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Investigating the localization mechanism of *Bsg25D* mRNA in *Drosophila melanogaster*

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Investigating the localization mechanism of *Bsg25D* mRNA in *Drosophila melanogaster*

Présentée par :

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a été évalué par un jury composé des personnes suivantes :

Dr. Vincent Archambault, président-rapporteur
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Résumé

Le transport subcellulaire et la traduction localisée des molécules d'ARNm semble être un processus très répandu et important pour contrôler la distribution asymétrique des protéines dans les cellules. L'ARNm, *Bsg25D*, connu pour se localiser aux centrosomes et aux microtubules astraux dans les embryons de drosophile au cours des premiers événements d'embryogenèse, a été sélectionné pour déterminer le rôle et l'importance du ciblage de l'ARNm à l'appareil mitotique lors de la division cellulaire. La localisation de *Bsg25D* aux centrosomes dans les embryons de drosophile est conservée entre espèces telles que *D. melanogaster*, *D. simulans* et *D. yakuba*. *Bsg25D* encode une protéine qui est étroitement liée à la Ninein (Nin) et à la Ninein-like protein (Nlp), deux protéines associées aux centrosomes présentes dans les cellules mammifères. L'analyse structure-fonction démontre que la région codante et la région 3'UTR de *Bsg25D* sont nécessaires pour son ciblage. Ceci suggère qu'un élément de régulation en *cis*, qui favorise sa localisation se situe dans la région codante + 3'UTR.

Mots-clés : Localisation des ARNm, Centrosomes, Hybridation *in situ* de fluorescence, Embryogenèse.

Abstract

The subcellular transport and localized translation of mRNA molecules is emerging as a highly prevalent and important process for controlling asymmetric protein distribution in cells. A candidate mRNA, *Bsg25D*, known to localize to centrosomes and astral microtubules in *Drosophila* embryos during early events of embryogenesis, was selected to determine the role and importance of mRNA targeting to the mitotic apparatus during cell division. The localization of *Bsg25D* to centrosomes in *Drosophila* embryos is conserved between species such as *D. melanogaster*, *D. simulans* and *D. yakuba*. *Bsg25D* encodes a protein closely related to centrosome-associated proteins Ninein (Nin) and Ninein-like protein (Nlp) in mammalian cells. Structure function analysis revealed that the coding and 3'UTR of *Bsg25D* are necessary for its targeting pattern, suggesting that a *cis*-regulatory motif that drives its localization, is in the coding + 3' UTR region.

Keywords: mRNA localization, Centrosomes, Fluorescent *in situ* hybridization, Embryogenesis.

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Liste des abréviations

3'UTR	3' Untranslated Region
A/P	Anterior/Posterior
Ash1	Achaete-Scute Homologue-1
Bub	Budding uninhibited by benomyl
CIN	Chromosomal Instability
C-terminus	Carboxyl terminus
D. melanogaster	*Drosophila melanogaster*
D/V	Dorsal/Ventral
DGRP84	Drosophila Gamma-Tubulin Ring Protein 84
DGRP91	Drosophila Gamma-Tubulin Ring Protein 91
DNA	Deoxyribonucleic acid
DAPI	4',6-Diamidino-2-phenylindole
EGFR	Epidermal growth factor receptor
G1	Gap 1
G2	Gap 2
Grk	Gurken
GFP	Green fluorescent protein
Hsp83	Heat shock protein 83kD
Hb	Hunchback
IF	Immunofluorescence
MTOC	Microtubule organizing centers
MTs	Microtubules
N-terminus	Amino terminus
NLP	Ninein-like protein
ORF	Open reading frame
PCM	Pericentriolar material
Plk	Polo-like kinase

P53	Tumour protein 53
RBP	RNA binding protein
RNA	Ribonucleic acid
S	Synthesis
SAC	Spindle Assembly Checkpoint
SPB	Spindle pole body
S. cerevisiae	*Saccharomyces cerevisiae*
TGFα	Transforming growth factor alpha
Tgf-β	Transforming growth factor beta
Top	torpedo
UTR	Untranslated regions
Vg1	Vestigial
wt	Wild type
γ- tubulin	Gamma tubulin
γ-TuRC	γ – Tublin ring complex

எனது குடும்பத்திற்கு

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I would like to thank all the wonderful people who have helped me get to where I am today. This has undeniably been a journey, one with a whirlwind of change throughout the entire process. I am fortunate to have the unconditional love and support of my family and friends.

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The best for last: Nitish. Thank you for never giving up on me. Thank you for giving me hope and always showing me how much I am loved. Love you forever and always.

Introduction

1 – Introduction

The central dogma of molecular biology was first described by Francis Crick (Crick, 1958). He explained the flow of genetic information from DNA being transcribed into RNA (transcription) and subsequently being translated from RNA to protein (translation) (Crick, 1970). As seen in **Figure 1**, DNA undergoes replication, forming two identical strands of DNA. This idea of the central dogma suggests that genes encode proteins and proteins alone are responsible for the expression of the genetic material inscribed in our DNA. At the time, only the lines that are solid were known interactions, and the dotted lines were predicted interactions. There are several mechanisms that have since come to light that involve regulation at the RNA level. The addition of 5' cap or 3' Poly-A tail, the splicing of introns and exons and alternate splicing, are a few examples of such mechanisms. These short segments of mRNA are subsequently translated into protein molecules of different shapes and sizes to serve as structural supports, chemical catalysts, and molecular motors, in order to enable cells to communicate with each other and regulate gene expression (Alberts, 2002; Ding et al., 1993).

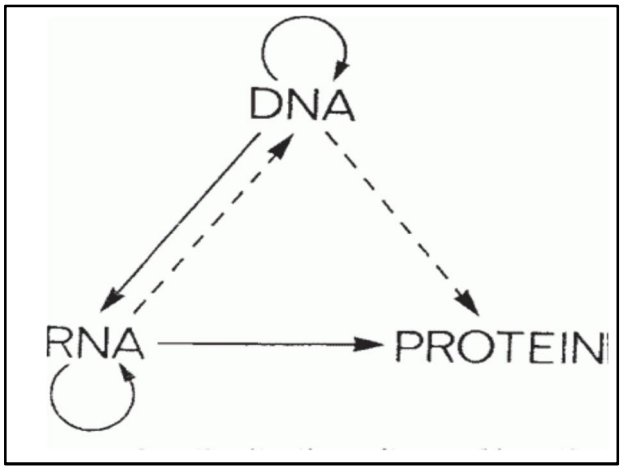


Figure 1. Central dogma of molecular biology

All living cells follow this fundamental process coined the central dogma of molecular biology. *Figure was adapted from (Crick, 1970).*

Following transcription, RNAs undergo several maturation steps. RNA maturation is controlled at various points including 5'-end capping and 3'-end cleavage/polyadenylation (Neve et al., 2017; Ramanathan et al., 2016), splicing of introns (Shi, 2017), RNA nuclear export through the nuclear pore (Sakuma and D'Angelo, 2017), cytoplasmic transport (Singh et al., 2015), translation and eventually degradation (Corbett, 2018). These steps are further regulated by RNA binding proteins (RBPs), which often control the trafficking, stability and translation of the mRNA (Gebauer et al., 2012; Glisovic et al., 2008; Keene, 2007).

2 - mRNA Localization

2.1 – General Importance and Functions of mRNA localization

Localized transcripts are found in numerous organisms ranging from unicellular fungi to animals and plants, and in a diverse array of cell types including oocytes, fibroblasts, glia, neurons and epithelial cells (Bashirullah et al., 1998; Chartrand et al., 2001; Jansen, 2001; Kloc et al., 2002; Lipshitz and Smibert, 2000; Palacios and St Johnston, 2001; Seydoux and Schedl, 2001). Asymmetric mRNA localization is an important mechanism by which cells achieve polarity and generate asymmetric protein distributions that are important for development both functionally and morphologically. Subcellular localization of mRNA is a post-transcriptional mechanism that allows for delicate regulation of when and where proteins are made and function, as well as confining target proteins to specific subcellular compartments (Bashirullah et al., 1998). There are highly regulated *cis* and *trans*-acting elements that mediate specific targeting of mRNAs spatially and temporally. Early discoveries and different studies of mRNA localization were restricted to a few transcripts in a small number of model organisms. Today, there is looming evidence that mRNA localization is a very widespread process in eukaryotes (Bashirullah et al., 1998; Blower et al., 2007; Cody et al., 2013; Diehn et al., 2000; Jansen, 2001; Lecuyer et al., 2007; Marc et al., 2002; Martin and Ephrussi, 2009; van Heesch et al., 2014; Wilk et al., 2016). Though the

process of mRNA localization was thought to be a very rare phenomenon, a previous study analyzing 3370 *Drosophila* genes has shown that more than 70% of mRNAs that are expressed are found to be localized subcellularly (Lecuyer et al., 2007). Several other such large genome wide studies have shown the importance of RNA localization subcellularly (Medioni et al., 2012; Mili et al., 2008; Rapoport, 2007; van Heesch et al., 2014; Wickner and Schekman, 2005; Wilk et al., 2016).

Among the earliest evidence of localized mRNAs was the finding by Jeffery and colleagues (1983) in the eggs of ascidians (sac-like marine invertebrate filter feeders), who showed that *β-actin* mRNA localizes to the myoplasm of ascidian eggs and participates in ooplasmic segregation (Jeffery, 1986). Our growing knowledge of mRNA localization in different model systems is derived from several different genetic, cytological and biochemical assays performed in various model systems. In budding yeast *Saccharomyces cerevisiae*, *Ash1* mRNA localizes to the bud tip during anaphase of the cell cycle, leading to an asymmetric distribution of *Ash1* to daughter cells, an important process for mating type switching (Chartrand et al., 2002; Long et al., 1997; Takizawa et al., 1997). Several well-characterized mRNAs are found to localize in early embryos and oocytes, where they work to regulate the development of the germline and control the formation of axis plan. Embryonic patterning is established during oogenesis in *Xenopus* and *Drosophila* through the localization of maternally provided mRNAs that are polarized (Bashirullah et al., 1998; Deshler et al., 1998; Ephrussi et al., 1991). Asymmetric subcellular localization of mRNAs is not limited to the germline. It is also observed in somatic cells like neurons where it is shown to play an important role in memory and learning (Amtul and Rahman, 2016). These studies collectively have provided us with the foundation to understand the biological and functional importance of mRNA localization.

2.2 - Why localize mRNAs?

Transcript localization can potentially serve many important biological functions (see **Figure 2** for summary). mRNA localization is a mechanism used to regulate expression of proteins in a temporal and spatial manner. Firstly, mRNA localization is an effective means to concentrate proteins synthesized at a specific site since each template can serve many rounds of translation. Local protein synthesis can also be regulated temporally in response to stimuli, which is efficient since synthesizing proteins that are not necessary is energetically costly. This allows cells to rapidly respond to local requirement making it possible to regulate gene expression (St Johnston, 2005). Each gene can be transcribed and translated at different efficiencies depending on the cells' needs. *Bicoid* mRNA, for example, localizes to the anterior end of a *Drosophila* embryo. There, it encodes Bicoid protein and induces cells to adopt anterior cell fates (Driever and Nusslein-Volhard, 1988). Normal *bicoid* gradient is necessary for establishing an anterior/posterior (AP) axis and proper development of the head and thorax (Nusslein-Volhard et al., 1987). Secondly, transcript localization can provide a way to prevent proteins from being targeted to the wrong compartment, where they could exert harmful or toxic effects. *Drosophila nanos* mRNA localizes to the posterior of the early embryo where its corresponding protein product induces posterior cell fates. When *nanos* transcripts are mislocalized to the anterior end of the embryo, it induces cells to adopt posterior fates thereby causing severe abnormalities, such as development of a second abdomen in place of the head and thorax (Gavis and Lehmann, 1992; Smith et al., 1992). This example illustrates how the presence of a protein in the wrong place at the wrong time can have harsh implications on a developing organism. This is particularly important in large or highly polarized cells. Thirdly, in certain cases, the localized mRNAs code for proteins that have their own targeting signals which allows these proteins to be sorted to organelles and subcellular domains such as the mitochondria, mitotic microtubules and endoplasmic reticulum (Pelham, 1990; Rapoport, 2007; Rothman and Orci, 1992; Wickner and Schekman,

2005). However, many of these proteins do not contain this sorting signal and therefore may solely rely on the subcellular localization of their transcripts.

