang, Baxter, & Van Doren, 2012). Sxl was found to recruit SETDB1, a chromatin writer, to deposit trimethylated H3K9 (H3K9me3) repressing transcription of *phf7* (Smolko, Shapiro-Kulnane, & Salz, 2018). 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 (Chau, Kulnane, & Salz, 2009). 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 (Chau et al., 2012). Loss of Sxl leads to an accumulation of excess of Nanos protein, which is thought to limit? GSC differentiation (Boerner & Becker, 2016; Chau et al., 2012; Li et al., 2013). 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 (Chau et al., 2009), 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 (Polydorides, Okano, Yang, Stefani, & Darnell, 2000; Romanelli, Diani, & Lievens, 2013). A PTB, *half pint* (*hfp*), a homolog of human PUF60, is important for oogenesis (Maniatis & Tasic, 2002). Loss of *hfp* results in missplicing of the *otu* transcripts described above (Van Buskirk & Schüpbach, 2002). In addition, *hfp* also regulates alternative splicing of *eukaryotic initiation factor 4E* (*eIF4E*) during development through 3' splice site selection (Reyes & Izquierdo, 2008). Hfp is required to increase the relative abundance of the longer *eIF4E* transcript (Van Buskirk & Schüpbach, 2002). Lastly, *hfp* also regulates splicing of *gurken*, a critical regulator of dorsal-ventral patterning (Kalifa, Armenti, & Gavis, 2009). 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.

3. 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 (Yi & Pan, 2011). There have been over 100 described RNA modifications that can alter stability, function and splicing of RNAs (Licht & Jantsch, 2016; Roundtree, Evans, Pan, & He, 2017). 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 (Mitchell et al., 2010; Mukherjee et al., 2012). A variety of RNA modifications have been linked to developmental transitions, such as those affecting GSC fate (Batista et al., 2014; Roundtree et al., 2017). Specifically during oogenesis, N6A-methyladenosine (m^6A) has been shown to be important for differentiation of germline stem cell daughter cells in females by ensuring proper female-specific splicing of *sxl* (Haussmann et al., 2016). 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 (Kiss, Fayet-Lebaron, & Jády, 2010; Morita, Ota, & Kobayashi, 2018).

m^6A is prevalent on mRNA and is mediated by a methyltransferase complex that deposits a methyl-group at the sixth nitrogen on adenosine (Yang, Hsu, Chen, & Yang, 2018). In *Drosophila,* m^6A is placed by a m^6A writer complex consisting of Xio, Virilizer (Vir), Spenito (Nito), female lethal d (fl(2)d), Methyltransferase like 3 (Mettl3) and Methyltransferase like 14 (Mettl14) (Yan & Perrimon, 2015). Some described roles of m^6A involve modulating RNA-structure, facilitating mRNA degradation, promoting translation initiation and mediating alternative splicing (Roundtree et al., 2017). Interestingly, the m^6A writer complex has been linked to *sxl* splicing during *Drosophila* oogenesis (Kan et al., 2017). miCLIP data revealed that m^6A must be placed at intergenic regions of the *sxl* mRNA in order to produce the female-specific isoform (Kan et al., 2017). Accordingly. loss of m^6A 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* (Mattox et al., 1990); (Kan et al., 2017). This suggests that m^6A 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 (Zhao & He, 2015). Although most commonly found on tRNAs, pseudouridine is also found on mRNAs as well as rRNA (Penzo & Montanaro, 2018). 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 (Cohn, 1960). 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 (De Zoysa & Yu, 2017). 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 (Morita et al., 2018). 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* (Morita et al., 2018). 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) (Morita et al., 2018). 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.

4. 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 (Granneman & Baserga, 2004; Nazar, 2004; Teng, Thomas, & Mercer, 2013; Yelick & Trainor, 2015). 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 (Phipps, Charette, & Baserga, 2011). 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 (Granneman & Baserga, 2004; Yelick & Trainor, 2015; You, Park, & Kim, 2015). 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 (Karpen, Schaefer, & Laird, 1988; Ritossa & Spiegelman, 1965; Schwarzacher & Wachtler, 1993). rDNA is transcribed into rRNA in the nucleolus and processing steps begin cotranscriptionally (Koš & Tollervey, 2010) to remove internal and external spacers found in immature rRNA (Granneman & Baserga, 2004; Granneman, Petfalski, Tollervey, & Hurt, 2011; Schäfer, Strauß, Petfalski, Tollervey, & Hurt, 2003; Tafforeau et al., 2013). As these processing steps occur, the rRNA is covalently modified and ribosomal proteins begin to interact with the partially processed rRNA (Agalarov, Sridhar, Funke, Stout, & Williamson, 2000; Deshmukh, Tsay, Paulovich, & Woolford, 1993; Gumienny et al., 2017; Jády & Kiss, 2001; Kiss, Jady, Bertrand, & Kiss, 2004). 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 (Lo et al., 2010; Schäfer et al., 2003; Sloan et al., 2017; Tschochner & Hurt, 2003; Zemp & Kutay, 2007). Errors at any of these steps can result in ribosome biogenesis defects which in humans result in disease states known as ribosomopathies (Armistead & Triggs-Raine, 2014; Barlow et al., 2010; Brooks et al., 2014; Higa-Nakamine et al., 2012; Mills & Green, 2017; Sloan et al.,

2017).

Curiously, despite the presence of ribosomes across cell types and sharing similar molecular origins, ribosomopathies manifest as tissue specific defects rather than pleiotropic phenotypes (Brooks et al., 2014; Higa-Nakamine et al., 2012; Mills & Green, 2017; Pereboom, van Weele, Bondt, & MacInnes, 2011; Yelick & Trainor, 2015). 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 (Brooks et al., 2014; Morgado-Palacin, Llanos, & Serrano, 2012; Pereboom et al., 2011; Watanabe-Susaki et al., 2014). 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 (Sanchez et al., 2016; Zhang, Shalaby, & Buszczak, 2014). 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 (Fichelson et al., 2009; Zhang et al., 2014). 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 (Zhang et al., 2014). 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 (Spradling et al., 1997), 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 (D. Chen & McKearin, 2003a, 2003b; Lin & Spradling, 1997).Consistent with this loss of *wcd* results in premature differentiation of GSCs (Fichelson et al., 2009). 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) (Zhang et al., 2014). Additionally, it has been observed that certain ribosome biogenesis components are expressed at high levels specifically in the germline (Kai et al., 2005). In particular, RNA exonuclease 5 (Rexo5) is an RNA exonuclease that facilitates ribosome biogenesis by trimming snoRNAs as well as rRNAs (Gerstberger et al., 2017). 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 (Gerstberger et al., 2017). 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 (Mathieu et al., 2013; Matias, Mathieu, & Huynh, 2015; Sanchez et al., 2016) . 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 (Grewal, Evans, & Edgar, 2007), results in a failure of germ cells to differentiate, causing a marked overproliferation of undifferentiated GSC daughters (Zhang et al., 2014). 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 (Belin et al., 2009; Deisenroth & Zhang, 2010; Vlachos & Muir, 2010).

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 (Cramton & Laski, 1994). 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 (Cramton & Laski, 1994). Incomplete loss of function alleles for another ribosomal protein, *ribosomal protein S3*, result in a similar repeating egg chamber phenotype [(**???**)¦bÃ¸e-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 (Xue & Barna, 2012). 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 (Kai et al., 2005). 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 (Kong et al., 2019). *rps5b* is most highly expressed in ovaries in contrast to its paralog, *ribosomal protein S5a* (*rps5a*), which is expressed at high levels ubiquitously (Kong et al., 2019). 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 (Kong et al., 2019). 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 (Kong et al., 2019). In accordance with the binding data, *rps5b* depleted ovaries expressed lower levels of proteins involved in oxidative phosphorylation and mitochondrial respiration (Kong et al., 2019). 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 (Dinman, 2016).

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 (Chymkowitch, Aanes, Robertson, Klungland, & Enserink, 2017; Magnuson, Ekim, & Fingar, 2012; Wei & Zheng, 2009; Yerlikaya et al., 2016) TOR is a kinase that is part of two distinct subcomplexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Wullschleger, Loewith, & Hall, 2006). These complexes have distinct biological roles. TORC2 has been shown to function as an important regulator of the cytoskeleton (Wullschleger et al., 2006). 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 (Kim, Goraksha-Hicks, Li, Neufeld, & Guan, 2008; Magnuson et al., 2012; Texada et al., 2019). TORC1 activity also helps to coordinate the transcription and translation of the components required for ribosome biogenesis (Grewal et al., 2007; Magnuson et al., 2012; Martin, Powers, & Hall, 2006). 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 (Wei, Bettedi, Kim, Ting, & Lilly, 2019). Interestingly, the landscape of TORC1 activity resembles the landscape of ribosome biogenesis, but not global translation (Figure 3) (Sanchez et al., 2016; Zhang et al., 2014). However, loss of TORC1 components does not phenocopy perturbation of ribosome biogenesis (Sanchez et al., 2016). 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 (Kim et al., 2008; S. Li et al., 2009; Moreno-Torres, Jaquenoud, & De Virgilio, 2015; Noda, 2017; Wei & Zheng, 2009). 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 (Fonseca et al., 2015; Hong et al., 2017; Lahr et al., 2017; Tcherkezian et al., 2014); 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 (Blagden et al., 2009; Ichihara, Shimizu, Taguchi, Yam-

aguchi, & Inoue, 2007). In contrast, in males Larp is required for proper spindle pole formation as well as proper cytokinesis (Blagden et al., 2009). 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(Sanchez et al., 2016; Zhang et al., 2014). 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 (Sanchez et al., 2016; Zhang et al., 2014). 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.

5. 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 (Fukao et al., 2009; Martineau et al., 2008; Preiss & Hentze, 1998). This interaction is mediated by cap binding proteins such as eukaryotic initiation factor 4E (eIF4E) and the poly-A binding protein (PABP)(Eichhorn et al., 2016; Kronja et al., 2014; Subtelny, Eichhorn, Chen, Sive, & Bartel, 2014; Tarun Jr, Wells, Deardorff, & Sachs, 1997). A longer poly-A tail and uninhibited access to the 5' cap for eIF4E is believed to promote efficient translation (Jalkanen, Coleman, & Wilusz, 2014). A major mode of translational regulation is that RNA binding proteins (RBPs) recognize cognate sequences in the 3' UTRs of their target mRNAs (Harvey et al., 2018). The binding of the RBP prevents circularization of the mRNA and inhibits efficient translation initiation, leading to reduced translation (Mazumder, Seshadri, Imataka, Sonenberg, & Fox, 2001). RBP binding to the 3' UTR can mediate translation inhibition by recruiting cofactors to inhibit circularization (Szostak & Gebauer, 2013).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 (Rissland, 2017). In some cases, RBPs can both block initiation as well as mediate shortening of the poly-A tail (Neve, Patel, Wang, Louey, & Furger, 2017).