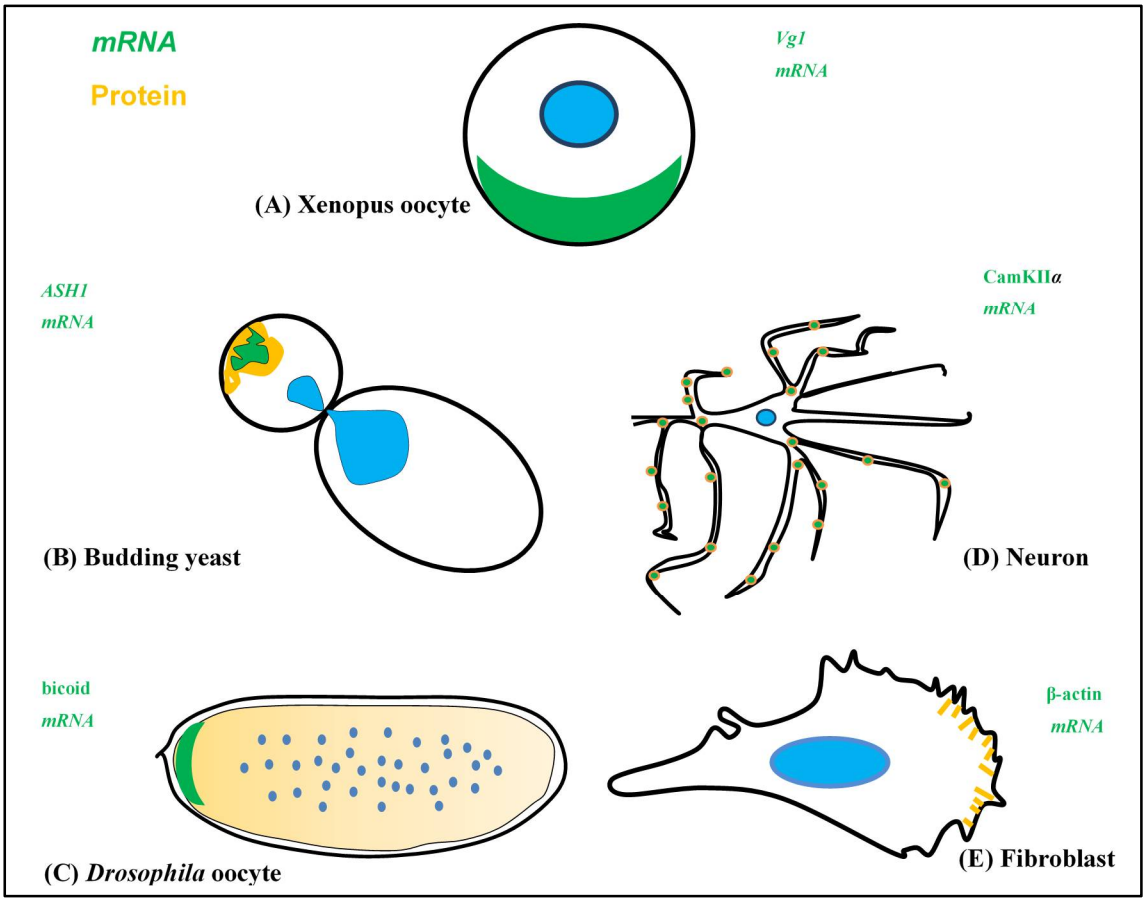


Figure 2: Examples of Localized mRNA

Various examples of RNA localization playing an important role in asymmetric cell division, synaptic plasticity, morphogen gradient formation and cell migration. **(A)** In *Xenopus* oocytes, *Vg1* mRNA localizes to the vegetal pole. **(B)** In budding yeast, *S. cerevisiae*, *Ash1* mRNA localizes to the bud tip. **(C)** In *Drosophila* embryos, *bicoid* mRNA localizes to the anterior pole of the developing embryo. (**D**) In neurons, *CamKIIα* mRNA localizes to the dendrites of the axon. **(E)** In fibroblasts, β-actin localizes to the lamellipodia.

2.3 Mechanisms of mRNA localization

There are several different mechanisms by which RNAs get localized, including active transport, localized synthesis, diffusion and localized entrapment, localized degradation, and polarised nuclear export (Lipshitz and Smibert, 2000). A combination of these processes may take place to localize a transcript. Active transport along the cytoskeletal filaments is considered the major localization mechanism in most cells (Jansen, 2001; Kloc et al., 2002; Palacios and St Johnston, 2001; Tekotte and Davis, 2002). The transcripts can be moved from the site of synthesis to their destination within the cytoplasm on tracks made by microtubules or microfilaments since the cytoskeleton is composed of actin and microtubule networks that are important in short-distance and long-distance transport, respectively. They are important in the transport of RNA cargos (Kloc et al., 2002) which are transported along the cytoskeleton by molecular motors such as kinesin, kinesin-like and dynein as well as ribonucleoprotein (RNP) complexes that are necessary to associate with these molecular motors (Kloc et al., 2002).

Local synthesis, though rare, is a simple way to target mRNA to a specific area in the cell. Transcripts can also diffuse from where they are made until they become anchored at a specific site, thereby creating a gradient. *Drosophila Nanos* is a well-characterized example of diffusion and entrapment, which is perhaps the easiest form of localization. *Nanos* localizes to the posterior pole of an embryo during late oogenesis. There, it translationally represses *hunchback* (*hb*) RNA and promotes abdominal development (Tautz, 1988). *hb* RNA is initially maternally expressed and evenly distributed in the embryo (Tautz, 1987; Tautz, 1989). *Nanos* then inhibits maternally derived *hb* from being translated at the posterior end thereby forcing it to congregate in the anterior end (Tautz, 1987; Tautz, 1989). Although *nanos* mRNA is known to localize to the posterior of the *Drosophila* embryo, only 4% of the total *nanos* mRNA concentrates to the posterior pole while the rest of the *nanos* mRNA is found throughout the embryo (Bergsten and Gavis, 1999) and the anchoring is established

through actin filaments present at the posterior pole (Forrest and Gavis, 2003). This entrapment or anchoring allows for a stable association of mRNA with parts of the cellular architecture, which in this case is required for translation (Gavis and Lehmann, 1992). Embryos that are mutant for *nanos* fail to develop an abdomen (Lehmann and Nusslein-Volhard, 1991). Therefore, *nanos* is able to establish a concentration gradient of both *hb* and itself from anterior to posterior pole of a mature oocyte through its inhibition of Hb translation (Pelegri and Lehmann, 1994). Germline localization of *nanos* appears to be an evolutionarily conserved mechanism even in primordial germ cells of zebrafish (Gavis et al., 2008).

Localized degradation is a process by which transcripts that were widely distributed throughout the cytoplasm are degraded everywhere but at the site of localization, where it is said to be protected. Many RNAs in *Drosophila,* like Heat Shock protein 83kD (*Hsp83*) localize to precursors of germ cells called pole cells at the posterior of the embryo (Palacios, 2007). *Hsp83* mRNA is uniformly distributed in a mature oocyte where upon fertilization, *Hsp83* is degraded everywhere except at the posterior pole plasm where it is localized (Kelley, 1993). Each nuclear division results in a decrease in the level of total RNAs with the exception of the pole plasm where they are protected (Ding et al., 1993). This degradation-protection mechanism accounts for the removal of *Hsp83* during the first two hours of embryogenesis when 95% of Hsp83 is degraded (Bashirullah et al., 1999). In mutants that do not have a pole plasm, *Hsp83* RNA is degraded throughout (Bashirullah et al., 1999).

2.4 Localization Signals within the RNAs

Conserved mechanisms of mRNA localization and localized translation exist in all branches of eukaryotes in order to regulate gene expression, by localizing transcripts to a specific region and activating translation of those localized mRNA (Du et al., 2007; Kraut-

Cohen and Gerst, 2010; Micklem et al., 2000). This in turn results in an asymmetric distribution of RNAs and proteins. *Cis*-regulatory motifs, often called "zipcodes", are localization elements usually found in the untranslated regions of transcripts where they are not constrained to have protein coding sequences (Holt and Bullock, 2009). They mediate interactions between mRNAs and their binding partners to set up mRNA targeting pathways (Andreassi and Riccio, 2009; Jambhekar and Derisi, 2007). Zipcodes are recognized by transacting factors that specifically bind to them (Czaplinski and Singer, 2006). RNA binding proteins (RBPs) are the main players involved in recognizing, binding and mediating the translocation of the transcript to its appropriate destination. In some cases, the interaction is mediated by several RBPs forming complexes such as in the example of *Vg1* mRNA in *Xenopus laevis*. *Vg1* is a signalling molecule that belongs to transforming growth factor-β (tgf-beta) superfamily and is important for mesoderm induction during embryogenesis. Several RBPs are required to promote *Vg1* mRNA localization to the vegetal pole in *Xenopus* oocytes (King et al., 2005; Rand and Yisraeli, 2001).

In some cases, a single localization element will efficiently localize the transcript (Serano and Cohen, 1995). This, however, is not usually the case. Multiple localization elements and sometimes, several copies of the same element, are required for proper mRNA localization. For many localized mRNAs characterized to date, *cis*-regulatory sequences are found within the 3'UTR. However, the coding region and 5'UTR can also harbour localization signals (Gonzalez et al., 1999). For example, in budding yeast *S. Cerevisiae*, *Ash1* behaves as a transcriptional repressor of HO endonuclease and is crucial for asymmetric distribution of *Ash1* (Bobola et al., 1996; Sil and Herskowitz, 1996). *Ash1* localizes to the bud tip thereby restricting Ash1 protein expression in daughter cell to promote opposite mating types between mother and daughter (Long et al., 2001; Takizawa et al., 1997). Functional *cis*-acting elements are found in both 3'UTR and the coding region of *Ash1* mRNA: one that spans the end of the coding sequence and the 3'UTR while three others are in the coding sequence (Chartrand et al., 1999; Gonzalez et al., 1999). It was demonstrated

that the 3'UTR contains a stem-loop structure that is necessary for the localization of this RNA (Chartrand et al., 1999; Gonzalez et al., 1999). *Gurken (grk)* mRNA encodes a protein called transforming growth factor alpha-like protein (TGFα-like protein). *Gurken* is important for establishing the Anterior/Posterior (A/P) and Dorsal/Ventral (D/V) axes of the oocyte and embryo. Localization signals for *grk* RNA are found in the 5' and 3' UTRs. While the zipcode in the 5'UTR is important for *grk* mRNA localization within the oocyte in early stage egg chambers, the localization signal in the coding sequence is required for mid to late stage egg chamber localization (Neuman-Silberberg and Schupbach, 1993; Thio et al., 2000). The 3'UTR localization signal is necessary to confine the message to the dorsal-anterior end (Neuman-Silberberg and Schupbach, 1993; Thio et al., 2000). Defects in *grk* lead to consequences in the polarity of the oocyte and the embryo where the embryos become ventralized through an expansion of ventral structures (Kelley, 1993; Manseau and Schupbach, 1989; Schupbach, 1987; Schupbach and Wieschaus, 1991; Wieschaus, 1978).

2.5 Translational regulation

Translational regulation is a means by which mRNAs on route to their destination are repressed from undergoing translation until it is necessary. This prevents the encoded proteins from being ectopically expressed during transport. However, the RNAs need to be actively translated once they reach their destination and therefore the repression is alleviated through de-repression. The overall mechanism is an interplay between repression and activation of translation governed by RNA localization. In certain instances, like *Ash1* transcripts in budding yeast, this translational repression is essential to properly localize the RNA to the bud tip (Gu et al., 2004; Irie et al., 2002). Once the RNA reaches the bud tip, its translation is necessary for anchorage. However, it is not clear how this transition from repression to activation is achieved.

Our understanding of the relationship between RNA localization and translation has been gleaned from studies done in *Drosophila* focusing on four maternal transcripts critical for axis determination: *bicoid*, *gurken*, *nanos*, and *oskar* during oogenesis and embryogenesis. *Cis*- and *trans*-acting factors are necessary not only for proper localization of mRNA but for translational control of these mRNAs as well. *Bicoid* transcript localizes to the anterior of the oocyte and is translationally regulated through polyadenylation, a post-transcriptional modification made to the 3'end of the transcript upon exiting the nucleus (Salles et al., 1994). *Nanos* and *oskar* localize to the posterior end of the oocyte, and are controlled translationally via transcript localization (Kloc et al., 2002; Lipshitz and Smibert, 2000; St Johnston, 2005).

Translational regulation is not limited to the embryo and oogenesis. Studies have shown that mRNAs localized to dendrites of neurons have to be translationally regulated to achieve synaptic plasticity (Steward and Schuman, 2001), important in memory and learning (Farris et al., 2014; Kejiou and Palazzo, 2017).

3 - The Cell Cycle and Mitosis

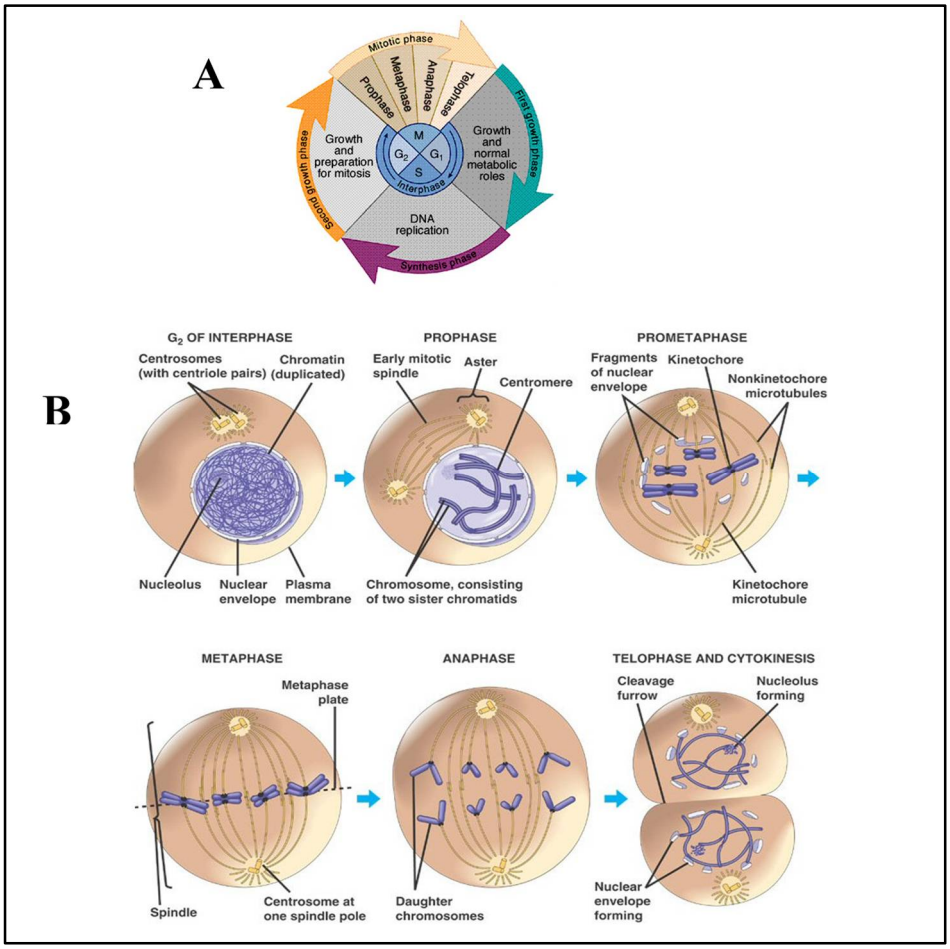


Figure 3: Overview of the Cell Cycle and Mitosis

The cell cycle allows for the doubling of genetic material **(A)** and division of cells into two daughter cells. The cycle has two main phases: *Interphase*, where the DNA is replicated (S) and the cell grows (G1 and G2); and *Mitosis* where the cell divides into two identical cells (M) *(Image adapted from Morgan, D., 2007. The Cell Cycle).* (**B)** Mitosis consists of five phases: prophase, prometaphase, metaphase, anaphase and telophase. The mitotic spindle, made up of microtubules, pulls apart the chromosomes during mitosis. Once mitosis is complete, the cytoplasm is divided into two through a process called cytokinesis (Image adapted from Alberts et al., 2002. *Molecular Biology of the Cell*).

3.1 The Cell Cycle

Cells undergo a series of events collectively known as the cell cycle. These steps allow cells to grow, copy their genetic material, prepare for cell division and finally divide. The cell cycle is divided into two phases: interphase and mitosis (see **Figure 3**). Cells spend most of their time in interphase, consisting of three subphases - G1, S and G2 phases. The DNA is replicated during the S phase (synthesis phase), which is also when the centrosomes undergo duplication (Morgan, 2007). The G1 and G2 phases (Gap 1 and Gap 2, respectively) separate DNA synthesis and mitosis. Mitosis or the M phase of the cell cycle is comprised of mitosis and cytokinesis (Morgan, 2007). The M phase is the shortest phase of the cell cycle, lasting about 30 minutes, during which sister chromosomes segregate into two daughter cells each with a complete set of chromosomes (Morgan, 2007). Cytokinesis occurs at the end of mitosis where the cytoplasm is cleaved to form two genetically identical cells (Alberts, 2002; Schafer, 1998).

Several hundred proteins coordinate the assembly and activity of the mitotic spindle and centromeres during the cell cycle (Vitre and Cleveland, 2012). Targeting RNA molecules to structures of the mitotic apparatus has long been suggested as a way to control cell cycle regulators through localized translation and to control asymmetric inheritance of genetic material during cell division (Suprenant, 1993; Vitre and Cleveland, 2012).

3.2 Cell Division

Mitosis is divided into 5 different subphases. During prophase, the chromosomes becomes very compact, condensing into visible chromosomes and the centrosomes separate while the mitotic checkpoint proteins, such as BUB1 and BUBR1 are recruited to the kinetochores (Elowe, 2011). At prometaphase, the nuclear envelope begins to break down and the kinetochore microtubules start to form and connect to the centrosomes through the kinetochores (see **Figure 3**). During metaphase, the chromosomes align along the equatorial plate with each chromatid attached to a kinetochore microtubule, at the centre of the cell

called the metaphase plate. The chromatids begin to separate during anaphase as they move toward the spindle poles, while tethered at the centromere. During telophase, the chromosomes reach the spindle pole and the nuclear envelope begins to re-form while chromatin decondenses. Cell division is completed with the division of the cytoplasm, during cytokinesis. Although mitosis is the shortest process during the cell cycle, there are many intricate steps and feedback mechanisms that help maintain the integrity of the genome (Alberts, B. et al, 2004).

3.3 Mitotic Apparatus

The mitotic apparatus consists of 1 spindle and 2 centrosomes (Flemming, 1882) (Wilson, 1902). For the purpose of this thesis, we will concentrate on the centrosome as a key player along with microtubules (MTs).

4 - Overview of the Centrosome

The centrosome was first discovered by Boveri and Van Beneden, in the late 19th century when it was coined the term “centrosome” as the division centre of the cell during mitosis (Boveri, 2008; Hamoir, 1992). In animal cells, the centrosome is the major microtubule-organizing centre (MTOC), responsible for nucleating, anchoring and releasing MTs (see **Figure 4**). Centrosomes are involved in several processes including cell motility, cell adhesion and polarity and cell division (Doxsey et al., 2005).

The centrosome, also called the spindle pole body in fungi, consists of two centrioles embedded within an electron-dense cloud called the pericentriolar matrix (PCM). The centriole provides structural stability in vertebrate cells. Each centriole is made up of a total of nine MT triplets, spanning 0.2 μm in width and on average 0.5 μm in length (Bornens, 2002; Paintrand et al., 1992), though this can vary depending on the species and cell type (Bornens, 2002; Paintrand et al., 1992). For example, in *D. melanogaster* the microtubules can be arranged as doublet or triplet MT (Bettencourt-Dias and Glover, 2007). Centrioles are

polarized along the proximo-distal axis (Bettencourt-Dias and Glover, 2007; Nigg and Stearns, 2011).

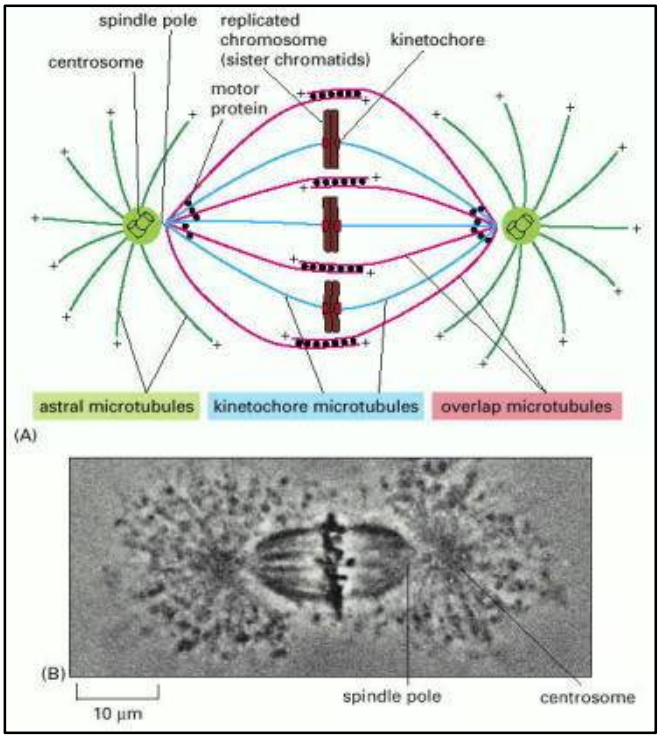


Figure 4: Centrosomes, Centrioles and Kinetochores

(A) Schematic diagram showing main players in cell division: microtubules, centrosomes, kinetochores, chromosomes and motor proteins. The chromosomes are aligned at the metaphase plate, ready for division. The microtubules are attached by kinetochores and will draw apart the sister chromatids to their respective spindle poles. **(B)** A phase-contrast micrograph depicting a similar image to (A) the schematic. (E.D. Salmon and R.R. Segall, *J. Cell Biol.* 86:355–365, 1980. © The Rockefeller University Press) *(Both Images were taken from Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York.*)

4.1 Centrioles

Centrioles are stable structures and their loss has been shown to lead to PCM breakdown (Basto, 2006; Bettencourt-Dias et al., 2005; Janke et al., 2005). It is believed that post-translational modifications, such as polyglutamylation of the centriolar tubulin confers its stability (Bobinnec et al., 1998; Janke et al., 2005). Tektins and ribbon proteins are other structural components of the centriole, which might also provide structural stability (Hinchcliffe and Linck, 1998; Steffen and Linck, 1988).

To form a bipolar mitotic spindle the centriole duplicates using the mother or mature centriole as a template to give rise to the daughter. The centrosome duplicates during the S phase of the cell cycle, matures during G2 phase by accumulating PCM material, then the duplicated centrosomes separate at the beginning of M phase (Alvey, 1985; Kochanski and Borisy, 1990; Kuriyama and Borisy, 1981a; Robbins et al., 1968; Sluder and Rieder, 1985; Vorobjev and Chentsov Yu, 1982). The mother centriole has subdistal and distal appendages that dock cytoplasmic MTs. If depleted using a laser, centrioles can reform *de novo* (La Terra et al., 2005; Marshall et al., 2001; Riparbelli and Callaini, 2003).

Proteomic studies revealed that there are more than 200 centrosome-associated proteins, many that have yet to be characterized (Andersen et al., 2003). The PCM contains proteins such as pericentrin and AKAP450, a family of coiled-coil domain proteins that anchor MTs in the cytoplasm during interphase and mitosis (Gillingham and Munro, 2000). Gamma (γ) tubulin is found on the walls of the centrioles and has been shown to increase in concentration prior to mitosis (Martin et al., 1997). γ- tubulin is found in a complex called γ-TuSC or γ-tubulin small complex. In *D. melanogaster*, each complex has two tubulin molecules and a molecule of DGRP84 and DGRP91. These four proteins together are known as the γ-tubulin ring complex (γ-TuRC), which holds the subcomplexes of γ-TuSC. The removal of these molecules in *D. melanogaster* has been shown to give rise to spindles that are abnormal (Colombie et al., 2006).

MTs are anchored onto subdistal appendages of centrioles. Ninein is part of the subdistal appendage of the mother centriole, which interacts with the centriole at its C-terminus while connecting to the γ-TuRC through its N-terminus (Mogensen et al., 2000). Several cells depend on centrioles and spindle pole body (SPB) for accurate cell division in early embryonic systems. However, many other cell types such as higher plants cells and oocytes divide even in the absence of centrioles. Centrioles have been implicated in the regulation of cytokinesis and G1-S transition. The removal of the centrosome has been shown to disrupt cytokinesis (Hinchcliffe et al., 2001), even though most cells form a furrow. SPB also helps concentrate molecules that are important for mitosis exit. In some species, centrioles assure mitotic fidelity and contribute to spindle orientation (Khodjakov and Rieder, 2001). Interestingly, in *D. melanogaster,* the removal of centrosomes is not sufficient to halt mitosis, as cells still transition from G1 to S in their absence (Basto et al., 2008). However, if the proteins associated with the centrosome are also removed along with the centrosome, then the cells are halted at G1 of the cell cycle. This could simply be a mechanism that has evolved to prevent cells from becoming aneuploid or from developing defects that can lead to disease. The increase in centrosome number leads to multipolar spindles, as well as tumorigenesis (Basto et al., 2008; Levine et al., 2017; Timonen and Therman, 1950). Indeed, chromosome instability is a common feature of tumour cells, characterized by an elevated rate of gain or loss of whole chromosomes.

Even though the centrioles do not seem to be a universal requirement in cell division and are absent during female meiosis, they seem to be a requirement for the assembly of cilia and are important in male meiotic divisions. Centrioles serve as basal bodies when they are attached to the membrane, and they form cilia and flagella, which are involved in sensory perception, propagation of morphogenetic signals and motility. In most cells, cilia are found in their immotile form as primary cilia, while in other cells, such as sperm and gut cells, they exist in their motile form (Alberts, 2002). Both forms are equipped with sensory functions. A study done by Jonassen *et al.* in 2008 suggests that cilia might be important in determining

the axis of cell division and the positioning of the centrosome in kidney cells, associating two separate functions of the centriole in a process (Jonassen et al., 2008).

The proper control of centrosome and centriole number is crucial for the development of a healthy organism. Since centrosomes facilitate the organization of the spindle poles during mitosis, any errors in this process can lead to cancer and cause genomic instability (Nigg, 2002) as a consequence of aberrant cell division (Sluder and Nordberg, 2004). The increase in centrosome number could be a source of chromosomal instability thereby leading to cancer. There is an increasing number of studies being performed in the 21st century many years after the discovery of the centrosome and its link to several human diseases such as ciliopathies and diseases involved in brain development. The knockdown of p53, a tumour suppressor, leads to the increase in centrosome number in mouse fibroblasts and skin tumours (Fukasawa, 2007), but this link between centrosome abnormalities and cancer is not limited to mice. In fact, it is common in several prevalent human cancers such as breast cancer, prostate cancer, lung cancer, colon cancer and brain cancer (Lingle et al., 2002; Pihan et al., 1998).

4.2 Ninein and Ninein-like proteins

The minus-end of the microtubules are nucleated and anchored from the pericentriolar material (PCM). Elongation occurs by the addition of tubulin subunits at the distal plus-end of microtubules. Ninein is a centrosomal protein important for microtubules minus-end anchorage and plays a role in docking γ-tubulin containing complexes (Delgehyr et al., 2005; Stillwell et al., 2004). Ninein has been shown to localize to the ends of the subdistal appendages of the mother centriole as well as to the minus-ends of both centrioles (Mogensen et al., 2000; Ou et al., 2002). The centrosomal protein, Ninein-like protein (Nlp) plays a major role in centrosome maturation via γ-TuRC recruitment during interphase. Nlp has also been shown to be an important substrate for many mitotic kinases such as Aurora B, Plk1, Cdc2, Nek2 (Bornens, 2002; Casenghi et al., 2005; Casenghi et al., 2003; Rapley et al., 2005; Zhao

et al., 2010). During the cell cycle, Nlp is associated with the maturing centrosome leading up to the G2/M transition where it dissociates via phosphorylation. This is a very important step for the maturation process and mitotic spindle formation. When Nlp is overexpressed, it forms large aggregates that subsequently induce aberrations in mitotic spindle formation and the depletion of Nlp gives rise to lagging chromosomes and multinucleated phenotypes (Casenghi et al., 2003; Jin et al., 2009; Zhao et al., 2010). Understandably, since both Ninein and Nlp play a crucial role during microtubule anchoring, γ-tubulin docking and centrosomal maturation, the perturbation of these proteins during cell division can induce mitotic aberrations, spindle checkpoint defects, chromosomal missegregation and failure of cytokinesis. This can induce chromosomal instability potentially leading to tumorigenesis (Thompson et al., 2010; Thompson and Compton, 2008).

5 - Pathways of Control

The accurate transmission of genetic information from one cell to its daughters is crucial for the survival of an organism. Proper segregation of chromosomes during mitosis is therefore necessary to maintain the genomic integrity of a cell (Hartwell and Weinert, 1989; O'Farrell et al., 2004). There are several checkpoints within the cell cycle that help maintain the fidelity of each step allowing the overall organism to remain healthy.

During the cell cycle, the cell must make decisions on whether or not to proceed based on the faithfulness of each process (Ciccia and Elledge, 2010; Litwin et al., 2018). These checkpoints are highly conserved and as soon as they encounter a problem, the cycle is halted allowing the error to be corrected prior to proceeding to the next step. Since each step must be highly regulated, when these checkpoints are turned on for an excessive period of time, it may give rise to an abnormal number of chromosomes, thereby inducing apoptosis or leading to a disease state (Hanahan and Weinberg, 2011).