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) (Joshi, Riddle, Djabrayan, & Rothman, 2010; Slaidina & Lehmann, 2014a). 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 (Eichhorn et al., 2016; Flora, Wong-Deyrup, et al., 2018). To add an additional layer of complexity, the expression or abundance of several RBPs that regulate translational control oscillate as oogenesis progresses (Figure 4) (Flora, Wong-Deyrup, et al., 2018; Rangan et al., 2009; Richter & Lasko, 2011). As the levels of RBPs decrease, their bound mRNA targets are licensed for translation (Flora, Wong-Deyrup, et al., 2018; Lasko, 2000; Linder & Lasko, 2006). 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) (Flora, Wong-Deyrup, et al., 2018). The feedback mechanism has been extensively reviewed elsewhere and is not the focus of this chapter (Flora, Wong-Deyrup, et al., 2018; Slaidina & Lehmann, 2014a). 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) (Forbes & Lehmann, 1998; Gilboa & Lehmann, 2004; Joly, Chartier, Rojas-Rios, Busseau, & Simonelig, 2013; Lin & Spradling, 1997). 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 (Forbes & Lehmann, 1998; Kai et al., 2005). 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 (Sonoda & Wharton, 1999; Tadauchi, Matsumoto, Herskowitz, & Irie, 2001). Pum is known to have dynamic interactions with two critical regulators, Nos in GSCs, and Brain tumor (Brat) in CBs (Figure 4) (Arvola, Weidmann, Tanaka Hall, & Goldstrohm, 2017; Goldstrohm et al., 2018; Harris et al., 2011; Reichardt et al., 2018; Sonoda & Wharton, 1999, 2001). 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 (Arvola et al., 2017). 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 (Bhandari, Raisch, Weichenrieder, Jonas, & Izaurralde, 2014; Raisch et al., 2016; Temme, Simonelig, & Wahle, 2014). While in some systems Pum can directly recruit the CCR4-Not complex, activity of *nos* is required for this interaction in the *Drosophila* germline (Joly et al., 2013; Temme et al., 2014). 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) (Flora, Wong-Deyrup, et al., 2018). 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-Nakamura, Sonobe-Nojima, Tanigawa, Lasko, & Nakamura, 2008). A single pulse of expression of Pgc protein in the CB allows for epigenetic and transcriptomic reprogramming during differentiation (Flora, Schowalter, et al., 2018). 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 (Flora, Wong-Deyrup, et al., 2018). 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) (Flora, Wong-Deyrup, et al., 2018). In addition to *pgc*, Pum/Nos and Twin also regulate Brain tumor (Brat) (Joly et al., 2013). 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) (Arvola et al., 2017; Harris et al., 2011; Sonoda & Wharton, 2001). 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 (Joly et al., 2013). Thus, in the GSCs, a combination of Pum, Nos and CCR4-Not complex are required for repressing translation of several critical differentiation promoting mRNAs (Flora, Wong-Deyrup, et al., 2018; Lasko, 2000, 2012; Slaidina & Lehmann, 2014a).

Subsequent differentiation of the GSC daughters relies on several factors to repress expression of *nos* mRNA (Lasko, 2000, 2012). 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) (Y. Li et al., 2009; McCarthy, Deiulio, Martin, Upadhyay, & Rangan, 2018). This repression mechanism includes deadenylation activity by Twin, which works in conjunction with Bam and Bgcn (Fu et al., 2015). As Nos protein levels decrease in the CB, *pgc* and *brat* mRNAs are translated (Flora, Wong-Deyrup, et al., 2018). The expressed Brat protein now partners with Pum to repress translation of GSC self-renewal genes (Figure 4) (Harris et al., 2011). 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 (Li et al., 2013; Reichardt et al., 2018). 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) (Flora, Wong-Deyrup, et al., 2018). 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-Ha, Kerr, & Macdonald, 1995; Schupbach & Wieschaus, 1989, 1991; Webster, Liang, Berg, Lasko, & Macdonald, 1997). Downregulation of Pum expression is critical for the transition from GSC to an oocyte (Carreira-Rosario et al., 2016; Forbes & Lehmann, 1998). 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-Rosario et al., 2016). Loss of Rbfox1 leads to an expansion of Pum protein expression and a disruption of differentiation (Carreira-Rosario et al., 2016). Repression of Pum levels by Rbfox1 allows for Bru expression (Carreira-Rosario et al., 2016). Surprisingly, Bru can bind to a sequence in the 3' UTR that is very similar to Pum binding sequence (Figure 4)(Reveal, Garcia, Ellington, & Macdonald, 2011). Bruno blocks translation initiation by interacting with Cup, a conserved eIF4E binding protein (Kim et al., 2015; Nakamura, Sato, & Hanyu-Nakamura, 2004). In fact, Bru binds the same sequence in the *pgc* 3' UTR as Nos/Pum to prevent *pgc* translation (Flora, Wong-Deyrup, et al., 2018). 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 (Flora, Wong-Deyrup, et al., 2018).

6. 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*.

0.2 A translation control module coordinates germline stem cell differentiation with ribosome biogenesis during *Drosophila* oogenesis

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Summary: Ribosomal defects perturb stem cell differentiation, causing diseases called ribosomopathies. How ribosome levels control stem cell differentiation is not fully known. Here, we discovered three RNA helicases are required for ribosome biogenesis and for *Drosophila* oogenesis. Loss of these helicases, which we named Aramis, Athos and Porthos, lead to aberrant stabilization of p53, cell cycle arrest and stalled GSC differentiation. Unexpectedly, Aramis is required for efficient translation of a cohort of mRNAs containing a 5'-Terminal-Oligo-Pyrimidine (TOP)-motif, including mRNAs that encode ribosomal proteins and a conserved p53 inhibitor, Novel Nucleolar protein 1 (Non1). The TOP-motif co-regulates the translation of growth-related mRNAs in mammals. As in mammals, the La-related protein co-regulates the translation of TOP-motif containing RNAs during *Drosophila* oogenesis. Thus, a previously unappreciated TOP-motif in *Drosophila* responds to reduced ribosome biogenesis to co-regulate the translation of ribosomal proteins and a p53 repressor, thus coupling ribosome biogenesis to GSC differentiation.

Introduction

All life depends on the ability of ribosomes to translate mRNAs into proteins. Despite this universal requirement, ribosome biogenesis is not universally equivalent. Stem cells, the unique cell type that underlies the generation and expansion of tissues, in particular have a distinct ribosomal requirement (Gabut, Bourdelais, & Durand, 2020; Sanchez et al., 2016; Woolnough, Atwood, Liu, Zhao, & Giles, 2016; Zahradkal, Larson, & Sells, 1991; Zhang et al., 2014). Ribosome production and levels are dynamically regulated to maintain higher amounts in stem cells (Fichelson et al., 2009; Gabut et al., 2020; Sanchez et al., 2016; Woolnough et al., 2016; Zahradkal et al., 1991; Zhang et al., 2014). For example, ribosome biogenesis components are often differentially expressed, as observed during differentiation of embryonic stem cells, osteoblasts, and myotubes (Gabut et al., 2020; Watanabe-Susaki et al., 2014; Zahradkal et al., 1991). In some cases, such as during *Drosophila* germline stem cell (GSC) division, ribosome biogenesis factors asymmetrically segregate during asymmetric cell division, such that a higher pool of ribosome biogenesis factors is maintained in the stem cell compared to the daughter cell (Blatt et al., 2020; Fichelson et al., 2009; Zhang et al., 2014). Reduction of ribosome levels in stem cells causes differentiation defects. In *Drosophila,* perturbations that reduce ribosome levels in the GSCs result in differentiation defects causing infertility (Sanchez et al., 2016). Similarly, humans with reduced ribosome levels are afflicted with clinically distinct diseases known as ribosomopathies, such as Diamond-Blackfan anemia, that often result from loss of proper differentiation of tissue-specific progenitor cells (Armistead & Triggs-Raine, 2014; Barlow et al., 2010; Brooks et al., 2014; Higa-Nakamine et al., 2012; Lipton, Kudisch, Gross, & Nathan, 1986; Mills & Green, 2017). However, the mechanisms by which ribosome biogenesis is coupled to proper stem cell differentiation remain incompletely understood.

Ribosome production requires the transcription of ribosomal RNAs (rRNAs) and of mRNAs encoding ribosomal proteins (Bousquet-Antonelli, Vanrobays, Gélugne, Caizergues-Ferrer, & Henry, 2000; de la Cruz, Karbstein, & Woolford, 2015; Granne-

man, Bernstein, Bleichert, & Baserga, 2006; Granneman et al., 2011; Tafforeau et al., 2013; Venema, Cile Bousquet-Antonelli, Gelugne, Le Caizergues-Ferrer, & Tollervey, 1997). Several factors, such as helicases and endonucleases, transiently associate with maturing rRNAs to facilitate rRNA processing, modification, and folding (Granneman et al., 2011; Sloan et al., 2017; Tafforeau et al., 2013; Watkins & Bohnsack, 2012). Ribosomal proteins are imported into the nucleus, where they assemble with rRNA to form the small 40S and large 60S ribosome subunits, which are then exported to the cytoplasm (Baxter-Roshek, Petrov, & Dinman, 2007; Decatur & Fournier, 2002; Granneman et al., 2006, 2011; Koš & Tollervey, 2010; Nerurkar et al., 2015; Tafforeau et al., 2013; Zemp & Kutay, 2007). Loss of RNA Polymerase I transcription factors, helicases, exonucleases, large or small subunit ribosomal proteins, or other processing factors all compromise ribosome biogenesis and trigger diverse stem cell-related phenotypes (Brooks et al., 2014; Calo et al., 2018; Mills & Green, 2017; Sanchez et al., 2016; Yelick & Trainor, 2015; Zhang et al., 2014).

Nutrient availability influences the demand for *de novo* protein synthesis and thus ribosome biogenesis (Anthony, Anthony, Kimball, Vary, & Jefferson, 2000; Hong, Mannan, & Inoki, 2012; Mayer & Grummt, 2006; Shu, Swanda, & Qian, 2020). In mammals, nearly all of the mRNAs that encode the ribosomal proteins contain a Terminal Oligo Pyrimidine (TOP) motif within their 5' untranslated region (UTR), which regulates their translation in response to nutrient levels (Fonseca et al., 2015; Hong et al., 2017; Lahr et al., 2017; Tcherkezian et al., 2014). Under growth-limiting conditions, La related protein 1 (Larp1) binds to the TOP sequences and to mRNA caps to inhibit translation of ribosomal proteins (Fonseca et al., 2015; Jia et al., 2021; Lahr et al., 2017; Philippe, Vasseur, Debart, & Thoreen, 2018). When growth conditions are suitable, Larp1 is phosphorylated by the nutrient/redox/energy sensor TOR complex 1 (TORC1), and does not efficiently bind the TOP sequence, thus allowing for translation of ribosomal proteins (Fonseca et al., 2018, 2015; Hong et al., 2017; Jia et al., 2021). In some instances, Larp1 binding can also stabilize TOP-containing mRNAs (Aoki et al., 2013; Berman et al., 2020; Gentilella et al., 2017; Ogami, Oishi, Nogimori, Sakamoto, & Hoshino, 2020), linking mRNA translation with mRNA stability to promote ribosome biogenesis (Aoki et al., 2013; Berman et al., 2020; Fonseca et al., 2018, 2015; Hong et al., 2017; Lahr et al., 2017; Ogami et al., 2020; Philippe et al., 2018). Cellular nutrient levels are known to affect stem cell differentiation and oogenesis in *Drosophila* (Hsu, LaFever, & Drummond-Barbosa, 2008), however whether TOP motifs exist in *Drosophila* to coordinate ribosome protein synthesis is unclear. The *Drosophila* ortholog of Larp1, La related protein (Larp) is required for proper cytokinesis and meiosis in *Drosophila* testis as well as for female fertility, but its targets remain undetermined (Blagden et al., 2009; Ichihara et al., 2007).

Germline depletion of ribosome biogenesis factors manifests as a stereotypical GSC differentiation defect during *Drosophila* oogenesis (Sanchez et al., 2016). Female *Drosophila* maintain 2-3 GSCs in the germarium (**Figure 1A**) (Kai et al., 2005; Twombly et al., 1996; Xie & Li, 2007; Xie & Spradling, 1998, 2000a). Asymmetric cell division of GSCs produces a self-renewing daughter GSC, and a differentiating daughter, called the cystoblast (CB) (D. Chen & McKearin, 2003b; McKearin & Ohlstein, 1995). This asymmetric division is unusual: following mitosis, the abscission of the

GSC and CB is not completed until the following G2 phase (**Figure 1A'**) (De Cuevas & Spradling, 1998; Hsu et al., 2008). The GSC is marked by a round structure called the spectrosome, which elongates and eventually bridges the GSC and CB, similar to the fusomes that connect differentiated cysts (**Figure 1A'**). During abscission the extended spectrosome structure is severed and a round spectrosome is established in the GSC and the CB (De Cuevas & Spradling, 1998; Hsu et al., 2008). Ribosome biogenesis defects result in failed GSC-CB abscission, causing cells to accumulate as interconnected cysts marked by a fusome-like structure called "stem cysts" (**Figure 1A'**) (Mathieu et al., 2013; Sanchez et al., 2016). In contrast with differentiated cysts (McKearin & Spradling, 1990; McKearin & Ohlstein, 1995; Ohlstein & McKearin, 1997), these stem cysts lack expression of the differentiation factor Bag of Marbles (Bam), do not differentiate, and typically die, resulting in sterility (Sanchez et al., 2016). How proper ribosome biogenesis promotes GSC abscission and differentiation is not known.