5.1 Aneuploidy and Chromosomal Instability

When the integrity of the genome is threatened, the cell has many feedback mechanisms to try and protect the genome while preventing the accumulation of mutations to protect future generations. However, genes that are involved in these checkpoints are also susceptible to mutations giving rise to various human diseases (Suprenant, 1993; Vitre and Cleveland, 2012). Most cancers are a result of mutations in one or more genes associated in the checkpoint pathway(s) (Lengauer et al., 1997). A few examples of defects leading to chromosomal instability include failed mitotic checkpoint signalling, defects in chromosome cohesion or attachment and assembly of multipolar mitotic spindles.

Checkpoints that guard the cell from errors can often be defective as is the case for many aneuploid cancers including types of leukemia, breast, colorectal, ovarian and lung cancers (Weaver and Cleveland, 2009). In these diseases the signalling of the Spindle Assembly Checkpoint (SAC) is inadequate as a result of an altered expression or mutations of components of the mitotic checkpoints. A rare genetic disorder called mosaic variegated aneuploidy has also been associated with mutations in BubRI (or BublB), a component of the mitotic checkpoint (Schmid et al., 2014). This disease causes growth retardation, microcephaly, and childhood cancer, which often results in death at a young age (Hanks et al., 2004; Matsuura et al., 2006). Defects in sister chromatid cohesion might also promote aneuploidy, as many studies have demonstrated that overexpression of securin or separase regulators important for the control of chromatid cohesion gives rise to aneuploidy and cell transformations (Pei and Melmed, 1997; Yu et al., 2003; Zhang et al., 2008). Merotelic attachment results from one kinetochore attaching improperly to microtubules extending from both spindle poles, leading to improper segregation of chromosomes (Thompson and Compton, 2008). Even though in most cases, the cell corrects this error prior to moving onto anaphase, it can promote aneuploidy. If aneuploidy is left undetected in the cell, it leads to lagging anaphase chromosomes at the spindle midzone, which subsequently do not segregate into daughter cells (Zhang et al., 2008). Chromosomal instability (CIN) can be caused by

missegregation of a whole chromosome. However, it is not limited to this, as structural rearrangements such as translocation, deletions and inversions can also give rise to CIN. CIN has been associated with poor patient prognosis in tumors (Choi et al., 2009; Gao et al., 2007; Heilig et al., 2010) and resistance to chemotherapeutic agents, thereby leading to tumor evolution (Kuukasjarvi et al., 1997; McClelland et al., 2009; Swanton et al., 2009). As such, it is important to supress CIN to target tumor cell adaptability as a means of therapeutically targeting the tumor.

6 - mRNA localization and the Mitotic Apparatus

The components of the mitotic apparatus were first successfully isolated as a single unit from dividing sea urchin eggs (Mazia and Dan, 1952). These components were physically associated with each other and were called the mitotic apparatus (MA). In later years, spindles were isolated from sea urchin eggs and it was determined that they were mainly composed of microtubules with chromosomes attached to them (Robbins et al., 1968).

A very early observation of mRNA associating with the cytoskeleton was documented when several polysomes were present in the cytoskeleton even after the cells were treated with detergents (Kuriyama and Borisy, 1981b). They demonstrated that the cytoskeleton retains almost all the active polyribosomes and they were attached to the cytoskeleton via mRNA (Kuriyama and Borisy, 1981b). Interestingly they ruled out microtubules (MT) of playing any part in this process, since polysomes were retained in the cytoplasm even when the MT were depleted by using detergents that created an environment unfavourable for stabilizing MT. However, it was later shown that under specific stabilization conditions it was possible to also preserve MTs even after detergent extraction (Kuriyama and Borisy, 1981a).

Microtubules are approximately 25 nm in diameter. The basic unit of a microtubule is the tubulin dimer composed of alpha and beta subunits. Microtubule arrays are not fixed and can be polymerized or depolymerized through GTP hydrolysis and have a rapid turnover

due to their dynamic instability (Kuriyama and Kanatani, 1981). They act as transport vehicles and roadways for mRNA to travel directionally (Suprenant, 1993).

When Salmon and Segall (1980) isolated spindles, they made observations using both a light microscope as well as an electron microscope (Robbins et al., 1968). Under the light microscope, they observed globular material, approximately 0.5 um diameter aligned in a circular fashion around astral fibres on the central spindle. They were able to distinguish features such as the centrosome and centriole at opposite poles, chromosomes and MT. A closer look using high magnitude electron microscopy showed particles that were spread along the central spindle MT, adhering to a thin filament, in large clumps. These particles corresponded with the globular material seen with the light microscope. At the time, they were not able to identify the thin filament and particles. They did however postulate that the particles were ribosomes or other ribonuclear proteins.

Early evidence of ribosomes associating or interacting with MTs came from morphological evidence gained through electron microscopy (EM). High voltage EM observations suggested that polyribosomes interact with microtubules. Heuser and Kirschner (1980) documented a very interesting observation they discovered through freeze-dried cell samples (Heuser and Kirschner, 1980). They reported grape-like clusters of ribosomes surrounding microtubules (Heuser and Kirschner, 1980). This data was further confirmed when Ris (1985) observed the presence of several polyribosomes, with the help of a short 2-3 nm filament, highly crosslinked to microtubules, intermediate filaments and actin filaments in cultured mammalian cells (Ris, 1985).

RNAs are not only found associated with the mitotic apparatus. Other organelles and structures have also been shown to contain RNAs. One such example is the association of RNA in muscle. Early evidence from histochemical studies of RNA distribution in muscle (Clavert et al., 1949) have later been confirmed using immunohistochemical studies with anti-ribosomal antibodies (Horne and Hesketh, 1990) to show that ribosomes are present

along the myofibrils. In some studies, the associated mRNAs have been found to be present in polysomes and are therefore being actively translated (Bag and Pramanik, 1987; Bird, 1986).

Work by Peterson and Berns (1978) using psoralens, a nucleic acid-binding drug that is light activated, demonstrated that RNA present at the centriolar region is responsible for forming spindle in dividing PTK2 cells (Peterson and Berns, 1978). This was not the first time RNA was suspected to be present in the centriolar region. Prior studies using staining agents, such as acridine orange (Randall, 1965) and ethidium bromide (McGill et al., 1976) had also suggested the presence of nucleic acid in the centriolar region, as well as in basal bodies. Other studies in the 1970s suggested a similar notion, indicating high levels of RNA were found at microtubule organising centers (Bielek, 1978; Rieder, 1979). This RNA is believed to be important in nucleating microtubules. This was concluded through a study done by Heidermann *et al.*, (1977) where basal bodies isolated from *Chlamydomonas* and *Tetrahymena* were treated with enzymes and their ability to nucleate aster formation in *Xenopus laevis* was examined (Heidemann et al., 1977). Through their work, they showed that centrioles have RNA that is required and is important for aster formation.

More recently, Blower *et al.*, (2007) performed a global study of mRNAs bound to microtubules during metaphase in *X. laevis* egg extracts and human cell extracts revealing conserved groups of mRNA enrichment on MTs (Blower et al., 2007). Only a select group of mRNAs were associated with MT-bound polyribosomes suggesting that mRNA that are both translationally active and inactive are present on the mitotic spindles. Blower and his colleagues proposed that mRNA subcellular trafficking to the microtubules is a mechanism for enhancing protein localization and for dividing translationally inactive transcripts to daughter cells during cell division. The evidence from this article and others mentioned above set the basis for the presence of mRNA and its association with components of the mitotic apparatus.

RNA localization was thought to be a rare phenomenon due to poor detection methods leading to poor resolution. As such, in an attempt to improve the ability to visualize the mRNA localization patterns in developing *Drosophila* embryos, Dr. Lecuyer and his colleagues refined their protocol to add a few crucial steps to ameliorate signal detection. The improved *in situ* hybridization technique was subsequently used to uncover a whole new plethora of mRNAs that localize to various parts of the cells, each potentially having different functional roles in the cell. During a genome-wide global analysis of RNA localization patterns in early *Drosophila* embryogenesis, Lecuyer *et al.* (2007) discovered that more than 70 % of RNAs are localized, a phenomenon that was otherwise thought to occur rarely (Lecuyer et al., 2007). The high-resolution fluorescent *in situ* hybridization protocol was instrumental in uncovering these patterns of mRNA trafficking and their importance during embryogenesis (Lecuyer et al., 2007).

Approximately 4000 genes in the *Drosophila* genome were analyzed and 71% of the genes that were expressed encode subcellular localized mRNAs. This was a ground breaking discovery since it was previously estimated that less than 1% of mRNAs were localized (Tomancak et al., 2002). Lecuyer *et al*., also uncovered several striking new patterns of localization as well as diverse subcellular locations (e.g. membranes, cytoskeleton, mitotic apparatus, chromatin, nuclei, etc.), indicating that there is more to mRNA localization than originally conceived. These patterns have been documented into a web resource (http://fly-fish.ccbr.utoronto.ca). The number of different patterns also suggests that there are probably several different mechanisms associated with the observed localization patterns. Furthermore, there was a strong correlation between the transcript distribution and protein localization/function, which suggests that mRNA localization plays a vital role in organizing cellular protein networks (Lecuyer et al., 2007).

6.1 Candidate mRNA

Among the list of mRNAs found to have a specific localization pattern in *Drosophila* embryos, an interesting group of mRNAs localize to parts of the cell division apparatus, including centrosomes, astral MTs, and mitotic spindles (**Table 1**). This raises the possibility that mRNA targeting, and localized translation might play a role in the regulation of mitosis. A subset of about 30 mRNAs was found to localize to the mitotic apparatus and they were found to be functionally enriched for transcripts encoding regulators of cytoskeleton organization and cell division-related processes (see **Figure 8**). Although it has long been thought that localized translation of mRNAs may occur along the mitotic apparatus as a means of targeting regulators of mitosis or asymmetric cell division, this has not been investigated thoroughly.

To elucidate the function and the mechanism of mitotic mRNA localization, we selected a candidate mRNA with a dynamic and diverse localization pattern that we are interested in characterizing. The candidate mRNA, *Blastoderm-specific gene 25D* (*Bsg25D*), localizes to centrosomes and astral microtubules in *Drosophila* embryos during early events of embryogenesis (see **Figure 5**).

Ultimately, we want to test whether mRNA targeting to the mitotic apparatus is important for the regulation of cell division.

Table 1: mRNA localized to components of the cell division apparatus in *Drosophila* (adapted from Lecuyer *et al.*, 2007)

mRNA Localization Pattern	Gene	Protein Function
Microtubule/Centrosomal	Bicaudal D (BicD)	Dynein Binding/RNA Transport
"	**Bsg25D/Ninein**	**Microtubule Nucleation/Centrosomal**
"	Cyclin B	Cell-Cycle Regulation/Kinase Activator
"	CG14897	Unknown
"	CG1962	Unknown/SMC Domain Protein
"	CG9977	Unknown
"	CP309/AKAP450	Microtubule Nucleation/Centrosomal
"	diminutive (dm)	Transcription Factor
"	Downstream of kinase (Dok)	Insulin Receptor Binding
"	Ensconsin/E-MAP-115	Recruitement of Kinesin to Microtubules
"	IkB-kinase like 2 (IK2)	Kinase/Actin Filament Organization
"	l(1)dd4/dGrip91	Microtubule Binding/Centrosomal
"	rapsynoid (raps)	Establishment of Spindle Orientation
"	stonewall (stwl)	Transcription Factor
Spindle-Associated	CG14408	Unknown/SH3 Domain Binding Protein
"	CG15634	Unknown
"	CG5077	Unknown/Oxysterol Binding
"	CG6045	Unknown/FAD Binding
"	hiiragi (hrg)	RNA-Binding/PolyA Polymerase
"	kugelkern (kuk)	Nuclear Membrane/Nucleus Organization
"	odd skipped (odd)	Transcriptional Repressor
"	Proximal to Ras (Ptr)	Unknown
"	ribbon (rib)	Transcription Factor
"	Runt (run)	Transcription Factor
Centriolar	CG14438	Unknown/Multi-Zinc Finger Protein
"	trio	Rho GEF/Actin Cytoskeleton Regulation
Other Cell Division	Ady43A	Adenosin Kinase Activity
"	CG8654	Unknown
"	dappled (dpld)	Cell Cycle Regulation

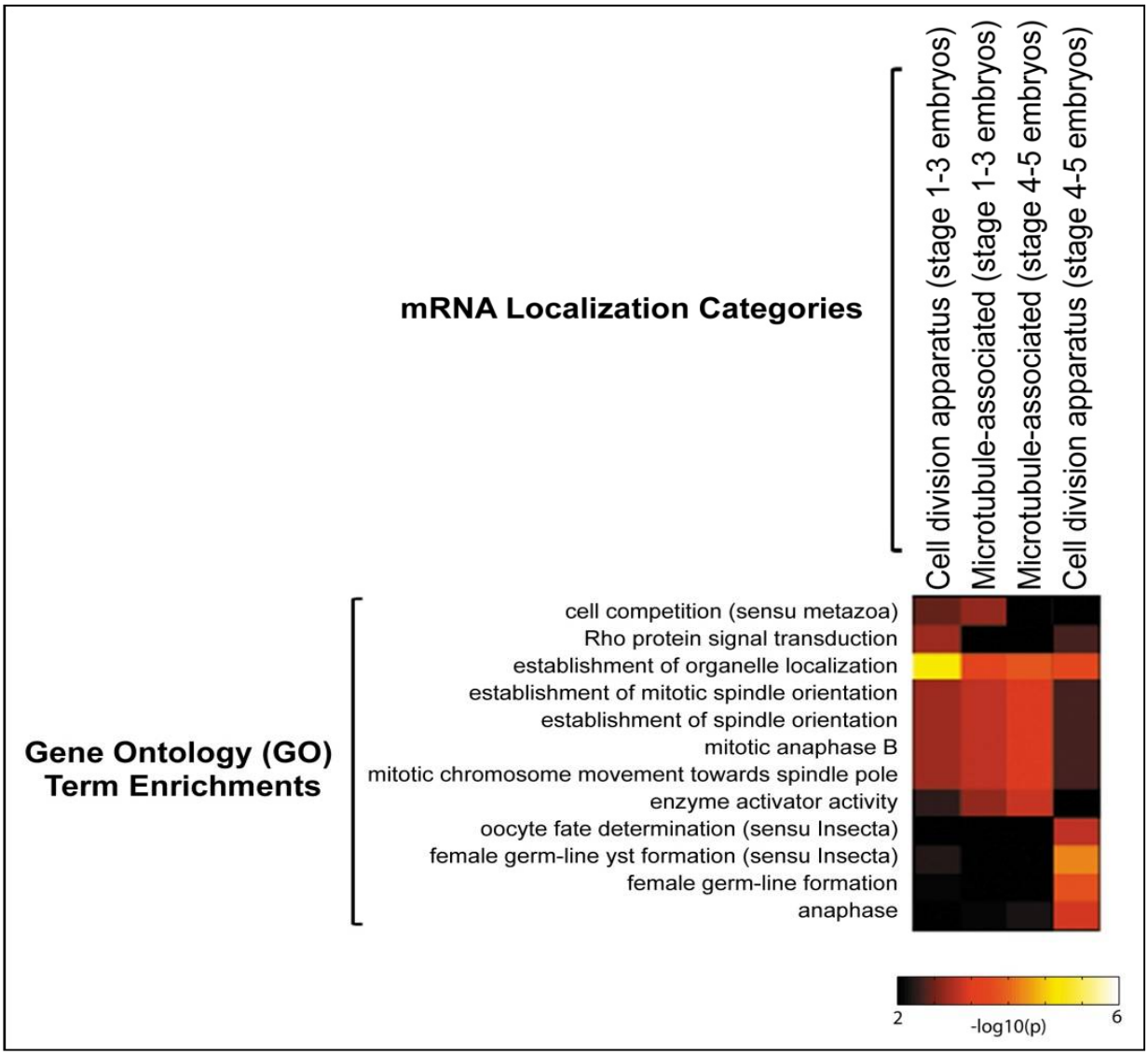


Figure 5: Functional enrichment of mRNAs localized to the cell division apparatus

GO term enrichments exhibited by mRNAs classified within the cell division apparatus and microtubule-associated localization categories in stage 1-5 *Drosophila* embryos. The "hot metal" color scale reflects statistical significance (-log 10 of the p-value) of the GO term enrichments (Figure adapted from Lecuyer *et al*., 2007).