By characterizing three RNA helicases that promote ribosome biogenesis, we identified a translational control module that is sensitive to proper ribosome biogenesis and coordinates ribosome levels with GSC differentiation. When ribosome biogenesis is optimal, ribosomal proteins and a p53 repressor are both efficiently translated allowing for proper GSC cell cycle progression and its differentiation. However, when ribosome biogenesis is perturbed, we observe diminished translation of both ribosomal proteins and the p53 repressor. As a consequence, p53 is stabilized, cell cycle progression is blocked and GSC differentiation is stalled. Thus, our work reveals an elegant tuning mechanism that links ribosome biogenesis with cell cycle progression checkpoint and thus stem cell differentiation. Given that ribosome biogenesis defects in humans result in ribosomopathies, which often result from stem cell differentiation defects, our data lay the foundation for understanding the etiology of developmental defects that arise due to ribosomopathies.

Results

Three conserved RNA helicases are required in the germline for GSC differentiation

We performed a screen to identify RNA helicases that are required for female fertility in *Drosophila*, and identified three predicted RNA helicases with previously uncharacterized functions, *CG5589*, *CG4901,* and *CG9253* (**Figure 1B-C**) (**Supplemental Table 1**) (Blatt, Wong-Deyrup, et al., 2020). We named these candidate genes *aramis*, *athos*, and *porthos,* respectively, after Alexandre Dumas' three musketeers who fought in service of their queen. To further investigate how these helicases promote fertility, we depleted *aramis*, *athos*, and *porthos* in the germline using the germline-driver *nanos-GAL4* (*nosGAL4*) in combination with RNAi lines. We detected the germline and spectrosomes/fusomes in ovaries by immunostaining for Vasa and 1B1, respectively. In contrast to controls, *aramis*, *athos*, and *porthos* germline RNAi flies lacked spectrosome-containing cells, and instead displayed cells with fusome-like structures proximal to the self-renewal niche **(Figure 1D-H; Figure S1A-A"')**. The cells in this cyst-like structure contained ring canals, a marker of cytoplasmic bridges, suggesting that they are indeed interconnected (**Figure S1B-B"'**) (Zhang et al., 2014). In addition to forming cysts

in an aberrant location, the *aramis*, *athos*, and *porthos* germline RNAi ovaries failed to form egg chambers **(Figure 1D-H)**.

Aberrant cyst formation proximal to the niche could reflect stem cysts with GSCs that divide to give rise to CBs but fail to undergo cytokinesis or differentiated cysts that initiate differentiation but cannot progress further to form egg chambers. To discern between these possibilities, first we detected the expression of a marker of GSCs, phosphorylated Mothers against decapentaplegic (pMad). We observed pMad expression in the cells closest to the niche, but not elsewhere in the germline cysts of *aramis*, *athos*, and *porthos* germline RNAi flies (**Figure S1C-F')** (Kai & Spradling, 2003). Additionally, none of the cells connected to the GSCs in *aramis*, *athos*, and *porthos* germline RNAi flies expressed the differentiation reporter *bamGFP* (**Figure 1D-G")** (McKearin & Ohlstein, 1995). Thus, loss of *aramis*, *athos*, or *porthos* in the germline results in the formation of stem cysts, however with variable severity. This variability could be due to a differential requirement for these genes or different RNAi efficiencies. Overall, we infer that Aramis, Athos, and Porthos are required for proper GSC cytokinesis to produce a stem cell and differentiating daughter.

Athos, Aramis, and Porthos are required for ribosome biogenesis

We found that Aramis, Athos, and Porthos are conserved from yeast to humans (**Figure 1B**). The closest orthologs of Aramis, Athos, and Porthos are Rok1, Dhr2, and Rrp3 in yeast and DExD-Box Helicase 52 (DDX52), DEAH-Box Helicase 33 (DHX33), and DEAD-Box Helicase 47 (DDX47) in humans, respectively (Hu et al., 2011). Both the yeast and human orthologs have been implicated in rRNA biogenesis (Bohnsack, Kos, & Tollervey, 2008; Khoshnevis et al., 2016; Martin et al., 2014; O 'day, Chavanikamannil, & Abelson, 1996; Sekiguchi, Hayano, Yanagida, Takahashi, & Nishimoto, 2006; Tafforeau et al., 2013; Venema et al., 1997; Venema & Tollervey, 1995; Vincent, Charette, & Baserga, 2017; Zhang, Forys, Miceli, Gwinn, & Weber, 2011). In addition, the GSC-cytokinesis defect that we observed in *aramis*, *athos*, and *porthos* RNAi flies is a hallmark of reduced ribosome biogenesis in the germline (Sanchez et al., 2016). Based on these observations, we hypothesized that Aramis, Athos, and Porthos could enhance ribosome biogenesis to promote proper GSC differentiation.

Many factors involved in rRNA biogenesis localize to the nucleolus and interact with rRNA (Arabi et al., 2005; Grandori et al., 2005; Henras et al., 2008; Karpen et al., 1988). To detect the subcellular localization of Aramis and Athos, we used available lines that express Aramis::GFP::FLAG or Athos::GFP::FLAG fusion proteins under endogenous control. For Porthos, we expressed a Porthos::FLAG::HA fusion under the control of UASt promoter in the germline using a previously described approach (DeLuca & Spradling, 2018). We found that in the germline, Aramis, Athos and Porthos colocalized with Fibrillarin, which marks the nucleolus, the site of rRNA synthesis (**Figure 2A-C"'**) (Ochs, Lischwe, Spohn, & Busch, 1985). Aramis was also in the cytoplasm of the germline and somatic cells of the gonad. To determine if Aramis, Athos, and Porthos directly interact with rRNA, we performed immunoprecipitation (IP) followed by RNA-seq. We found that rRNA immunopurified with Aramis, Athos, and Porthos **(Figure 2D-D", Figure S2A-A").** Thus, Aramis, Athos, and Porthos are present in the nucleolus and interact with rRNA, suggesting

that they might regulate rRNA biogenesis.

Nucleolar size, and in particular nucleolar hypotrophy, is associated with reduced ribosome biogenesis and nucleolar stress (Neumüller et al., 2008; Zhang et al., 2011). If Aramis, Athos, and Porthos promote ribosome biogenesis, then their loss would be expected to cause nucleolar stress and a reduction in mature ribosomes. Indeed, immunostaining for Fibrillarin revealed hypotrophy of the nucleolus in *aramis, athos,* and *porthos* germline RNAi flies compared to in control flies, consistent with nucleolar stress (**Figure S2B-C**). Next, we used polysome profile analysis to evaluate the ribosomal subunit ratio and translation status of ribosomes in S2 cells depleted of *aramis*, *athos*, or *porthos* (Boamah, Kotova, Garabedian, Jarnik, & Tulin, 2012; Õunap, Käsper, Kurg, & Kurg, 2013). We found that upon the depletion of all three helicases, the heights of the polysome peaks were reduced (**Figure 2E-E"**). We found that depletion of *aramis* and *porthos* diminished the height of the 40S subunit peak compared to the 60S subunit peak, characteristic of defective 40S ribosomal subunit biogenesis (**Figure 2E, E"**, **Figure S2D)** (Cheng et al., 2019), whereas *athos* depletion diminished the height of the 60S subunit peak compared to the 80S peaks, characteristic of a 60S ribosomal subunit biogenesis defect (**Figure 2E', Figure S2D'**) (Cheng et al., 2019)**.** RNAi-mediated depletion of the orthologs of these helicases in HeLa cells similarly affected the polysome profiles (**Figure 2F'-F", Figure S2E-G**). Taken together our findings indicate that these helicases promote ribosome biogenesis in *Drosophila* and mammalian cells.

Aramis promotes cell cycle progression via p53 repression

Our data so far indicate that Aramis, Athos and Porthos promote ribosome biogenesis, which is known to be required for GSC abscission (Sanchez et al., 2016). Yet the connections between ribosome biogenesis and GSC abscission are poorly understood. To explore the connection, we further examined the *aramis* germline RNAi line, as its defect was highly penetrant but maintained sufficient germline for analysis (**Figure 1E**). First, we compared the mRNA profiles of *aramis* germline RNAi ovaries to *bam* germline RNAi to determine if genes that are known to be involved in GSC abscission have altered expression. We used *bam* depletion as a control because it leads to the accumulation of stem cell daughters (Flora, Schowalter, et al., 2018; Gilboa, Forbes, Tazuke, Fuller, & Lehmann, 2003; McKearin & Ohlstein, 1995; Ohlstein & McKearin, 1997)..

We performed RNA-seq and found that 607 RNAs were downregulated and 673 RNAs were upregulated in *aramis* germline RNAi versus *bam* germline RNAi (cut-offs for differential gene expression were $\log_2$(foldchange) $>|1.5|$, FDR < 0.05) (**Figure S3A, Supplemental Table 2**). Gene Ontology (GO) analysis for biological processes on these genes encoding these differentially expressed mRNAs (Thomas et al., 2003) revealed that the genes that were downregulated upon *aramis* germline depletion were enriched for GO terms related to the cell cycle, whereas the upregulated genes were enriched for GO terms related to stress response (**Figure 3A, Figure S3B**). The downregulated genes included *Cyclin A*, which is required for cell cycle progression, *Cyclin B* (*CycB*) and *aurora B*, which are required for both cell cycle progression and cytokinesis; in contrast the housekeeping gene *Actin 5C* was unaffected (**Figure 3B-C, Figure S3C-C'**) (Mathieu et al., 2013; Matias et al., 2015).

We confirmed that CycB was reduced in the ovaries of *aramis* germline RNAi flies compared to *bam* germline RNAi flies by immunofluorescence (**Figure 3D-F**). These results suggest that *aramis* is required for the proper expression of key regulators of GSC abscission.

CycB is expressed during G2 phase after asymmetric cell division to promote GSC abscission (Flora, Schowalter, et al., 2018; Mathieu et al., 2013). To test if the loss of germline *aramis* leads to GSC abscission defects due to diminished expression of CycB, we attempted to express a functional CycB::GFP fusion protein in the germline under the control of a UAS/GAL4 system (**Figure S3D-D'**) (Mathieu et al., 2013). Unexpectedly, the CycB::GFP fusion protein was not expressed in the *aramis*-depleted germline, unlike the wild type (WT) germline (**Figure S3E-E'**) (Glotzer, Murray, & Kirschner, 1991; Mathieu et al., 2013; Zielke et al., 2014). We considered the possibility that progression into G2 is blocked in the absence of *aramis*, precluding expression of CycB. To monitor the cell cycle, we used theÂ FluorescenceÂ Ubiquitin-basedÂ CellÂ CycleÂ Indicator (FUCCI) system. *Drosophila* FUCCI utilizes a GFP-tagged degron from E2f1 to mark G2, M, and G1 phases and an RFP-tagged degron from CycB to mark S, G2, and M phases (Zielke et al., 2014). We observed cells in different cell cycle stages in both WT and *bam*-depleted germaria, but the *aramis*-depleted germaria did not express GFP nor RFP (**Figure S3F-H"**). Double negative reporter expression is thought to indicate early S phase, when expression of E2f1 is low and CycB is not expressed (Hinnant, Alvarez, & Ables, 2017). The inability to express FPs is not due to a defect in translation as *aramis*-depleted germline can express GFP that is not tagged with the degron (**Figure S3I-I'**). Taken together, we infer that loss of *aramis* blocks cell cycle progression around late G1 phase/early S phase and prevents progression to G2 phase, when GSCs abscise from CBs.

In mammals, cells defective for ribosome biogenesis stabilize p53, which is known to impede the G1 to S transition {Formatting Citation}. Therefore, we hypothesized that the reduced ribosome biogenesis in the *aramis*-depleted germline leads to p53 stabilization in undifferentiated cells, driving cell cycle arrest and GSC abscission defects. To test this hypothesis, we detected p53 and Vasa in the germline by immunostaining. A hybrid dysgenic cross that expresses p53 in undifferentiated cells was utilized as a positive control, and *p53* null flies were used as negative controls (**Figure S3J-K**) (Moon et al., 2018). In WT, we observed p53 expression in the meiotic stages of germline but p53 expression in GSCs and CBs was attenuated as previously reported (**Figure 3G-G"**) (Lu, Chapo, Roig, & Abrams, 2010). However, compared to WT, we observed p53 expression in the stem cysts of the *aramis*-depleted germline (**Figure 3G-I**). Similarly, we observed p53 expression in the stem cysts of *athos-* and *porthos*-depleted germlines **(Figure S3L-M)**, further supporting that reduced ribosome biogenesis stabilizes p53. To determine if p53 stabilization is required for the cell cycle arrest in *aramis*-depleted germline cysts, we depleted *aramis* in the germline of *p53* mutants. We observed a partial but significant alleviation of the cyst phenotype, such that spectrosomes were restored (**Figure 3J-L**). This finding indicates that p53 contributes to cytokinesis failure upon loss of *aramis*, but that additional factors are also involved. Taken together, we find that *aramis*-depleted germ cells display reduced ribosome biogenesis, aberrant expression of p53 protein

and a block in cell cycle progression. Reducing p53 partially alleviates the cell cycle block and GSC cytokinesis defect.

Aramis promotes translation of Non1, a negative regulator of p53, linking ribosome biogenesis to the cell cycle

Although p53 protein levels were elevated upon loss of *aramis* in the germline, *p53* mRNA levels were not significantly altered ($\log_2$ fold change: -0.49; FDR: 0.49). Given that ribosome biogenesis is affected, we considered that translation of p53 or one of its regulators was altered in *aramis*-depleted germlines. To test this hypothesis, we performed polysome-seq of gonads depleted for *aramis* or *bam* in the germline (Flora, Wong-Deyrup, et al., 2018). We plotted the ratios of polysome-associated RNAs to total RNAs (**Figure 4A-A", Supplemental Table 3**) and identified 87 mRNAs with a reduced ratio upon depletion of *aramis*, suggesting that they were translated less efficiently. Loss of *aramis* reduced the levels of these 87 downregulated transcripts in polysomes, without significantly affecting their total mRNA levels (**Figure 4B, Figure S4A-A'**). These 87 transcripts encode proteins mostly associated with translation including Ribosomal proteins (**Figure 4C).** To validate that Aramis regulates translation of these target mRNAs, we utilized a reporter line for the aramis-regulated transcript encoding Ribosomal protein S2 (RpS2) that is expressed in the context of the endogenous promoter and regulatory sequences (Buszczak et al., 2007; Zhang et al., 2014). We observed reduced levels of RpS2::GFP in germlines depleted of *aramis* but not in those depleted of *bam* **(Figure 4D-F)**. To ensure that reduced RpS2::GFP levels did not reflect a global decrease in translation, we visualized nascent translation using O-propargyl-puromycin (OPP). OPP is incorporated into nascent polypeptides and can be detected using Click-chemistry (Sanchez et al., 2016). We observed that global translation in the germlines of ovaries depleted of *aramis* was not reduced compared to *bam* **(Figure 4G-I)**. Thus, loss of *aramis* results in reduced translation of a subset of transcripts.

None of these 87 translational targets have been implicated in directly controlling abscission (Mathieu et al., 2013; Matias et al., 2015). However, we noticed that the mRNA encoding Novel Nucleolar protein 1 (Non1/CG8801) was reduced in polysomes upon loss of *aramis* in the germline (**Figure 4C**). The human ortholog of Non1 is GTP Binding Protein 4 (GTPBP4), and these proteins are known to physically interact with p53 in both *Drosophila* and human cells and have been implicated in repressing p53 (mentioned as CG8801 in Lunardi et al.) (Li et al., 2018; Lunardi et al., 2010). To determine if translation of Non1 is reduced upon depletion of *aramis,* we monitored the abundance of Non1::GFP, a transgene that is under endogenous control (Sarov et al., 2016), and found that Non1::GFP was expressed in the undifferentiated GSCs and CBs (**Figure 5A-A")**. Non1::GFP levels were reduced in the *aramis*-depleted stem cysts compared to the CBs that accumulated upon *bam*-depletion (**Figure 5B-D**), suggesting that Aramis and ribosome biogenesis promote efficient translation of Non1.

During normal oogenesis, p53 is expressed in cyst stages in response to recombination-induced double strand breaks (Lu et al., 2010). We found that Non1 was highly expressed at undifferentiated stages and in two- and four-cell cysts when p53 levels were low, whereas its expression was attenuated at eight- and 16-cell cyst

stages when p53 levels were high (**Figure 5A-A"**, **Figure S5A-B'**). Non1 was highly expressed in egg chambers, which express low levels of p53. To determine if Non1 regulates GSC differentiation and p53, we depleted *Non1* in the germline. We found that germline-depletion of *Non1* results in stem cyst formation and loss of later stages, as well as increased p53 expression, phenocopying germline-depletion of *aramis*, *athos*, and *porthos* (**Figure 5E-F, H, Figure S5C-E**). In addition, we found that loss of *p53* from *Non1*-depleted germaria partially suppressed the phenotype (**Figure 5F-H**). Thus, *Non1* is regulated by *aramis* and is required for p53 suppression, cell cycle progression, and GSC abscission.

To determine if Aramis promotes GSC differentiation via translation of Non1, we restored *Non1* expression in germ cells depleted of *aramis*. Briefly, we cloned *Non1* with heterologous UTR elements under the control of the UAS/GAL4 system (see Methods) (Rørth, 1998). We found that restoring *Non1* expression in the *aramis*-depleted germline significantly attenuated the stem cysts and increased the number of cells with spectrosomes (**Figure 5I-K**). Taken together, we conclude that Non1 can partially suppress the cytokinesis defect caused by germline *aramis* depletion.

Aramis-regulated targets contain a TOP motif in their 5'UTR

We next asked how *aramis* and efficient ribosome biogenesis promote the translation of a subset of mRNAs, including Non1, to regulate GSC differentiation. We hypothesized that the 87 mRNA targets share a property that make them sensitive to rRNA and ribosome levels. To identify shared characteristics, we performed *de novo* motif discovery of target genes compared to non-target genes (Heinz et al., 2010) and identified a polypyrimidine motif in the 5'UTRs of most target genes (UCUUU; E-value: $6.6e^{-094}$). This motif resembles the previously described TOP motif at the 5' end of mammalian transcripts (Philippe et al., 2018; Thoreen et al., 2012). Although the existence of TOP-containing mRNAs in *Drosophila* has been speculated, to our knowledge their presence has not been explicitly demonstrated (Chen & Steensel, 2017; Qin, Ahn, Speed, & Rubin, 2007). This observation motivated us to precisely determine the 5' end of transcripts, so we analyzed previously published cap analysis of gene expression sequencing (CAGE-seq) data that had determined transcription start sites (TSS) in total mRNA from the ovary (**Figure 6A, Figure S6A-A'**) (Boley, Wan, Bickel, & Celniker, 2014; Chen et al., 2014; dos Santos et al., 2015). Of the 87 target genes, 76 had sufficient expression in the CAGE-seq dataset to define their TSS. We performed motif discovery using the CAGE-seq data and found that 72 of 76 Aramis-regulated mRNAs have a polypyrimidine motif that starts within the first 50 nt of their TSS (**Figure 6B-C**). In mammals, it was previously thought that the canonical TOP motif begins with an invariant 'C' (Meyuhas, 2000; Philippe, van den Elzen, Watson, & Thoreen, 2020). However, systematic characterization of the sequence required in order for an mRNA to be regulated as a TOP containing mRNA revealed that TOP mRNAs can start with either a 'C' or a 'U' (Philippe et al., 2020). Thus, mRNAs whose efficient translation is dependent on *aramis* share a terminal polypyrimidine-rich motif in their 5'UTR that resembles a TOP motif.

In vertebrates, most canonical TOP-regulated mRNAs encode ribosomal proteins and translation initiation factors that are coordinately upregulated in response to growth cues mediated by the Target of Rapamycin (TOR) pathway and the TOR

complex 1 (TORC1) (Hornstein, Tang, & Meyuhas, 2001; Iadevaia, Liu, & Proud, 2014; Kim et al., 2008; Meyuhas & Kahan, 2015; Pallares-Cartes, Cakan-Akdogan, & Teleman, 2012) Indeed, 76 of the 87 Aramis targets were ribosomal proteins, and 9 were known or putative translation factors, consistent with TOP-containing RNAs in vertebrates (**Figure 4C, Supplemental Table 4**). To determine if the putative TOP motifs that we identified are sensitive to TORC1 activity, we designed "TOP reporter" constructs. Specifically, the germline-specific *nanos* promoter was employed to drive expression of an mRNA with 1) the 5'UTR of the *aramis* target RpL30, which contains a putative TOP motif, 2) the coding sequence for a GFP-HA fusion protein and 3) a 3'UTR (K10) that is not translationally repressed (Flora, Wong-Deyrup, et al., 2018), referred to as the WT-TOP reporter (**Figure S6B**). As a control, we created a construct in which the polypyrimidine sequence was mutated to a polypurine sequence referred to as the Mut-TOP reporter (**Figure S6B**).

In *Drosophila*, TORC1 activity increases in 8- and 16-cell cysts (Hong et al., 2012; Kim, Jang, Yang, & Chung, 2017). We found that the WT-TOP reporter displayed peak expression in 8 cell cysts, whereas the Mutant-TOP reporter did not **(Figure 6D-E")**, suggesting that the WT-TOP reporter is sensitive to TORC1 activity. Moreover, depletion of *Nitrogen permease regulator-like 3* (*Nprl3*), an inhibitor of mTORC1 (Wei et al., 2014), led to a significant increase in expression of the WT-TOP reporter but not the Mutant-TOP reporter **(Figure S6C-G)**. Additionally, to attenuate mTORC1 activity, we depleted *regulatory-associated protein of mTOR (raptor)*, one of the subunits of the mTORC1 complex (Hong et al., 2012; Loewith & Hall, 2011). Here we found that the WT-TOP reporter had a significant decrease in reporter expression while the Mutant-TOP reporter did not show a decrease in expression **(Figure S6H-L)**. Taken together, our data suggest that Aramis-target transcripts contain TOP motifs that are sensitive to TORC1 activity. However, we note that our TOP reporter did not recapitulate the pattern of Non1::GFP expression, suggesting that Non1 may have additional regulators that modulate its protein levels in the cyst stages.

TOP mRNAs show increased translation in response to TOR signaling, leading to increased ribosome biogenesis (Jefferies et al., 1997; Jia et al., 2021; Powers & Walter, 1999; Thoreen et al., 2012). However, to our knowledge, whether reduced ribosome biogenesis can coordinately diminish the translation of TOP mRNAs to balance and lower ribosome protein production and thus balance the levels of the distinct components needed for full ribosome assembly is not known. To address this question, we crossed the transgenic flies carrying the WT-TOP reporter and Mutant-TOP reporter into *bam* and *aramis* germline RNAi backgrounds. We found that the expression from the WT-TOP reporter was reduced by 2.9-fold in the germline of *aramis* RNAi ovaries compared to *bam* RNAi ovaries **(Figure 6F-G, J)**. In contrast, the Mutant-TOP reporter was only reduced by 1.6-fold in the germline of *aramis* RNAi ovaries compared to *bam* RNAi ovaries **(Figure 6H-J)**. This suggests that the TOP motif-containing mRNAs are sensitive to ribosome biogenesis.