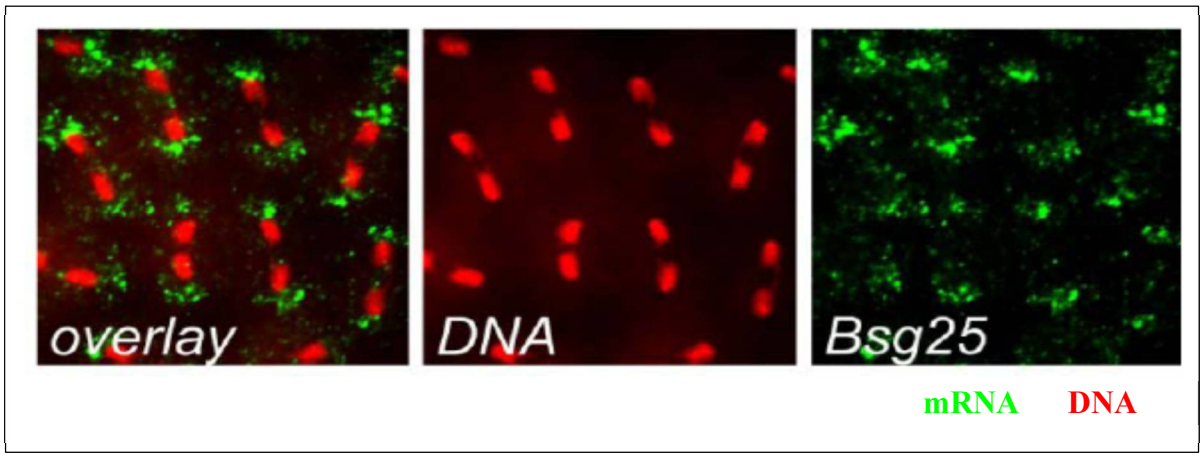


Figure 6: Preliminary finding of *Bsg25D* mRNA

Bsg25D exhibits targeting to centrosome and astral microtubules during *Drosophila* embryogenesis. The Green is *Bsg25D* mRNA while the Red stain is DAPI (Adapted from *Lecuyer et al.*, 2007).

7 - An Overview of the Model Organism: *Drosophila melanogaster*

Drosophila melanogaster, or the common fruit fly, was initially introduced in the context of evolutionary biology but has since developed into a very powerful tool in biological research, particularly in genetics and developmental biology (Arias, 2008; Hallem et al., 2004; Kohler, 1994). Several attributes have made *D. melanogaster* a good model system. These flies are easily maintained at standard laboratory conditions (25º C). They are inexpensive and allow easy observation and manipulation at most developmental stages. They produce large numbers of offspring. They are small and have a rapid generation time of 10 days from an embryo to an adult fly. Most importantly, we now have well-established techniques that facilitate genetic manipulation of these flies (Brookes, 2001; Sturtevant, 1961).

7.1 Life Cycle

The life cycle of *Drosophila*, from egg fertilization to adult life, takes about 10 days at 25°C. Female flies produce the most number of eggs between the fourth and seventh day after they emerge (Lodish et al., 2000). They can lay several hundred eggs in their lifetime. A few hours after copulation, the female starts laying fertilized eggs. The egg is surrounded by a thin inner lining called the vitelline membrane and an outer lining called a chorion (Lodish et al., 2000). After a day of embryonic development at 25º C, the egg hatches (see **Figure 7 A-B-C**). Over the next several days, the larvae undergo two molts from the first instar larvae to the second and third instar larva. During this time, they primarily feed and grow. The larva then molts into puparium. As the pupa develops the pupal casing becomes increasingly darker (Lodish et al., 2000). The final stage of pupation is metamorphosis into an adult fly and eclosion (Lodish et al., 2000).

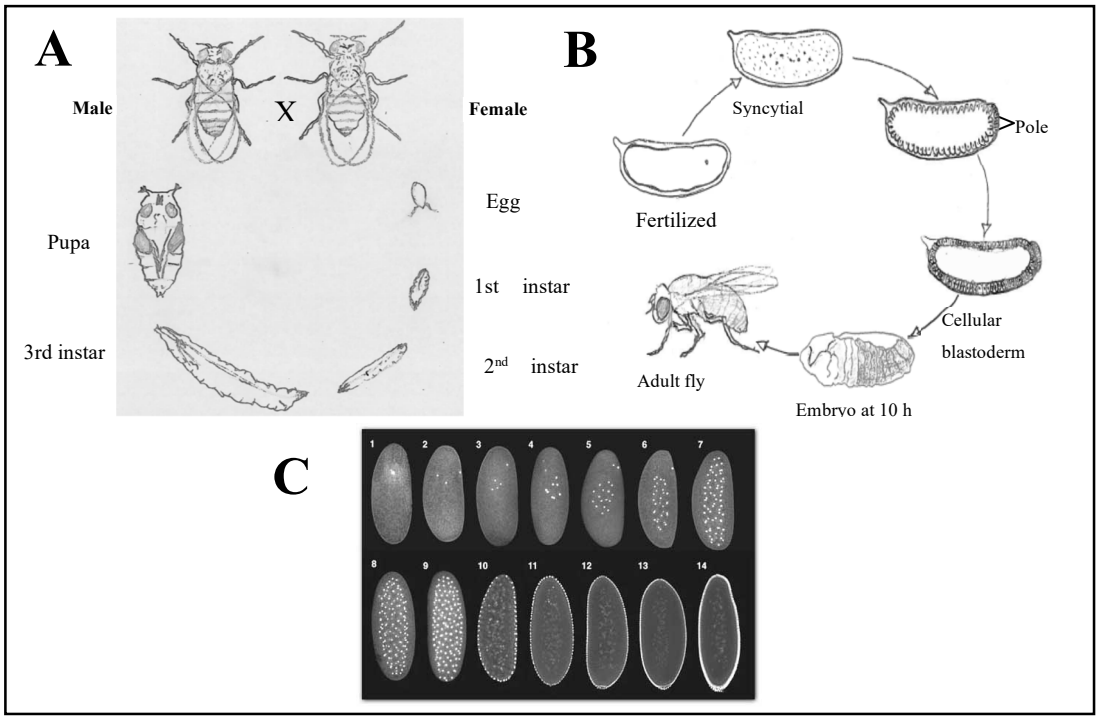


Figure 7: Life Cycle, Development and Embryogenesis of *Drosophila melanogaster*

The life cycle of *Drosophila*, from egg fertilization to adult life, takes about 10 days at 25°C. **(A)** Adult male and female mate and produce an egg, which undergoes a multitude of changes from larva (4 days) to pupa (4 days) and finally leading to eclosion of the adult fly. (*Image adapted from Carolina Biological Supply Company).* **(B)** Fertilized egg undergoes several changes as it develops into an adult fly: 24h after fertilization, embryogenesis, formation of a syncytium, cellularization, blastoderm formation, gastrulation and the emergence of an adult fly. *(Image adapted from Life: The Science of Biology, Purves et al., 1998)* **(C)** *Drosophila* embryogenesis: the zygote undergoes many rounds of mitotic divisions during embryogenesis. Looking closely at fluorescently labelled nuclei through confocal microscopy, by the end of 8 consecutive nuclear divisions each lasting about 8 minutes, a total of 256 nuclei are present in the centre (yolk) of the egg. The pole cells form after the ninth division, when ~5 nuclei migrate to the posterior pole and become enclosed. The remaining nuclei migrate to the periphery and become enclosed after 13 divisions, thereby forming the blastoderm. (*Image adapted from Gilbert SF. Developmental Biology. 7th edition. Sunderland (MA): Sinauer Associates; 2003*.)

7.2 Early Development

During fertilization, the male and female pronuclei appose next to each other eliciting several rapid and synchronous zygotic divisions (Zalokar and Erk, 1976). Interestingly cytokinesis does not occur in the first 13 rounds of division upon fertilization (see **Figure 5 C**). Instead, a multinucleated cell is formed, sharing a single large cytoplasm (syncytium). A regular *D. melanogaster* embryo is an ovoid shape approximately 500 um by 180 um and the first nuclear divisions take place in the yolk located in the centre of the embryo (Greenspan, 1997).

The nuclei then migrate to the periphery of the embryo forming the syncytial blastoderm (Foe and Alberts, 1983) while a few dozen nuclei remain in the centre forming the yolk nuclei (vitellophages). A couple nuclei are also incorporated into the posterior pole plasm to form the polar buds (Campos-Ortega, 1997; Mahowald, 1963). These pole cells form the primordial germ cells, which give rise to gametes. The embryo undergoes cellularization whereby the nuclei migrate to the periphery, and cell membranes are laid down between each nucleus. The embryo undergoes a series of asynchronous cell division creating the cellular blastoderm. Studies have shown early stages of cell division in a developing embryo are attributable to the maternal contribution of information (RNA and proteins) present in the egg prior to fertilization. Zygotic transcription only begins thereafter during the 14^{th} division where the embryo relies on the production of its own transcripts and proteins to properly develop into an adult fly. Zygotic gene mutations therefore may not produce an apparent phenotype until the maternal gene product has been consumed. Therefore, in order to study a gene's function in a fly embryo, it is important to generate mothers that are mutant for the gene of interest thereby giving rise to eggs that are deficient in specific maternal gene products.

7.3 *Delta 2-3(Δ2-3)*

An example of a transposase used for P-element mutagenesis is the P (Delta) 2-3 element (Robertson et al., 1988). The name of the line is derived from the location of the intron between exon 2 and 3, which is spliced solely in germ cells (Laski et al., 1986). Normally, P-element transposition does not occur in somatic cells as it is repressed at the level of RNA processing (Robertson et al., 1988). In somatic cells, however, a protein binds to exon 2, thereby preventing the intron from being spliced (Chain et al., 1991; Siebel and Rio, 1990; Tseng et al., 1991). The intron can be removed artificially, which creates a transposase that is capable of mobilizing P-elements in all tissues, somatic and germline cells (Laski et al., 1986). When the intron is not spliced, the resulting truncated protein behaves as a repressor of P-element mobility (Gloor et al., 1993; Handler et al., 1993; Misra et al., 1993; Rio et al., 1986).

7.4 Gal4-UAS Driver

The GAL4 system is a technique used to specifically drive transgene expression in a tissue-specific and cell-specific manner in *Drosophila* and other model organisms (Brand and Perrimon, 1993). The eukaryotic transcriptional machinery is highly conserved across different species, thereby allowing GAL4 to activate transcription in other species (Kakidani and Ptashne, 1988; Ma et al., 1988; Webster N, 1988) The GAL4 system allows for the study of the effects of misexpressing a gene of interest on development, by selectively expressing the transgene in cells where GAL4 is expressed. Gal4 is a eukaryotic transcription factor that is responsible for activating genes involved in galactose metabolism in yeast (Hashimoto et al., 1983). The upstream activation sequence (UAS) controls the expression of the transgene.

Figure 8 depicts a cross between a transgenic line expressing the GAL4 driver (A) in a known spatiotemporal pattern and the second line (B) which contains the transgene downstream of UAS sequences. The transgene is expressed in progeny that have cells or

tissues expressing the GAL4 protein. The GAL4 system allows one to express a gene of interest ectopically which allows for inducing cell fate change (Davis et al., 1987), inducing altered cell fates in neighbouring cells, and altering the cell's physiology (Southall et al., 2008). This method of ectopic expression can be used to test whether a gene functions autonomously or non-autonomously (Brand and Perrimon, 1993), whether the gene is sufficient for cell identity and whether the cell or tissue responds to changes in signalling pathways (Huang and Rubin, 2000; Zhu et al., 2005). It has been proposed that nearly 60% of *Drosophila* genes have no loss-of-function phenotype (Miklos and Rubin, 1996). Therefore, to study the functional importance of a gene of interest in these cases, expressing this gene ectopically might be the only way.