Larp binds TOP sequences in *Drosophila*

Next, we sought to determine how TOP-containing mRNAs are regulated downstream of Aramis. In mammalian cells, Larp1 is a critical negative regulator of TOP-

containing RNAs during nutrient deprivation (Berman et al., 2020; Fonseca et al., 2015; Hong et al., 2017; Philippe et al., 2020; Tcherkezian et al., 2014). Therefore, we hypothesized that *Drosophila* Larp reduces the translation of TOP-containing mRNAs when rRNA biogenesis is reduced upon loss of *aramis*. First, using an available gene-trap line in which endogenous Larp is tagged with GFP and 3xFLAG, we confirmed that Larp was robustly expressed throughout all stages of oogenesis including in GSCs **(Figure S7A-A')**.

Next, we performed electrophoretic mobility shift assays (EMSA) to examine protein-RNA interactions with purified *Drosophila* Larp-DM15, the conserved domain that binds to TOP sequences in vertebrates (Lahr et al., 2017). As probes, we utilized capped 42-nt RNAs corresponding to the 5'UTRs of *RpL30* and *Non1*, including their respective TOP sequences. We observed a gel shift with these RNA oligos in the presence of increasing concentrations of Larp-DM15 **(Figure 7A-A', Figure S7B)**, and this shift was abrogated when the TOP sequences were mutated to purines **(Figure S7C-C').** To determine if Larp interacts with TOP-containing mRNAs *in vivo*, we immunopurified Larp::GFP::3xFLAG from the ovaries of the gene-trap line and performed RNA-seq (**Figure S7D**). We uncovered 156 mRNAs that were bound to Larp, and 84 of these were among the 87 *aramis* translation targets, including *Non1*, *RpL30*, and *RpS2* (**Figure 7B-C, Supplemental Table 5**). Thus, *Drosophila* Larp binds to TOP sequences *in vitro* and TOP-containing mRNAs *in vivo*.

To test our hypothesis that *Drosophila* Larp inhibits the translation of TOP-containing mRNAs upon loss of *aramis*, we immunopurified Larp::GFP::3xFLAG from germline *bam* RNAi ovaries and germline *aramis* RNAi ovaries. Larp protein is not expressed at higher levels in *aramis* RNAi compared to developmental control *bam* RNAi (**Figure S7E-G**). We found that Larp binding to *aramis* target mRNAs *Non1* and *RpL30* was increased in *aramis* RNAi ovaries compared to *bam* RNAi ovaries (**Figure 7D, Figure S7H**). In contrast, a non-target mRNA that does not contain a TOP motif, *alpha-tubulin* mRNA, did not have a significant increase in binding to Larp in *aramis* RNAi ovaries compared to *bam* RNAi ovaries. Overall, these data suggest that reduced rRNA biogenesis upon loss of *aramis* increases Larp binding to the TOP-containing mRNAs *Non1* and *RpL30*.

If loss of *aramis* inhibits the translation of TOP-containing mRNAs due to increased Larp binding, then overexpression of Larp would be expected to phenocopy germline depletion of *aramis*. Unphosphorylated Larp binds to TOP motifs more efficiently, but the precise phosphorylation sites of *Drosophila* Larp, to our knowledge, are currently unknown (Hong et al., 2017). To circumvent this issue, we overexpressed the DM15 domain of Larp which we showed binds the RpL30 and Non1 TOP motifs *in vitro* (**Figure 7A-A'**), and, based on homology to mammalian Larp1, lacks majority of the putative phosphorylation sites (Jia et al., 2021; Lahr et al., 2017; Philippe et al., 2018). We found that overexpression of a Larp-DM15::GFP fusion in the germline resulted in fusome-like structures extending from the niche (**Figure 7E-F').** Additionally, ovaries overexpressing Larp-DM15 had 32-cell egg chambers, which were not observed in control ovaries (**Figure S7I-I'**). The presence of 32-cell egg chambers is emblematic of cytokinesis defects that occur during early oogenesis

(Mathieu et al., 2013; Matias et al., 2015; Sanchez et al., 2016). Our findings indicate that these cells are delayed in cytokinesis and that over expression of Larp partially phenocopies depletion of *aramis*.

Discussion

During *Drosophila* oogenesis, efficient ribosome biogenesis is required in the germline for proper GSC cytokinesis and differentiation. The outstanding questions that needed to be addressed were: 1) Why does disrupted ribosome biogenesis impair GSC abscission and differentiation? and 2) How does the GSC monitor and couple ribosome abundance to differentiation? Our results suggest that germline ribosome biogenesis defect stalls the cell cycle, resulting a loss of differentiation and the formation of stem cysts. We discovered that proper ribosome biogenesis is monitored through a translation control module that allows for co-regulation of ribosomal proteins and a p53 repressor. Loss of *aramis*, *athos* and *porthos* reduces ribosome biogenesis and inhibits translation of a p53 repressor, leading to p53 stabilization, cell cycle arrest and loss of stem cell differentiation (**Figure 7G**).

Aramis, Athos, and Porthos are required for efficient ribosome biogenesis in *Drosophila*

We provide evidence that Aramis, Athos and Porthos play a role in ribosome biogenesis in *Drosophila*, similar to their orthologs in yeast (Bohnsack et al., 2008; Granneman et al., 2006; Khoshnevis et al., 2016; O 'day et al., 1996) and mammals (Sekiguchi et al., 2006; Tafforeau et al., 2013; Zhang et al., 2011). Their role in ribosome biogenesis is likely a direct function of these helicases as they physically interact with precursor rRNA. In yeast, Rok1, the ortholog of Aramis, binds to several sites on pre-rRNA, predominantly in the 18S region (Bohnsack et al., 2008; Khoshnevis et al., 2016; Martin et al., 2014). This is consistent with the small subunit ribosome biogenesis defect we observe upon loss of *aramis* in *Drosophila* (**Figure 2E**). Rrp3, the yeast ortholog of Porthos, promotes proper cleavage of pre-rRNA and is required for proper 18S rRNA production (Granneman et al., 2006; O 'day et al., 1996). DDX47, the mammalian ortholog of Porthos, binds to early rRNA precursors as well as proteins involved in ribosome biogenesis (Sekiguchi et al., 2006). Consistent with these findings, we find that Aramis and Porthos promote 40S ribosome biogenesis. DHX33, the mammalian ortholog of Athos, has been implicated in facilitating rRNA synthesis (Zhang et al., 2011). In contrast, we find that Athos promotes 60S ribosome biogenesis by directly interacting with rRNA. However, we cannot rule out that Athos also affects transcription of rRNA in *Drosophila* as it does in mammals (Zhang et al., 2011). Overall, we find that each mammalian ortholog of Aramis, Athos, and Porthos has consistent ribosome subunit defects, suggesting that the function of these helicases is conserved from flies to mammals. Intriguingly, DDX52 (Aramis) is one of the 15 genes deleted in 17q12 syndrome (Hendrix, Clemens, Canavan, Surti, & Rajkovic, 2012). 17q12 syndrome results in delayed development, intellectual disability, and, more rarely, underdevelopment of organs such as the uterus (Bernardini et al., 2009; Hendrix et al., 2012). Our finding that Aramis disrupts stem cell differentiation could explain some of the poorly understood defects in 17q12 syndrome.

Ribosome biogenesis defects leads to cell cycle defects mediated by p53

Here we report that three RNA helicases, *aramis*, *athos*, and *porthos,* that promote

proper ribosome biogenesis in *Drosophila* are required in the germline for fertility. Loss of *aramis*, *athos*, and *porthos* causes formation of a "stem cyst" and loss of later stage oocytes. Stem cysts are a characteristic manifestation of ribosomal biogenesis deficiency wherein GSCs are unable to complete cytokinesis and fail to express the differentiation factor Bam, which in GSCs is initiated at G2 of the cell cycle (Sanchez et al., 2016; Zhang et al., 2014). Our RNA seq and cell cycle analysis indicates that depletion of *aramis* blocks the cell cycle at G1, and that failure to progress to G2 prevents abscission and expression of Bam. Thus, our results suggest that ribosome biogenesis defects in the germline stall the cell cycle, resulting in formation of stem cysts and sterility.

In most tissues in *Drosophila,* p53 primarily activates apoptosis, however, in the germline p53 is activated during meiosis and does not cause cell death (Fan et al., 2010; Lu et al., 2010). Furthermore, p53 activation in the germline is required for germline repopulation and GSC survival after genetic insult, implicating p53 as a potential cell cycle regulator (Ma et al., 2016; Tasnim & Kelleher, 2018). Our observation that reduction of *p53* partially rescues a stem cyst defect caused by ribosome deficiency due to germline depletion of *aramis* indicates that the G1 block in GSCs is, in part, mediated by p53 activation. Thus, in the *Drosophila* GSCs, p53 blocks the GSC cell cycle and is sensitive to rRNA production. The developmental upregulation of p53 during GSC differentiation concomitant with lower ribosome levels parallels observations in disease states, such as ribosomopathies (Calo et al., 2018; Deisenroth & Zhang, 2010; Pereboom et al., 2011; Yelick & Trainor, 2015).

We find that p53 levels in GSCs are regulated by conserved p53 regulator Non1. In mammalian cells, increased free RpS7 protein due to nucleolar stress binds and sequesters MDM2, a repressor of p53, freeing p53, resulting in G1 cell cycle arrest (Deisenroth & Zhang, 2010; Zhang & Lu, 2009). *Drosophila* have no identified homolog to MDM2. It is not fully known how ribosome levels are monitored in *Drosophila* in the absence of MDM2 and how this contributes to cell cycle progression. In *Drosophila,* Non1 levels are high in the GSCs and p53 is low, and reciprocally Non1 levels are low during meiosis, but p53 is expressed. Our finding that loss of Aramis leads to diminished Non1 and elevated p53, and that either loss of p53 or elevated Non1 suppress differentiation defects caused by loss of Aramis, suggests that, in the female germline, Non1 may fulfill the function of Mdm2 by promoting p53 degradation during *Drosophila* oogenesis. While Non1 has been shown to directly interact with p53, how it regulates p53 levels in both humans and *Drosophila* is not known (Li et al., 2018; Lunardi et al., 2010). Overall, our data place Non1 downstream of ribosome biogenesis and upstream of p53 in controlling cell cycle progression and GSC differentiation. However, our data do not rule out that Non1 may also act upstream of or in parallel to Aramis.

The vertebrate ortholog of Non1, GTPBP4, also controls p53 levels and is upregulated in some cancers (Li et al., 2018; Lunardi et al., 2010; Yu, Jin, Zhang, & Xu, 2016). This suggests that there may be parallel pathways for monitoring ribosome levels via p53 in different tissue types. Unlike *Drosophila* Non1, its ortholog, GTPBP4 has not been identified as a TOP mRNA, so if it similarly acts as a mediator between ribosome biogenesis and the cell cycle it is likely activated in a somewhat

different manner (Philippe et al., 2020). Mammalian Larp1 is required for proper cell cycle progression and cytokinesis (Burrows et al., 2010; Tcherkezian et al., 2014). Excitingly several differentiation and cell cycle regulation genes in mammals are TOP mRNAs regulated by Larp1, including Tumor Protein, Translationally-Controlled 1 (TPT1) and Nucleosome Assembly Protein 1 Like 1 (NAP1L1) (Philippe et al., 2020). TPT1 is a cancer associated factor that has been implicated in activating pluripotency (Burrows et al., 2010; Qiao et al., 2018). Similarly, NAP1L1, a nucleosome assembly protein, is required to maintain proper cell cycle control as loss of NAP1L1 results in cell cycle exit and premature differentiation. Overall, although the specific targets of Larp1 in mammals may differ from those in *Drosophila*, the mechanism by which Larp modulates cell cycle and differentiation may be conserved.