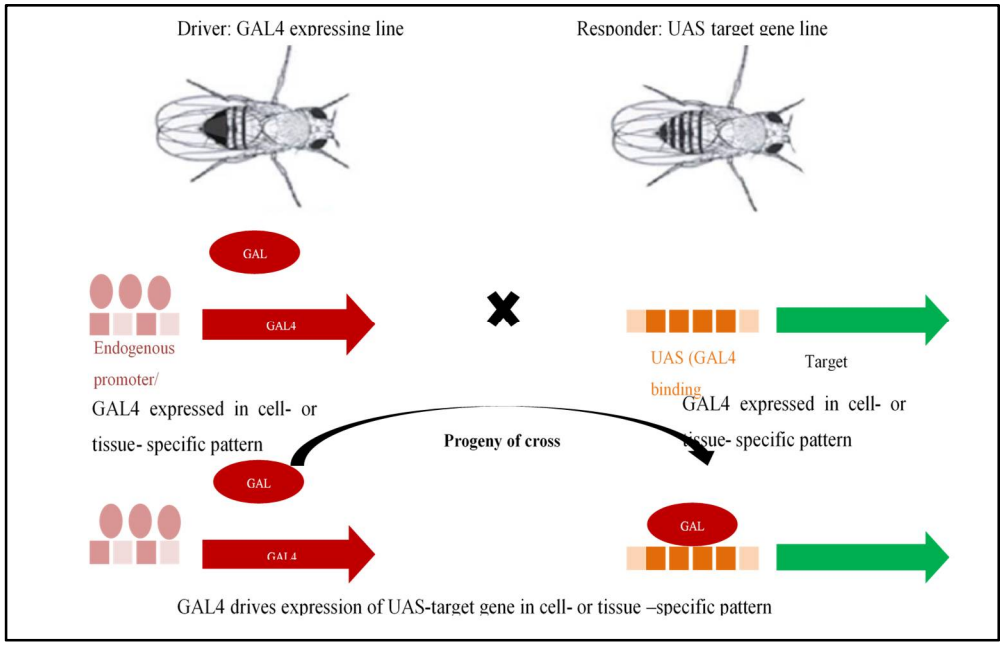


Figure 8: Schematic diagram of GAL4–UAS Driver

The expression line containing the GAL4 sequence is crossed to transgenic flies that contain the target gene under the control of UAS sequences. GAL4 is produced in the progeny and the expression of the transgene can be activated *in vivo* following the binding of the UAS sites (*Figure adapted from Phelps and Brand, 1998).*

7.5 Site-specific transgenesis

The ability to introduce and control the expression of transgenes in a model organism is a powerful tool for understanding how genes function.

The introduction of P-elements to induce mutations became an attractive technique to study gene function. Mutagenesis through P-element transposition, however, occurs in an unpredictable manner making it challenging to selectively target a gene of interest. They integrate several times throughout the genome and therefore it is almost impossible to generate integration at the same site with two different transgenes (Bischof et al., 2007; Brand and Perrimon, 1993). Also, it has been shown that in *Drosophila* the expression of transgenes is affected by positional effects especially when the transgene inserts into a heterochromatic region (Bischof et al., 2007; Brand and Perrimon, 1993). Finally, the frequency of integration of P-elements is relatively low in fertile adults.

The PhiC31 integrase system was developed for *D. melanogaster* by Groth *et al*. in 2004 to tweak the system previously shown to function in other model organisms such as *Xenopus laevis* embryos (Allen and Weeks, 2005; Groth et al., 2004), the mosquito *Aedes aegypti* (Nimmo et al., 2006) and even mammalian cells (Groth et al., 2000). The PhiC31 integrase is a bacteriophage (PhiC31) which mediates sequence-specific recombination between attB (donor) and attP (recipient) sites (see **Figure 9**). They have a stretch of 3 nucleotides in their central region that is common, which facilitates crossover events (Thorpe et al., 2000).

Therefore, the integrase catalyzes the insertion of the transgene in a sequence specific manner and integration is unidirectional. There are a hundred known attP lines that have been created with the attP insertion sites mapped. These lines are widely available to the fly community.

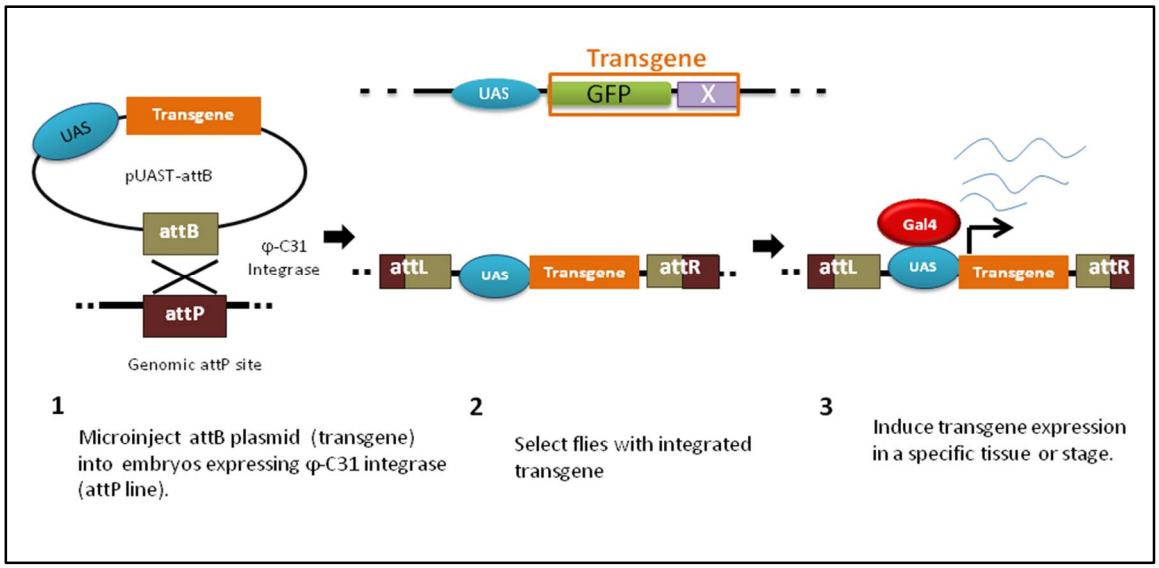


Figure 9: Site-Specific Transgenesis Using the Phi-C31 System

(1) The attB-pUAST plasmid is injected into embryos from a fly line expressing enzyme ϕC31 integrase and a defined attP landing site **(2)** The integration of the transgene destroys the recombination site and flies that have successfully integrated the transgene at a precise location are selected **(3)** To induce transgene expression, the transgenic fly line must be crossed to a GAL4-driver line with a known specific spatiotemporal expression pattern.

8 – Objectives, Hypothesis and Strategies

Given how important mRNA localization is for the asymmetric distribution of its encoded protein, their discovery two decades ago was followed by a period of intense research to gain insights into the mechanisms of mRNA localization and the biological function of mRNA targeting. During the genome-wide screen of ~4000 distinct mRNA transcripts during *Drosophila* development, a subset of 30 mRNAs was found to localize to the mitotic apparatus, raising the possibility that mRNA targeting and localized translation might play a role in the regulation of mitosis. Although it has long been thought that localized translation of mRNAs may occur along the mitotic apparatus as a means of targeting regulators of mitosis or asymmetric cell division, this theory has not been investigated thoroughly. We therefore formulate the **hypothesis that targeting *Bsg25D* mRNA to the mitotic apparatus is important for the proper coordination of cell division**.

The overall aim of my project was to develop a better understanding of the role of mRNA and its importance in the proper coordination of cell division, through the characterization of *Bsg25D* mRNA. The **first objective** was to characterize the mitotic defects of *Bsg25D* mutant flies through Fluorescent *in situ* Hybridization (FISH). The **second objective** was to identify *cis*-regulatory motifs (CRMs) required for *Bsg25D* mRNA targeting. To map localization of CRMs present in *Bsg25D* mRNA, we generated GFP constructs fused to the coding, the 3'UTR or the full-length sequence of *Bsg25D*. These chimeric mRNAs were then examined both *in vitro* (S2 cells) and *in vivo* (transgenic flies) to see their subcellular localization properties through high-resolution FISH using a probe to GFP to detect transgenic mRNAs. The **third objective** was to assess the functional role of *Bsg25D* mRNA localizing in the regulation of cell division *in vivo*.

9 - Materials and Methods

9.1 Fly Stocks and Genetics

The Bsg25D^{G13518}/CyO mutant stock and the deficiency stocks Df(2L)Exel6011/CyO and Df(2L)BSC693/CyO were obtained from Bloomington Drosophila Stock Center. P-element mediated deletion of the original Bsg25D stock was performed as described by Robertson *et al.* (1988). Unless otherwise indicated, all fly cultures and crosses were grown on standard fly medium at 25°C.

9.2 P-element mutagenesis

The P-element excision strategy consisted of an initial cross between males expressing active transposase (Δ2-3) and virgin females from original Bsg25D^{G13518}/CyO. The progeny (F1) is screened for male recombinants, with mosaic eyes. The original stock with the transposase source has white eyes, and the original Bsg25D^{G13518}/CyO has red eyes. When the transposase is active, it excises the P-element and the progeny has eyes that are mainly white with swirls of red, referred to as mosaic flies. Each pigment cell in the eye represents an independent cell and the mosaic phenotype indicates active transposition. These flies are therefore selected for subsequent crosses and each male progeny represents an independent excision event. The frequency of recombination is quite low with only about 0.5-1% of the progeny being recombinant. The next cross is to ensure that the transposase is eliminated to prevent further transposition. This is accomplished through a cross between the F1 mosaic males to virgin females of Adv/CyO. The F2 males will lack the stubble (sb) marker and have white eyes. Stubble is found on the third chromosome and is easily identified by its dominant phenotype, giving rise to shortened hairs on the back of the fly. The white-eye phenotype among the progeny indicates the loss or elimination of the transposase. Each F2 male is crossed separately, even if they are the progeny of the same P cross. These F2 progeny males are crossed again to Adv/CyO to balance the stock. Finally,

brothers and sisters of F3 generation, or Bsg*/CyO, are crossed to themselves to maintain the stock.

9.3 Nanos Gal4-VP16

Transgenic flies were created using pUAST-attB plasmid in which GFP-Bsg25D fusions were cloned. These plasmids were then injected into embryos with a white eye phenotypic background and flies that successfully integrated the transgenes were identified though the red eye colour conferred by the white mini-gene. These flies were subsequently crossed to ensure that the genes were balanced and stable prior to further genetic manipulation and analysis. Once we had a balanced stock carrying the UAS transgene, they were crossed to the GAL4 transcription factor which can induce the expression of the transgene of interest. The eggs laid by these flies, induced to express the GFP::Bsg Full, coding and 3'UTR respectively, were used to perform a high resolution FISH experiment to see whether localization of *Bsg25D* RNA was perturbed. The staining only revealed that the driver was expressed much too late to see early localization dynamics of *Bsg25D*.

9.4 Embryo Collection, Harvesting and Fixation

Plexiglass egg-laying cages with a fly screen on one end and an apple juice agar plate with yeast paste mounted onto the other end were set up. The yeast paste was made and left at room temperature prior to being used. The container or beaker with yeast paste was properly sealed to prevent contamination of other flies. Flies were added to the cage and allowed to adjust to the new environment a few days. The cages were precleared, and embryos staged at 0-4 hours were collected. This allowed for early developmental events in *Drosophila* embryogenesis to be studied. These embryos were subsequently harvested prior to fixation.

Harvesting embryos is described in detail by Lecuyer *et al.*, 2007. The embryos were gently rinsed to remove debris and the eggs were subsequently brushed off of the agar plates onto a thin porous mesh (basket), dechorionated with 50% bleach for 90 seconds and rinsed thoroughly with lukewarm water. These embryos were then fixed in a scintillation vial for 20 minutes with 7.5 ml of heptane, 2.5 ml PBS and 250ul of 37% formaldehyde that was freshly prepared, shaking at setting 1-2 on the vortex. The embryos were then aspirated into a glass Pasteur pipette and added to a biphasic eppendorf with 1:1 methanol:heptane. The embryos were vigorously shaken through the interphase to crack the vitelline membrane. The heptane was then aspirated off and methanol was added. The mixture was again shaken until the majority of embryos sunk to the bottom. The embryos were washed two more times with 1ml of fresh methanol and stored at -20 ºC in methanol.

9.5 Fluorescence in Situ Hybridization

Probe (template)

The probes were synthesized via PCR, amplified from overnight cultures of bacterial glycerol stocks as detailed by Lecuyer *et al.,* 2007. Using universal primers T7 and Sp6, the amplified *Bsg25D* products were subsequently agarose gel purified through Biobasic columns. The eluted product was then ethanol precipitated to clean and concentrate prior to resuspension in nuclease-free water.

Transcription

The *Bsg25D* PCR product was subsequently used as a template for *in vitro* transcription, using 2 ul DIG labelled UTPs, in the transcription mixture consisting of NTPS, 10X transcription buffer, RNA polymerase, RNAse out and Nuclease-free water in a total volume of 20 ul. The reaction was incubated for 4 hours at 37 ºC. The volume was subsequently brought up to 50 ul and the probes were precipitated with 3M sodium acetate

and 100% cold ethanol. The samples were then incubated overnight at -80 °C, spun down and the pellet was washed with 70% ethanol, and resuspended in RNAse-free water. Validation of size and concentration was done through gel electrophoresis. The probes were stored at -80°C for later use.

9.6 Western blotting and Immunofluorescence microscopy

Antibody production

Guinea pig antibodies were raised against the N-terminal domain of Bsg25D, expressed in *E. coli*. GST-N-term Bsg25D were isolated and purified over a Ni2+ column under denaturing conditions. The purified fragments were injected into Guinea pigs over 4-week intervals. Antibodies against the N-terminal domain of Bsg25D were affinity-purified.

Western Blot

For western blotting, S2 cells cultured for three days after transfection at 25°C were homogenized in sampling buffer and the protein extracts were loaded on a polyacrylamide gel for western blotting and probed with anti-Bsg25D and mouse anti-GFP.

9.7 Immunofluorescence microscopy

S2 cells

Cells were grown in 8-well slides and fixed with 4% PF for 10 minutes. For immunostaining of Bsg25D, the cells were fixed and washed three times in PBS and incubated at 4°C in blocking solution (BSA). All subsequent antibody incubations were performed in blocking solution.

Embryos

Freshly laid embryos from fly crosses of 2-3 days old were collected and fixed with methanol/heptane as described in Lecuyer *et al.*, 2007 and stained with antibodies diluted to 1/250 anti-Bsg25D B (Lecuyer et al., 2007).

9.8 DNA constructs/Expression vectors

1. pGEM-GFP constructs

The initial step of this cloning strategy was to create an *Eco*RI site upstream of the GFP cassette to allow for *Bsg25D* to be cloned downstream of GFP. This was done in order to excise and subclone the transgene into expression vectors pAC 5.1 (for expression in S2 cells) and attB-pUAST (for microinjection for *in vivo* expression in *Drosophila melanogaster)*.

Once the modified vector, pGEM-GFPV2 (version 2) was constructed, the inserts were prepared from bacterial glycerol stocks at -80 °C. *Bsg25D* was PCR amplified using Hot Start Polymerase (KOD from novogen) and specific primers that annealed to *Bsg25D.* This introduced a unique restriction site not present in the original sequence. The amplified products of *Bsg25D*'s full length gene (*Bsg25D* Full), *Bsg25D* Coding region (*Bsg25D* coding) and *Bsg25D* 3'UTR region (*Bsg25D* 3'UTR) were subsequently digested with restriction enzymes *Bgl*II and *Kpn*I and gel purified. Once the inserts were prepared, they were ligated into pGEM-GFPV2 downstream of GFP, using *Bam*HI and *Hind*III restriction sites, before being transformed into DH5α cells *(E. coli)* and plated onto LB agar plates with ampicillin (amp^R). The clones were screened to confirm successful uptake of constructs in the correct orientation using restriction enzymes and mapping. Positive clones were sequenced for mutations before being stored at -80°C as bacterial glycerol stocks.

2. pAC constructs

For construction of pAC 5.1 expression vectors, the positive clones harbouring the GFP cassette and region of *Bsg25D* of interest, were excised using *Eco*RI and subcloned into pAC 5.1 which was also cut with *Eco*RI. Once digested, the vector was dephosphorlyated with *Calf Intestinal Alkaline Phosphatase* (*CIAP from Invitrogen*) for 1 hour at 37°C. The CIAP was then heat inactivated at 65 °C for 15 minutes and products were finally agarose gel purified. The ligation was performed overnight at room temperature, plated on amp^R LB agar plates and subsequently screened for positive clones.

3. attB-pUAST constructs

Standard methods, similar to those discussed above to create pAC expression vectors, were used to generate transgenic lines using nos-Gal4VP16 to express pUASt-Bsg25D transgenes (*Bsg25D* coding, coding + 3'UTR and 3'UTR fused to GFP) in ovaries and embryos.

4. attB-pUASP construct

The signal from Bsg25D-GFP fusions was not strong in the embryos via FISH since the pUAST constructs express very poorly in the germ line. We decided to use pUASP-attB instead. The vector did not contain an *Eco*RI site necessary to subclone constructs discussed in #3 above. Therefore, the initial step was to introduce an *Eco*RI restriction site using a short oligonucleotide linker, formed by annealing sequences (similar to a transcription reaction) that are complementary to each other on one end. Specific restriction sites were included in this linker region including *Eco*RI to allow easy manipulation for future cloning. The linker was digested with *Kpn*I and *Xba*I, while the attB-pUASP vector was linearized with the same enzymes to create cohesive ends. They were ligated together, and the resulting plasmid contained a unique *Eco*RI site which will allow for the subcloning of GFP fusion constructs from attB-pUAST to attB-pUASP.

9.9 Cell Culture

Schneider S2 cells are *Drosophila* tissue culture cells that were generated from late stage *Drosophila melanogaster* embryos of Oregon R wildtype flies (Schneider, 1972).

The cells were split every 5-7 days into fresh Schneider's medium with 10% FBS/PS and serum (fetal bovine serum and penicillin-streptomycin). The cells were grown at 23-25°C in the absence of CO_2.

10 – Results

10.1 Bsg25D mRNA localization properties in early Drosophila embryogenesis

Our first objective was to define more clearly the localization features of *Bsg25D* mRNA during *Drosophila* embryogenesis. For this, we collected wildtype (WT) Oregon R (OreR) embryos between 0-4h of development to perform FISH. *Bsg25D* mRNA was found to localize to the pole plasm of the embryos at the posterior end (**Figure 10A**) in early stages of development. As the embryonic nuclei start dividing to form a syncytial embryo, a prominent localization pattern around each cortical nucleus was observed, more strongly at the pole plasm and diffusely around the syncytial nuclei. In the cellular blastoderm embryos (nuclear divisions 10-13), we observe a more perinuclear, diffuse pattern of *Bsg25D* mRNA localization around yolk nuclei as well as the peripheral blastoderm.

At higher resolution (**Figure 10B**), *Bsg25D* mRNA localizes to peri-nuclear clouds on both sides of the nuclei, which is suggestive of a centrosomal and microtubule-like pattern. During mitosis, the mRNA concentrates to the centrosomes and spindle, both during metaphase and anaphase (**Figure 10 B**). These results confirmed and extended the previously characterized embryonic localization features of *Bsg25D* mRNA.

When examining *Bsg25D* mRNA localization patterns in S2 cells by FISH, the transcript was found to form defined cytoplasmic foci. These foci are not present in the "no probe" negative control sample, nor when conducting FISH with a probe for 18S rRNA, which exhibits a broad cytoplasmic localization (**Figure 11**). These cytoplasmic foci are reminiscent of a centrosome-like pattern, although co-labeling with mitotic marker antibodies, such as gamma Tubulin (γ-tub), would also help distinguish whether the dots are indeed centrosomes.

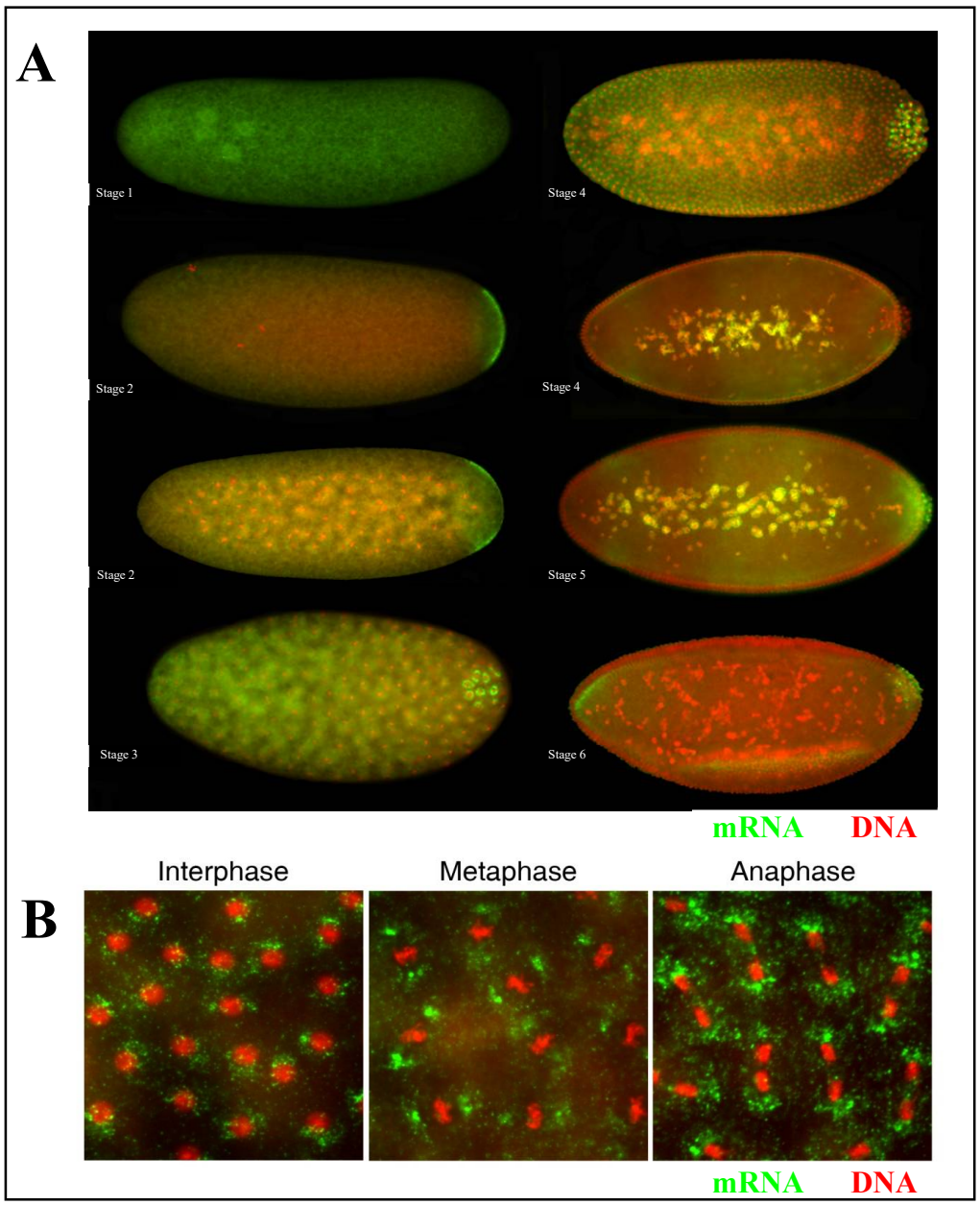


Figure 10: Diverse Localization of *Bsg25D* in *Drosophila* Embryos

(A) Dynamic and diverse *Bsg25D* localization in developing *Drosophila* embryos shown through Fluorescence *In Situ* Hybridization (FISH). *Bsg25D* mRNA is stained with Cy3 tyramide, false coloured in green. The nuclear DAPI stain is depicted in red. *Bsg25D* is found at the pole plasm at the posterior end of the embryo as well as perinuclear staining. The embryos are oriented anterior to posterior (right to left). **(B)** FISH experiments using a *Bsg25D* probe on WT *Drosophila* embryos show that *Bsg25D* is localized to centrosomes and astral microtubules during the different steps of mitosis (Interphase, Metaphase and Anaphase).

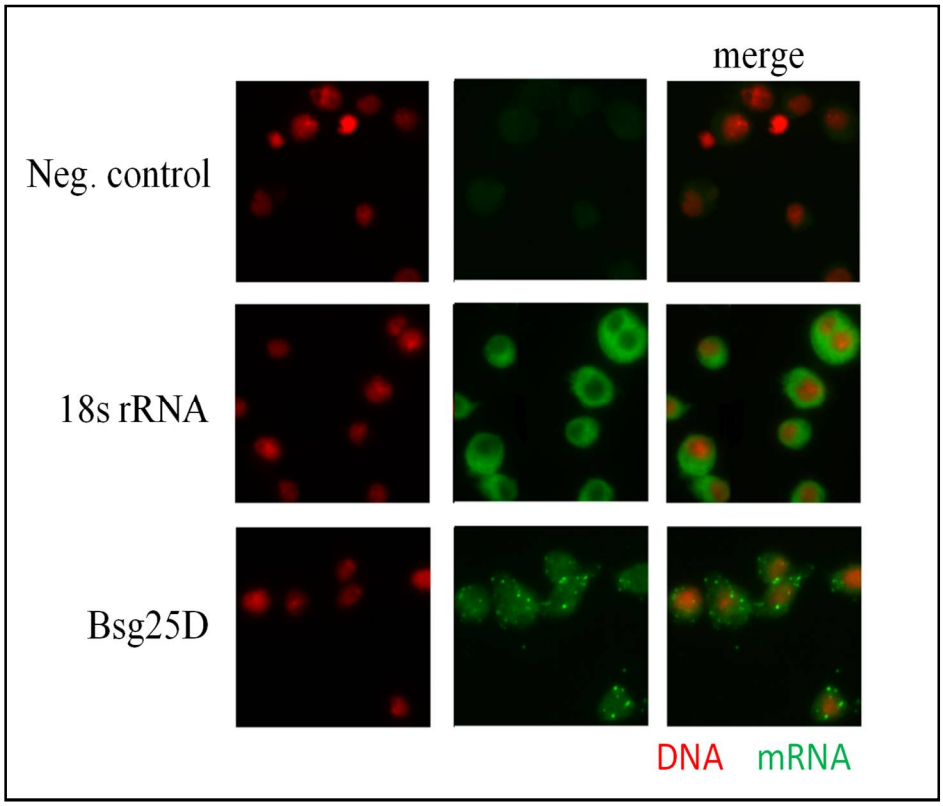


Figure 11: *Bsg25D* Localization in S2 cells

Drosophila S2 R+ cells were seeded into an 8-well chamber slide and incubated for 3 days at 25° C prior to fixation and FISH analyses. The control well with no probe shows low level of background; 18S ribosomal RNA (rRNA) shows diffuse cytoplasmic stain, while *Bsg25D* mRNA appears as distinct foci. The RNA in each sample is stained with Cy3 tyramide, false colored Green while the DAPI stains the nucleus, shown here in Red.

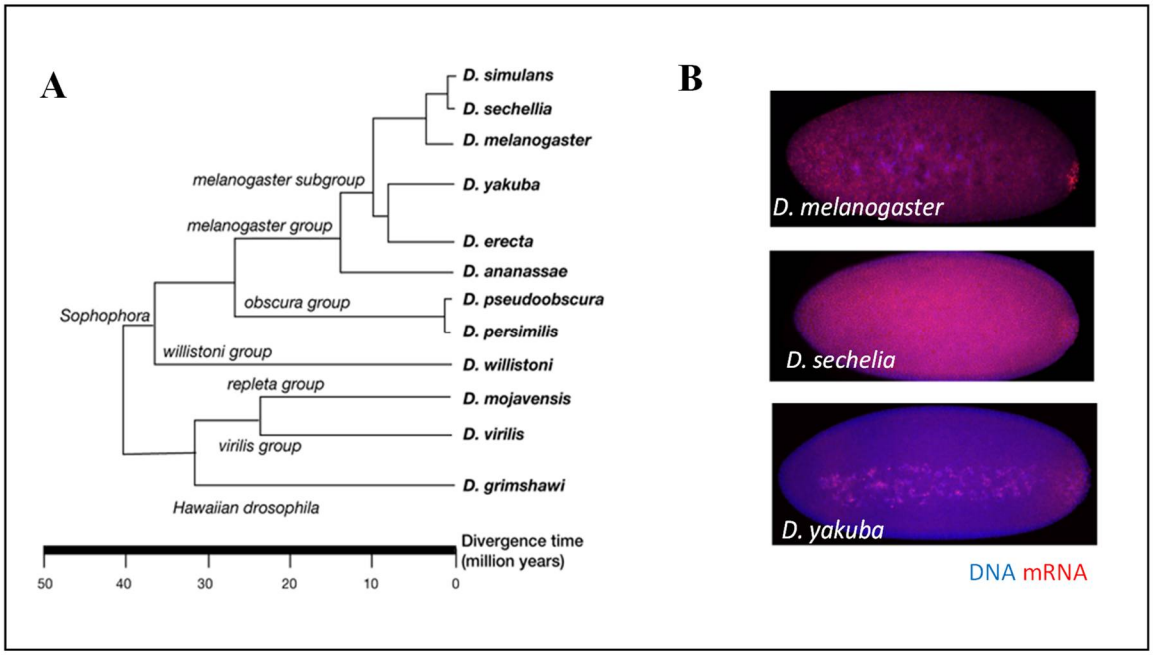


Figure 12: Evolutionary conservation of *Bsg25D* within different *Drosophila* species

(A) *Drosophila* phylogenic tree depicting evolutionary relationships and estimated divergence time among species in the *Drosophila* genes (diagram from Gilbert, DG 2005). Horizontal lines represent branch lengths, within and between groups.