Ribosome biogenesis defects leads to repression of TOP-containing mRNA

TOP-containing mRNAs are known to be coregulated to coordinate ribosome production in response to nutrition or other environmental cues (Kimball, 2002; Meyuhas & Kahan, 2015; Tang et al., 2001). Surprisingly, our observation that loss of *aramis* reduces translation of a cohort of TOP-containing mRNAs, including Non1, suggests that the TOP motif also sensitizes their translation to lowered levels of rRNA. This notion is supported by TOP reporter assays demonstrating that reduced translation upon loss of *aramis* requires the TOP motif. We hypothesize that limiting TOP mRNA translation lowers ribosomal protein production to maintain a balance with reduced rRNA production. This mechanism would prevent the production of excess ribosomal proteins that cannot be integrated into ribosomes and the ensuing harmful aggregates (Tye et al., 2019). Additionally, it would coordinate rRNA production and ribosomal protein translation during normal germline development, where it is known that the level of ribosome biogenesis and of global translation are dynamic (Blatt et al., 2020; Fichelson et al., 2009; Sanchez et al., 2016; Zhang et al., 2014).

Larp transduces growth status to ribosome biogenesis targets

Recent work has shown that the translation and stability of TOP-containing mRNAs are mediated by Larp1 and its phosphorylation (Berman et al., 2020; Hong et al., 2017; Jia et al., 2021). We found that perturbing rRNA production and thus ribosome biogenesis, without directly targeting ribosomal proteins, similarly results in deregulation of TOP mRNAs. Our data show that *Drosophila* Larp binds the *RpL30* and *Non1* 5'UTR in a TOP-dependent manner *in vitro* and to nearly all of the translation targets we identified *in vivo.* Together these data suggest that rRNA production regulates TOP mRNAs via Larp. Furthermore, the cytokinesis defect caused by overexpression of Larp-DM15 in the germline suggests that Larp regulation could maintain the homeostasis of ribosome biogenesis more broadly by balancing the expression of ribosomal protein production with the rate of other aspects of ribosome biogenesis, such as rRNA processing, during development.

Previous studies indicate that unphosphorylated Larp1 binds to and represses its targets more efficiently than phosphorylated Larp1 (Fonseca et al., 2018; Hong et al., 2017; Jia et al., 2021). Thus, although we do not know the identity of the kinase that phosphorylates Larp in *Drosophila*, we hypothesize that Larp is not phosphorylated upon loss of *aramis, athos* and *porthos*, when ribosome biogenesis is perturbed. We

propose that until ribosome biogenesis homeostasis is reached, this kinase will remain inactive, continuously increasing the pool of dephosphorylated Larp. In this scenario, as dephosphorylated Larp accumulates, it begins to bind its targets. Initially, it will bind its highest affinity targets, presumably encoding ribosomal proteins and repress their translation to rebalance ribosomal protein production with rRNA production. Consistent with this model, the TOP motif in *RpL30* is bound by Larp even more tightly with a nearly 9-fold higher affinity compared to the *Non1* TOP site (**Figure S7B**). We propose that such differences in affinity may allow Larp to repress ribosomal protein translation to facilitate cellular homeostasis without immediately causing cell cycle arrest. However, if homeostasis cannot be achieved and sufficient dephosphorylated Larp accumulates, Larp will also bind and repress the translation of lower affinity targets. Repression of Non1 in this manner would result in cell cycle arrest and block differentiation as occurs upon *aramis* depletion.

Ribosome biogenesis in stem cell differentiation and ribosomopathies

Ribosomopathies arise from defects in ribosomal components or ribosome biogenesis and include a number of diseases such as Diamond-Blackfan anemia, Treacher Collins syndrome, Shwachman-Diamond syndrome, and 5q-myelodysplastic syndrome (Armistead & Triggs-Raine, 2014; Draptchinskaia et al., 1999; McGowan et al., 2011; Valdez, Henning, So, Dixon, & Dixon, 2004; Warren, 2018). Despite the ubiquitous requirement for ribosomes and translation, ribosomopathies cause tissue-specific disease (Armistead & Triggs-Raine, 2014). The underlying mechanisms of tissue specificity remain unresolved.

In this study we demonstrate that loss of helicases involved in rRNA processing lead to perturbed ribosome biogenesis and, ultimately, cell cycle arrest. Given that *Drosophila* germ cells undergo an atypical cell cycle program as a normal part of their development it may be that this underlying cellular program in the germline leads to the tissue-specific symptom of aberrant cyst formation (McKearin & Spradling, 1990). This model implies that other tissues would likewise exhibit unique tissue-specific manifestations of ribosomopathies due to their underlying cell state and underscores the need to further explore tissue-specific differentiation programs and development to shed light not only on ribosomopathies but on other tissue-specific diseases associated with ubiquitous processes. Although it is also possible that phenotypic differences arise from a common molecular cause, our data suggests two sources of potential tissue specificity: 1) tissues express different cohort of mRNAs, such as *Non1*, that are sensitive to ribosome levels. For example, we find that in *Drosophila* macrophages, RNAs that regulate the metabolic state of macrophages and influence their migration require increased levels of ribosomes for their translation (Emtenani et al., 2021). 2) p53 activation, as has been previously described, is differentially tolerated in different developing tissues (Bowen & Attardi, 2019; Calo et al., 2018; Jones et al., 2008). Together, both mechanisms could begin to explain the tissue-specific nature of ribosomopathies and their link to differentiation.

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Author Contributions

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Figure Legends

Figure 1: RNA helicases Aramis, Athos and Porthos are required for GSC differentiation. (**A**) Schematic of *Drosophila* germarium. Germline stem cells are attached to the somatic niche (dark red). The stem cells divide and give rise to a stem cell and a cystoblast (CB) that expresses the differentiation factor Bag-of-marbles (Bam). GSCs and CBs are marked by spectrosomes. The CB undergoes four incomplete mitotic divisions giving rise to a 16-cell cyst (blue). Cysts are marked by branched spectrosome structures known as fusomes (red). One cell of the 16-cell cyst is specified as the oocyte. The 16-cell cyst is encapsulated by the surrounding somatic cells giving rise to an egg chamber (green). (**A'**) Ribosome biogenesis promotes GSC cytokinesis and differentiation. Disruption of ribosome biogenesis results in undifferentiated stem cyst accumulation. (**B**) Representation of conserved protein domains for three RNA helicases in *Drosophila* compared to *H. sapiens* and *S. cerevisiae* orthologs. Percentage values represent similarity to *Drosophila* orthologs. (**C**) Egg laying assay after germline RNAi knockdown of *aramis*, *athos* or *porthos* indicating a loss of fertility compared to *nosGAL4*, driver control (n=3 trials). *** = $p < 0.001$, Tukey's post-hoc test after one-way ANOVA, $p < 0.001$. Error bars represent standard error (SE). (**D-G"**) Confocal micrographs of control (**D-D"**) and germline RNAi depletion targeting (**E-E"**) *aramis*, (**F-F"**) *athos* or (**G-G"**) *porthos* stained for 1B1 (red, middle grayscale), Vasa (green), and Bam-GFP (blue, right grayscale). Depletion of these genes results in a characteristic phenotype in which early germ cells are connected marked by a 1B1 positive, fusome-like structure highlighted by a yellow dotted line (**E-G"**) in contrast to the single cells present in controls (white arrow) or differentiating cysts (yellow dashed line) (**D-D"**). Bam expression, if present, is followed by loss of the germline. (**H**) Phenotype quantification of ovaries depleted of *aramis*, *athos* or *porthos* compared to control ovaries (n=50 ovarioles, df=2, *** = $p < 0.001$, Fisher's exact tests with Holm-Bonferroni correction). Scale bars are 15 micron.

Figure 2. Athos, Aramis, and Porthos are required for efficient ribosome biogenesis. (**A-C"**) Confocal images of ovariole immunostained for Fibrillarin (red, right grayscale), Vasa (blue), (**A-A"**) Aramis::GFP, (**B-B"**) Athos::GFP and (**C-C"**) Porthos::HA (green, middle grayscale). (**A"'-C"'**) Fluorescence intensity

plot generated from a box of averaged pixels centered around the punctate of Fibrillarin in the white box. R values denote Spearman correlation coefficients between GFP and Fibrillarin from plot profiles generated using Fiji, taken from the nucleolus denoted by the white box. Aramis, Athos and Porthos are expressed throughout oogenesis and localize to the nucleolus. Aramis also localizes to the cytoplasm and Athos is also present throughout the nucleus (**D-D"**) RNA IP-seq of (**D**) Aramis, (**D'**) Athos, and (**D"**) Porthos aligned to rDNA displayed as genome browser tracks. Bar height represents log scaled rRNA reads mapping to rDNA normalized to input and spike-in. Grey boxes outline rRNA precursors that are significantly enriched in the IP compared to the IgG control (bootstrapped paired t-tests, n=3, * = p-value < 0.05). (**E-E"**) Polysome traces from *Drosophila* S2 cells treated with dsRNA targeting (**E**) *aramis*, (**E'**) *athos*, (**E"**) *porthos* (red line) compared to a mock control (black line). *aramis* and *porthos* are required to maintain a proper 40S/60S ribosomal subunit ratio compared to control and have a smaller 40S/60S ratio. *athos* is required to maintain a proper 40S/60S ribosomal subunit ratio compared to control and has a larger 40S/60S ratio. Additionally, *aramis*, *athos*, and *porthos* are required to maintain polysome levels. (**F-F"**) Polysome preparations from HeLa cells depleted of *DDX52*, *DHX33*, *DDX47*, and control siRNA treated cells. *DDX52*, *DHX33*, and *DDX47* are required to maintain a proper 40S/60S ribosomal subunit ratio. Additionally, all three are required to maintain polysome levels. Scale bar for all images is 15 micron.

Figure 3. Athos, Aramis, and Porthos are required for cell cycle progression during early oogenesis. (**A**) Bar plot representing the most significant Biological Process GO terms of downregulated genes in ovaries depleted of *aramis* compared to *bam* RNAi control (FDR = False Discovery Rate from p-values using a Fisher's exact test). (**B-C**) Genome browser tracks representing the gene locus of (**B**) *Cyclin B* and (**C**) a*urora B* in ovaries depleted of *aramis* compared to the developmental control, *bam* RNAi. Y-axis represents the number of reads mapping to the locus in bases per million (BPM). (**D-E"**) Confocal images of germaria stained for Cyclin B (red, middle grayscale) and Vasa (blue, right grayscale) in (**D-D"'**) *bam* RNAi control ovaries and (**E-E"'**) *aramis* germline RNAi. (**F**) Boxplot of Cyclin B intensity in the germline normalized to Cyclin B intensity in the soma in *bam* RNAi and *aramis* RNAi (n=12-14 germaria per sample, *** = p$<$ 0.001, Welch t-test. (**G-H"**) Confocal images of germaria stained for p53 (red, middle grayscale) and Vasa (blue, right grayscale) in (**G-G"**) *nosGAL4*, driver control ovaries and (**H-H"**) germline depletion of *aramis*. Cells highlighted by a dashed yellow circle represent cell shown in the inset. Driver control *nosGAL4* ovaries exhibit attenuated p53 expression in GSCs and CBs, but higher expression in cyst stages as previously reported, while p53 punctate are visible in the germline of *aramis* RNAi in the undifferentiated cells. (**I**) Box plot of percentage of pixel area exceeding the background threshold for p53 in GSCs and CBs in driver control *nosGAL4* ovaries and the germline of *aramis* RNAi indicates p53 expression is elevated in the germline over the GSCs/CBs of control ovaries. (n=10 germaria per sample, *** = p < 0.001, Welch's t-test. (**J-K"**) Confocal images of germaria stained for 1B1 (red, middle grayscale) and Vasa (blue, right grayscale) in (**J-J"**) germline *aramis* RNAi in a wild type background and (**K-K"**)

germline *aramis* RNAi with a mutant, null, $p53^{5\text{-}A\text{-}14}$ background showing presence of spectrosomes upon loss of p53. (**L**) Quantification of stem cyst phenotypes demonstrates a significant rescue upon of loss of $p53^{5\text{-}A\text{-}14}$ in *aramis* germline depletion compared to the wild type control (n=43-55 germaria per genotype, df=2, Fisher's exact test $p< 0.05$). Scale bar for main images is 15 micron, scale bar for insets is 3.75 micron.

Figure 4. Aramis is required for efficient translation of a subset of mRNAs. (**A-A"**) Biplots of poly(A)+ mRNA Input versus polysome associated mRNA from (**A**) ovaries genetically enriched for GSCs (*UAS-tkv*), (**A'**) Undifferentiated GSC daughter cells (*bam* RNAi) or (**A"**) germline *aramis* RNAi ovaries. (**B**) Boxplot of translation efficiency of target genes in *UAS-tkv*, *bam* RNAi, and *aramis* RNAi samples (ANOVA $p<0.001$, post-hoc Welch's t-test, n=87, *** = $p < 0.001$). (**C**) Summary of downregulated target genes identified from polysome-seq. (**D-E'**) Confocal images of germaria stained for 1B1 (red), RpS2::GFP (green, grayscale), and Vasa (blue) in (**D-D'**) *bam* RNAi control and (**E-E'**) *aramis* RNAi (yellow dashed line marks approximate region of germline used for quantification). (**F**) A.U. quantification of germline RpS2::GFP expression normalized to RpS2::GFP expression in the surrounding soma in undifferentiated daughter cells of *bam* RNAi compared to *aramis* RNAi. RpS2::GFP expression is significantly lower in *aramis* RNAi compared to control (n=14 germaria per sample, Welch's t-test, *** = $p < 0.001$). (**G-H'**) Confocal images of germaria stained for 1B1 (red), OPP (green, grayscale), and Vasa (blue) in (**G-G'**) *bam* RNAi and (**H-H'**) *aramis* RNAi (yellow dashed line marks approximate region of germline used for quantification). (**I**) A.U. quantification of OPP intensity in undifferentiated daughter cells in *bam* RNAi and *aramis* RNAi (n=11-17 germaria per genotype, Welch's t-test, *** = $p < 0.001$). OPP intensity is not downregulated in *aramis* RNAi compared to the control. Scale bar for all images is 15 micron.

Figure 5. Non1 represses p53 expression to allow for differentiation. (**A-A'**) Confocal images of Non1::GFP germaria stained for 1B1 (red), GFP (green, grayscale), and Vasa (blue). (**A"'**) Boxplot of Non1::GFP expression over germline development in GSCs, CBs and Cyst (CC) stages (* = $p < 0.05$, ** = $p < 0.01$, ANOVA with Welch's post-hoc tests). (**B-C'**) Confocal images of (**B-B'**) *bam* RNAi and (**C-C'**) *aramis* RNAi germaria both carrying non1::GFP transgene stained for 1B1 (red), Vasa (blue), and Non1::GFP (green, grayscale). (**D**) Boxplot of Non1::GFP expression in the germline normalized to somatic Non1::GFP expression in *bam* RNAi and *aramis* RNAi (n=24 germaria per genotype, Welch's t-test, *** = $p < 0.001$). Non1 expression is significantly lower in the germline of *aramis* RNAi compared to *bam* RNAi control. (**E-G'**) Confocal images of germaria stained for 1B1 (red), and Vasa (blue) in (**E-E'**) *nosGAL4*, driver control ovaries, (**F-F'**) germline *non1* RNAi, and (**G-G'**) germline *non1* RNAi in a $p53^{5\text{-}A\text{-}1\text{-}4}$ background. Arrow marks the presence of a single cell (**E, G**), yellow dashed line marks cyst emanating from the niche (**F-F'**) or the presence of proper cysts (**E-E'**). (**H**) Quantification of percentage of germaria with no defect (black), presence of single cell (salmon), presence of a cyst emanating from the niche (brown-red), or germline loss (dark red) demonstrates a significant rescue of stem cyst formation upon of loss of *Non1* in $p53^{5\text{-}A\text{-}14}$ compared to the *p53* wild type control (n=35-55 germaria per genotype, df=3, Fisher's exact test

with Holm-Bonferroni correction ** = p< 0.01, *** = p< 0.001). (**I-J'**) Confocal images of germaria stained for 1B1 (red), and Vasa (blue) in (**I-I'**) *aramis* germline RNAi exhibiting stem cyst phenotype (yellow dashed line) and (**J-J**') *aramis* germline RNAi with *non1* overexpression exhibiting single cells (arrow). (**K**) Phenotypic quantification of *aramis* RNAi with *non1* overexpression demonstrates a significant alleviation of the stem cyst phenotype (n=33-57 germaria per genotype, df=2, Fisher's exact test, ** = p< 0.01). Scale bar for all images is 15 micron.

Figure 6. Aramis regulated mRNAs contain a TOP motif. (**A**) Genome browser tract of *RpL30* locus in ovary CAGE-seq data showing the proportion of transcripts that are produced from a given TSS (orange). Predominant TSSs are shown in orange and putative TOP motif indicated with a green box. The bottom blue and red graph represents sequence conservation of the locus across *Diptera*. The dominant TSS initiates with a canonical TOP motif. (**B**) Sequence logo generated from *de novo* motif discovery on the first 200 bases downstream of CAGE derived TSSs of *aramis* translation target genes resembles a canonical TOP motif. (**C**) Histogram representing the location of the first 5-mer polypyrimidine sequence from each CAGE based TSS of *aramis* translation target genes demonstrates that the TOP motifs occur proximal to the TSS (n=76 targets). (**D-E"**) Confocal images and quantifications of *WT-TOP-GFP* (**D-D'**) and *Mut-TOP-GFP* (**E-E'**) reporter expression stained for 1B1 (red), GFP (green), and Vasa (blue). Yellow dotted-line marks increased reporter expression in 8-cell cysts of *WT-TOP-GFP* but not in *Mut-TOP-GFP*. Reporter expression was quantified over germline development for *WT-TOP-GFP* (**D"**) and *Mut-TOP-GFP* reporter expression (**E"**) and normalized to expression in the GSC reveals dynamic expression based on the presence of a TOP motif. (**F-G'**) Confocal images of *WT-TOP-GFP* reporter ovarioles showing 1B1 (red), GFP (green), and Vasa (blue) in *bam* germline depletion as a developmental control (**F-F'**) and *aramis* germline depleted ovaries (**G-G'**). (**H-I'**) Confocal images of *Mut-TOP-GFP* reporter expression showing 1B1 (red), GFP (green), and Vasa (blue) in *bam* RNAi (**H-H'**) and *aramis* germline RNAi. (**I-I'**) Yellow dotted-lines indicates germline. (**J**) A.U. quantification of WT and Mutant TOP reporter expression in undifferentiated daughter cells in *bam* RNAi compared *aramis* RNAi demonstrates that the *WT-TOP-GFP* reporter shows significantly lower expression in *aramis* RNAi than the *Mut-TOP-GFP* relative to the expression of the respective reporters in *bam* RNAi (n=17-25 germaria per genotype, with Welch's t-test *** = p<0.001). Scale bar for all images is 15 micron.

Figure 7. Larp binds to TOP mRNAs and binding is regulated by Aramis. (**A-A'**) EMSA of Larp-DM15 and the leading 42 nucleotides of *RpL30* (**A**) and *Non1* (**A'**) with increasing concentrations of Larp-DM15 from left to right indicates that both RNAs bind to Larp-DM15. (**B**) Volcano plot of mRNAs in Larp::GFP::3xFLAG IP compared to input. Blue points represent mRNAs significantly enriched in Larp::GFP::3xFLAG compared to input, but not enriched in an IgG control compared to input. (**C**) Venn diagram of overlapping Larp IP targets and *aramis* RNAi polysome seq targets indicates that Larp physically associates with mRNAs that are translationally downregulated in germline *aramis* RNAi ($p < 0.001$, Hypergeometric Test). (**D**) Bar plot representing the fold enrichment of mRNAs

from Larp RNA IP in germline *aramis* RNAi relative to matched *bam* RNAi ovaries as a developmental control measured with qPCR (n=3, * = p<0.5, ** = p<0.01, NS = nonsignificant, One-sample t-test, mu=1) indicates that more of two *aramis* translation targets *Non1* and *RpL30* are bound by Larp in *aramis* RNAi. (**E-F"**) Confocal images of *nosGAL4*, driver control (**E-E"**) and ovaries overexpressing the DM15 region of Larp in the germline (**F-F"**) ovaries stained for 1B1 (red), Vasa (blue), and Larp-DM15::GFP (green). Overexpression of Larp results in an accumulation of extended 1B1 structures (highlighted with a dotted yellow line), marking interconnected cells when Larp-DM15 is overexpressed compared to *nosGAL4*, driver control ovaries. (**G**) In conditions with normal ribosome biogenesis Non1 is efficiently translated, downregulating p53 levels allowing for progression through the cell cycle. When ribosome biogenesis is perturbed Non1 is not translated to sufficient levels, resulting in the accumulation of p53 and cell cycle arrest. Scale bar for all images is 15 micron.

Supplemental Figure 1. Aramis, Athos, and Porthos are required for proper cytokinesis and differentiation, related to Figure 1. (**A-A"'**) Confocal images of *nosGAL4*, driver control (**A**) and germline RNAi knockdown using additional RNAis for *aramis* (**A'**) and *athos* (**A"**) stained for 1B1 (red) and Vasa (green). (**A"'**) Quantification of percentage of germaria with no defect (black), stem cysts (salmon), or germline loss (dark red) in ovaries depleted of *athos*, *aramis*, or *porthos* compared to control ovaries recapitulates the phenotypes with independent RNAi lines (n=50, df=2, *** = p<0.001, Fisher's exact test with Holm-Bonferroni correction). (**B-B"'**) Confocal images of germaria stained for 1B1 (red) and Phosphotyrosine (green). Ring canals, marked by Phosopho-tyrosine, are present between the interconnected cells of ovaries depleted of *athos*, *aramis*, and *porthos* with 1B1 positive structures going through the ring canals. (**C-F'**) Confocal images of germaria stained for pMad (red) and Vasa (green). In control ovaries (**C**) nuclear pMad staining occurs in cells proximal to the niche marking GSCs. Nuclear pMad staining in ovaries depleted of (**D**) *athos*, (**E**) *aramis*, and (**F**) *porthos* demonstrates that the observed cysts are not composed of GSCs. Scale bar for main images is 15 micron, scale bar for insets is 3.75 micron.

Supplemental Figure 2. Athos, Aramis, and Porthos are required for efficient ribosome biogenesis., related to Figure 2. (**A-A"**) Western blots of immunoprecipitations from ovaries for FLAG-tagged Aramis (**A**), Athos, (**A'**), and Porthos (**A"**). (**B-B"'**) Confocal images of (**B**) *nosGAL4*, driver control, (**B'**) *aramis* (**B"**) *athos* and *porthos* (**B"'**) germline RNAi germaria stained for Fibrillarin (red), DAPI (blue), and Vasa (green). (**C**) Quantification of nucleolar volume in GSCs of *aramis*, *athos*, and *porthos* RNAi, compared to control normalized to somatic nucleolar volume indicates loss of each helicase results in nucleolar stress (n=24 GSCs per genotype, One-way ANOVA, p<0.001, with Welch's t-test, * = p<0.05, ** = p<0.01). (**D-D'**) Polysome preparations from *Drosophila* S2 cells in cells treated with dsRNA targeting *RpS19a* (**D**) or *RpL30* (**D'**). (**E-G**) Western blot against proteins targeted for depletion by siRNA in HeLa cells. The human homologs of Aramis (DDX52) (**E**), Athos (DHX33) (**F**), and Porthos (DDX47) (**G**) are efficiently depleted with siRNA treatment after 72 hours (n=3, Welch's t-test, * = p<0.05).

Scale bar for all images is 15 micron.

Supplemental Figure 3. **Aramis is required to maintain proper cell cycle progression, related to Figure 3**. (**A**) Volcano plot of mRNA expression in *aramis* RNAi compared to *bam* RNAi. Blue points represent mRNAs significantly upregulated *aramis* RNAi compared to *bam* RNAi, red points represent mRNAs significantly downregulated *aramis* RNAi compared to *bam* RNAi. (**B**) Bar plot representing the most significant Biological Process GO terms of upregulated genes in ovaries depleted of *aramis* compared to the developmental control, *bam* RNAi. (**C-C'**) Genome browser tracks of mRNA expression at the *Cyclin A* (**C**) and *Actin 5C* (**C'**) loci indicate that the RNAseq target gene *Cyclin A* expression is downregulated, while a non-target, *Actin 5C* is not downregulated. (**D-E'**) Confocal images of germaria stained for 1B1 (red), DAPI (blue), and Cyclin B::GFP (green) in control (**D-D'**) and germline depletion of *aramis* (**E-E'**) demonstrates that functional Cyclin B::GFP cannot be efficiently expressed in germline depleted of *aramis*. (**F-H"**) Confocal images of germaria that express Fly-FUCCI in the germline stained for Vasa (blue). GFP-E2f1$^{\text{degron}}$ (green, right greyscale) and RFP-CycB$^{\text{degron}}$ (red, left greyscale) *nosGAL4*, driver control ovaries (**F-F"**), *bam* RNAi as a developmental control (**G-G"**), and ovaries with germline depletion *of aramis* (**H-H"**) demonstrates that the germline of *aramis* RNAi germline depleted ovaries are negative for both G1 and G2 cell cycle markers. (**I-I'**) Confocal images of *aramis* germline RNAi expressing GFP indicates productive translation of transgenes still occurs. (**J-M**) Confocal images of germaria stained for p53 (red) and Vasa (blue) in hybrid dysgenic, Harwich, ovaries (**J**) and p53$^{11\text{-}B1}$ ovaries (**K**) demonstrate the expected p53 staining patterns. (**L-M**) Confocal images of germaria immunostained for p53 (red) and Vasa (blue) in ovaries depleted of *athos* (**L**) or *porthos* (**M**) in the germline exibit p53 punctate staining. Cells highlighted by a dashed yellow circle represent cells shown in the inset. Scale bar for main images is 15 micron, scale bar for insets is 3.75 micron.

Supplemental Figure 4. The mRNA levels of Aramis polysome-seq targets are not significantly changing, related to Figure 4. (**A-A'**) Volcano plot of mRNA expression from poly(A)+ mRNA Input libraries in germline *aramis* RNAi compared to germline driven *UAS-tkv* (**A**) and *bam* RNAi (**A'**) of targets identified from polysome-seq. No target genes identified from polysome-seq meet the differential expression cutoff for mRNA in *UAStkv* compared to *aramis* RNAi or *bam* RNAi compared to *aramis* RNAi input libraries.

Supplemental Figure 5. Non1 and p53 expression are inversely related, related to Figure 5. (**A-B'**) Confocal images of ovarioles expressing Non1::GFP stained for p53 (red), Vasa (blue), and Non1::GFP (green). Quantifications of staining (**B-B'**), peak Non1 expression in control ovaries occurs in GSC-4 cell cyst stages and 16-cell cyst-region 2b stages where p53 expression is low. (**C-D'**) Confocal images of *nosGAL4*, driver control (**C-C'**) and germline *non1* RNAi germaria stained for p53 (red) and Vasa (blue). (**E**) Quantification of p53 punctate area above cutoff are markedly brighter in the germline of *Non1* RNAi depleted ovaries compared to the control. Cells highlighted by a dashed yellow circle represent cells shown in the inset. Scale bar for main images is 15 micron, scale bar for insets is 3.75 micron.

Supplemental Figure 6. mTorc1 activity positively regulates TOP ex-

pression, related to Figure 6. (**A-A'**) Genome browser tracks of the *Non1* (**A**) and RpS2 (**A'**) loci in ovary CAGE-seq data showing the proportion of transcripts that are produced from a given TSS (orange). Predominant TSSs are shown in orange and putative TOP motif beginning at the dominant TSS is indicated with a green box. The bottom blue and red graph represents sequence conservation of the locus across *Diptera*. The dominant TSS of *Non1* initiates with a canonical TOP motif and the *RpS2* TSS initiates at a sequence resembling a TOP motif. (**B**) Diagram of the *WT* and *Mut-TOP-GFP* reporter constructs indicating the TOP sequence that is mutated by transversion in the Mutant reporter (blue). (**C-D'**) Confocal images of *WT-TOP* reporter expression stained for 1B1 (red), GFP (green), and Vasa (blue) in *nosGAL4*, driver control ovaries (**C-C'**) and ovaries depleted of *Nprl3* (**D-D'**) in the germline. (**E-F'**) Confocal images of *Mut-TOP-GFP* reporter expression stained for 1B1 (red), GFP (green), and Vasa (blue) in *nosGAL4*, driver control ovaries (**E-E'**) and ovaries depleted of *Nprl3* (**F-F'**) in the germline. (**G**) A.U. quantification of WT and Mutant TOP reporter expression in GSCs of *nosGAL4*, driver control ovaries and GSCs of *Nprl3* germline depleted ovaries normalized to Vasa expression indicate that the relative expression of the *WT-TOP-GFP* reporter is higher than the *Mut-TOP-GFP* reporter (n=9-11 germaria per genotype, Welch's t-test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$). (**H-I'**) Confocal images of *WT-TOP* reporter expression stained for 1B1 (red), GFP (green), and Vasa (blue) in *nosGAL4*, driver control ovaries (**H-H'**) and ovaries depleted of *raptor* (**I-I'**) in the germline. (**J-K'**) Confocal images of *Mut-TOP-GFP* reporter expression stained for 1B1 (red), GFP (green), and Vasa (blue) in *nosGAL4*, driver control ovaries (**J-J'**) and ovaries depleted of *raptor* (**K-K'**) in the germline. (**L**) A.U. quantification of WT and Mutant TOP reporter expression in GSCs of *nosGAL4*, driver control ovaries and GSCs of *raptor* germline depleted ovaries normalized to Vasa expression indicate that the relative expression of the *WT-TOP-GFP* reporter is lower than the *Mut-TOP-GFP* reporter (n=10 germaria per genotype, Welch's t-test, * = $p<0.05$, ** = $p<0.01$). Scale bar for images is 15 micron.

Supplemental Figure 7. Larp binds specifically to TOP containing mRNAs and regulates cytokinesis, related to Figure 7. (**A-A'**) Confocal images of germaria stained for 1B1 (red), Vasa (blue), and *Larp GFP-3xFLAG* (green, greyscale) indicates Larp is expressed throughout early oogenesis. (**B**) Quantification of EMSAs and summary of K_d of the protein-RNA interactions. (**C-C'**) EMSA of Larp-DM15 and the leading 42 nucleotides of *RpL30* (**B**) and Non1 (**B'**) with their TOP sequence mutated to purines as a negative control with increasing concentrations of Larp-DM15 from left to right indicates that Larp-DM15 requires a leading TOP sequence for its binding. (**D**) Western of representative IP of Larp::GFP::FLAG from ovary tissue used for RNA IP-seq. (**E-F'**) Confocal images of *Larp::GFP::FLAG* reporter expression stained for 1B1 (red), GFP (green, greyscale), and Vasa (blue) in *bam* (**E-E'**) and *aramis* depleted germaria (**F-F'**). (**G**) A.U. quantification of Larp::GFP::FLAG reporter expression in the germline of *bam* RNAi and *aramis* RNAi demonstrates that the germline expression of Larp is not elevated in *aramis* germline RNAi compared to *bam* germline RNAi as a developmental control (n=10, $p>0.05$, Welch's t-test). (**H**) Western of representative IP of Larp::GFP::FLAG from ovary

tissue used for RNA IP qPCR. (**I-I'**) Confocal images of *nosGAL4*, driver control (**H**) and ovaries overexpressing the DM15 region of Larp in the germline (**I'**) ovaries stained for 1B1 (red), Vasa (blue), and Larp-DM15::GFP (green). Overexpression of Larp-DM15 results in the production of 32-cell egg chambers which indicates it causes a cytokinesis defect. Scale bar for all images is 15 micron.

Supplemental Table 1. Results of germline helicase RNAi screen on ovariole morphology.

Results of screen of RNA helicases depleted from the germline. Reported is the majority phenotype from n=50 ovarioles.

Supplemental Table 2. Differential expression analysis from RNAseq of ovaries depleted of aramis in the germline compared to a developmental control. DEseq2 output from RNAseq of ovaries depleted of *aramis* in the germline compared to ovaries depleted of bam in the germline as a developmental control. Sheet 1 (Downregulated Genes) contains genes and corresponding DEseq2 output meeting the cutoffs to be considered downregulated in aramis RNAi compared to bam RNAi. Sheet 2 (Upregulated Genes) contains genes and corresponding DEseq2 output meeting the cutoffs to be considered upregulated in aramis RNAi compared to bam RNAi. Sheet 3 (All Genes) contains DEseq2 output for all genes in the dm6 assembly.

Supplemental Table 3. Analysis of polysome-seq of ovaries depleted of aramis in the germline compared to developmental controls. Results of polysome-seq from ovaries depleted of *aramis* in the germline, ovaries depleted of *bam*, and ovaries overexpressing Tkv in the germline as developmental controls. Sheet 1 (Downregulated Genes) contains genes and corresponding polysome/input ratio values and values representing the difference in the polysome/input ratios between *aramis* RNAi and the developmental controls meeting the cutoffs to be considered downregulated in *aramis* RNAi. Sheet 2 (Upregulated Genes) contains genes and corresponding polysome/input ratio values and values representing the difference in the polysome/input ratios between *aramis* RNAi and the developmental controls meeting the cutoffs to be considered upregulated in *aramis* RNAi. Sheet 3 (All Genes) contains DEseq2 output for all genes in the dm6 assembly.

Supplemental Table 4. Aramis translation targets contain TOP sequences. List of *aramis* RNAi polysome downregulated targets and the position and sequence of the first instance of a 5-mer pyrimidine sequence downstream of the CAGE-defined TSS of each gene.

Supplemental Table 5. Enrichment analysis of Larp RNA IP mRNA-seq. Results of Larp::GFP::FLAG IP/IgG/Input mRNAseq. Each sheet contains the output of DEseq2. Sheet 1 (Larp Targets) contains Larp IP targets as defined in methods. Sheet 2 (IPvsIn Enriched) contains genes significantly enriched in the Larp IP samples compared to the input samples. Sheet 3 (IgGvsIn Enriched) contains genes significantly enriched (see methods) in the IgG samples compared to the input samples. Sheet 4 (IPvsIn All Genes) contains the DEseq2 output of all genes in the Larp IP samples compared to the input samples. Sheet 5 (IgGvsIn All Genes) contains the DEseq2 output of all genes in the IgG samples compared to the input samples.

Conclusion

If we don't want Conclusion to have a chapter number next to it, we can add the `{-}` attribute.

More info

And here's some other random info: the first paragraph after a chapter title or section head *shouldn't be* indented, because indents are to tell the reader that you're starting a new paragraph. Since that's obvious after a chapter or section title, proper typesetting doesn't add an indent there.

Appendix A

The First Appendix

This first appendix includes all of the R chunks of code that were hidden throughout the document (using the `include = FALSE` chunk tag) to help with readibility and/or setup.

In the main Rmd file

```
# This chunk ensures that the thesisdown package is
# installed and loaded. This thesisdown package includes
# the template files for the thesis.
if (!require(remotes)) {
  if (params$`Install needed packages for {thesisdown}`) {
    install.packages("remotes", repos = "https://cran.rstudio.com")
  } else {
    stop(
      paste('You need to run install.packages("remotes")",
            "first in the Console.')
    )
  }
}
if (!require(thesisdown)) {
  if (params$`Install needed packages for {thesisdown}`) {
    remotes::install_github("ismayc/thesisdown")
  } else {
    stop(
      paste(
        "You need to run",
        'remotes::install_github("ismayc/thesisdown")',
        "first in the Console."
      )
    )
  }
}
library(thesisdown)
```

```
# Set how wide the R output will go
options(width = 70)
```

In Chapter ??:

Appendix B

The Second Appendix, for Fun

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