(B) FISH experiments using a probe targeting a conserved area reveal a conserved pattern of *Bsg25D* mRNA localization to the posterior cytoplasm of *D. sechelia* and *D. yakuba* embryos.

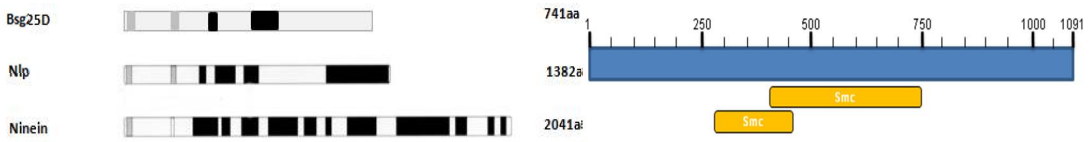
A cross-species comparison revealed that Bsg25D protein sequence is evolutionarily conserved within the *Drosophila* genus (**Figure 12 A-B**). Sequence conservation may indicate that Bsg25D is functionally conserved as well. To further investigate the level of conservation at the functional level, we designed RNA probes specific to a highly conserved stretch within the coding region of the gene. This would ensure that a single probe could be used to study differences in localization patterns between the different species, namely: *D. melanogaster, D. sechelia,* and *D. yakuba.* The conservation of *Bsg25D* at the level of the DNA sequence as well as RNA localization could help identify functional elements within the gene (Dermitzakis et al., 2002; DeSilva et al., 2002).

To evaluate the localization features of *Bsg25D* mRNA orthologs in different *Drosophila* species, we next performed comparative FISH analyses on *D. melanogaster*, *D. sechelia* and *D. yakuba* embryos (**Figure 12 B**), which revealed that the subcellular transcript localization of *Bsg25D* pattern is generally similar between species, in particular with regards to enrichment within posterior pole plasm.

A **NCBI Blast Protein Sequence Alignments for *Drosophila* Bsg25D:**

Sequences producing significant alignments:	Score (Bits)	E Value
ref|NP 7230721.1| Blastoderm-specific gene 25D, isoform B [Droso...	2210	0.0
gb|AAI44914.1| Ninein [Mus musculus]	63.9	1e-07
gb|AAF23015.2| Ninein [Homo sapiens]	62.4	3e-07
ref|NP 001086424.1| Ninein-like protein [Xenopus laevis]	58.2	6e-06
Gb|AAI54339.1| Nin protein [Danio rerio]	55.1	5e-05

B



C

Predicted Interacting Proteins of Bsg25D	
Known	Predicted
Rab6	aur
gnu	Mcm10
Tektin-A	caz
betaTub56D	Pka-C1
rogdi	Mad1
Mlh1	Dyn-p25
Beat-IIIa	polo
CG7757	chm
Msb1l	CG7172
crc	CG7164
CG3272	Jra

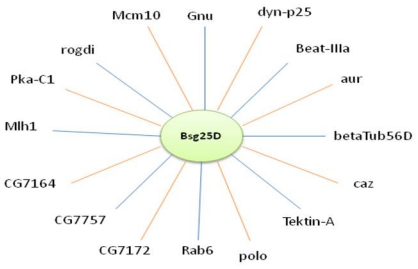


Figure 13: Bsg25D an ortholog of Ninein/Ninein-like proteins

(A) Protein sequence alignment using Blastp suggests that Bsg25D is an ortholog of Ninein and Ninein-like proteins. **(B)** Schematic representation of approximate sequence comparison of Bsg25D to hNLP and hninein (left) (adapted from Casenghi, M. 2003) where the dark bars indicate predicted coiled-coil domains and the light bars indicate potential EF-hand Ca $^{2+}$ binding domains. Bsg25D contains two Structural Maintenance of Chromosome (SMC) domains (right) shown through an output from the Conserved Domains Database (CDD) tool. These domains are found in many regulators of chromatin dynamics and mitosis. **(C)** List of predicted interactors of Bsg25D (Orange) and some known interactors (Blue) using the DroID database.

10.2 Bsg25D is an ortholog of human Ninein and Ninein-like protein

To gain insights into the potential function of Bsg25D, we next performed a BLAST search which revealed that *Bsg25D* is an ortholog of human Ninein and Nlp proteins (**Figure 13 A**). The E-value for *Bsg25D* for Ninein in *Mus musculus* was 1e-07, in *Homo sapiens* 3e-07 while E-value for *Bsg25D* for Nlp was 6e-06 in *Xenopus laevis* and 5e-05 in *Danio rerio*. BLAST searches for regions of similarity within the sequence - the smaller the E-value, the better the match. Casenghi *et al.* performed a comparative sequence analysis of Ninein and Nlp, showing that while the C-terminal domains did not share much sequence similarity, the N-terminal domains were 37% similar (Casenghi et al., 2003). *Bsg25D* also shows sequence similarity to the N-terminal domain of Ninein and Nlp (**Figure 13 B**). The conserved domains database suggests that Bsg25D has two structural maintenance of chromosome (SMC) domains (**Figure 13 B**). These SMCs are proteins that are important for chromosomes to be properly segregated and transferred during replication. They also share putative coiled-coil domains and EF-hand calcium binding domains.

A protein interaction database designed specifically for *Drosophila*, called *Drosophila* Interactions Database (DroID), was used to find potential interactors of *Bsg25D* (**Figure 13 C**). Understanding protein partners and interactors provides a wealth of information on potential cellular functions and physiological processes *Bsg25D* could be involved in. This is especially important because many biological functions require the assembly of protein complexes. Interestingly, Bsg25D interacts with several proteins that are either involved in cell division or components of the cell division apparatus such as microtubules. Many of the listed interacting proteins play key roles in cell division and other important cellular processes. Rab6, for example, is a GTPase important for transport pathways that are microtubule-dependent. Rab6 recruits dynactin protein complex important in diverse processes within the cell that involve motility (Short et al., 2002). Gnu encodes a small protein that is part of a larger protein kinase complex and is required for the transition from the completion of meiosis and entry into mitosis (Freeman and Glover, 1987; Freeman

et al., 1986; Shamanski and Orr-Weaver, 1991). Females with mutations in gnu produce eggs in which DNA replication occurs continuously with no cell division. As a result, "giant nuclei" are formed where the nuclei become very large and polyploid. Tektins are cytoskeletal proteins, a component of microtubules, found in cilia and flagella. Mcm or mini-chromosome maintenance proteins is involved in replication. Aurora-A, one of the predicted interactors, is a member of mitotic serine/threonine-protein kinases important in mitosis and meiosis. It is involved in actin-dependent asymmetric playing a vital role in cell proliferation (Berdnik and Knoblich, 2002). Aurora-A kinase regulates actin and microtubule dependent processes during mitosis. Taken together, these interactions suggest that Bsg25D may also play a role in cell division.

10.3 Bsg25D Protein Localization

To build up additional tools for the study of Bsg25D, we next raised our own Bsg25D specific antibodies in guinea pigs and sought to verify their specificity through Western blotting. Three different animals were injected with a recombinantly purified protein fragment corresponding to the N-terminal half of Bsg25D protein sequence. These antibodies were purified from the fifth bleed sera, just prior to sacrificing the animals.

To test the specificity of these antisera, we next performed Western blotting analyses with protein extracts generated from parental *Drosophila* S2 cells, or from cells treated with a double-stranded RNA (dsRNA) complementary to *Bsg25D* mRNA for RNA interface (RNAi) mediated depletion of Bsg25D.

Western blotting analyses of these samples revealed that these Bsg25D antisera indeed specifically recognize Bsg25D protein isoforms in S2 cell extracts (**Figure 14A**). Lanes 1-2 represent the preimmune samples, lanes 3-4 represent Antisera A, lanes 5-6 represent Antisera B and lanes 7-8 represent Antisera C, respectively. Comparative analyses

were conducted for control (lanes 1, 3, 5 and 7) and Bsg25D RNAi (lanes 2, 4, 6 and 8) cellular extracts.

The results obtained with each antiserum revealed a consistent detection of 2 Bsg25D isoforms at ~150 kDa and ~100 kDa, which are indicated by the arrowheads to the left of the film. In all 3 RNAi treated samples, these bands are lost, suggesting that these bands indeed correspond to Bsg25D isoforms, while an additional prominent band migrating ~70 kDa is not affected by the RNAi treatment and thus appears to represent a cross-reactive protein. With antisera C, there is an additional isoform that is shorter migrating at ~50 kDa (lanes 7, 8) which also appears to be a cross-reactive protein. Since antisera B appeared to be the cleanest of the samples, we selected this antisera to study Bsg25D protein localization using IF in fly embryos and S2 cells.

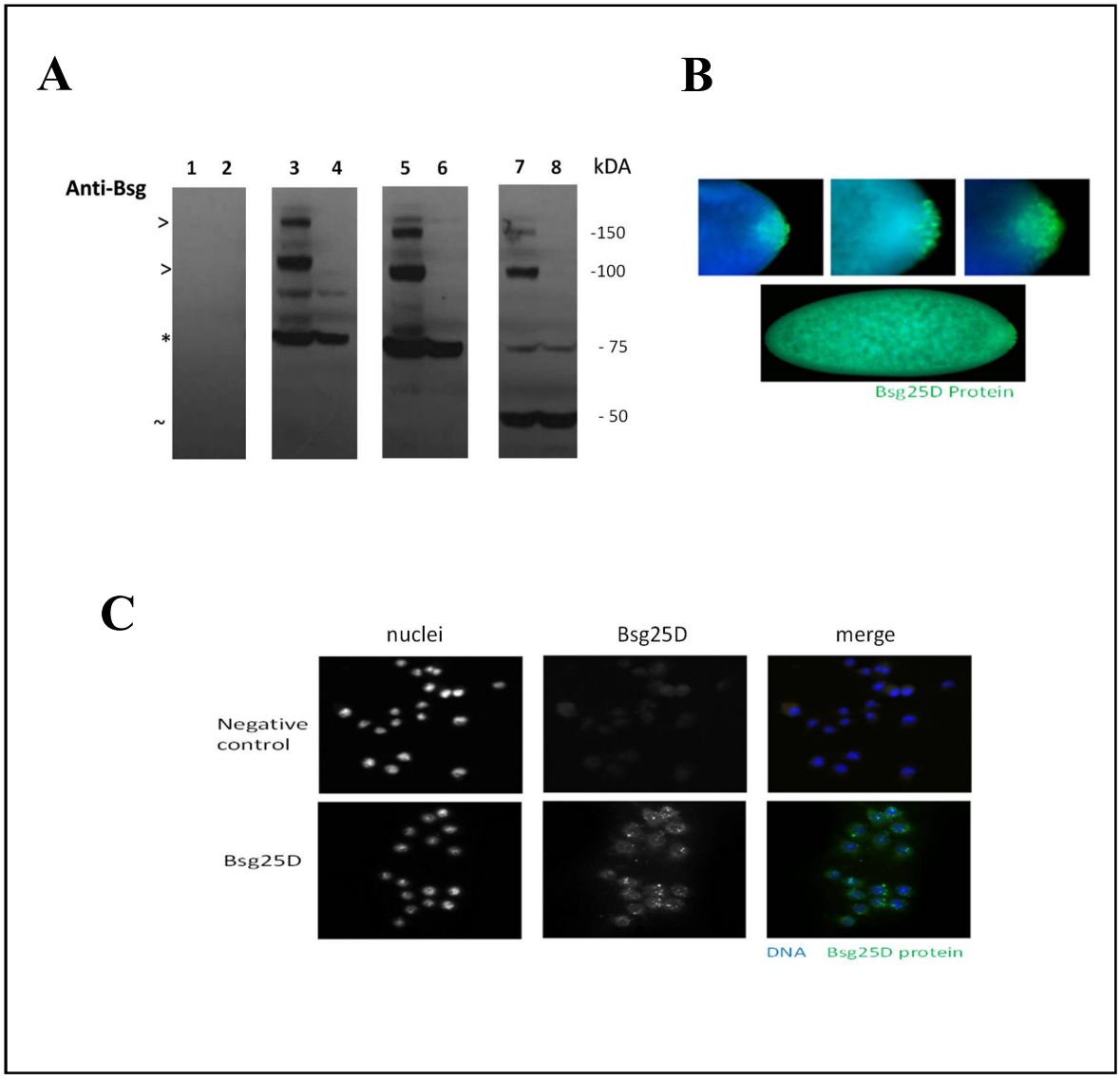

A
B
C
Anti-Bsg
1 2 3 4 5 6 7 8
kDA
-150
-100
- 75
- 50
Bsg25D Protein
nuclei
Bsg25D
merge
Negative control
Bsg25D
DNA
Bsg25D protein

Figure 14: Characterization of Bsg25D antibodies

Western Blot **(A)** testing three Bsg25D antisera on protein extracts from parental S2 cells, or cells treated with dsRNA for Bsg25D to assess antibody specificity of endogenous Bsg25D protein. There are 8 lanes in total: preimmune samples from guinea pig A (1, 2), antisera A (3, 4), antisera B (5, 6) and antisera C (7, 8), respectively. Lanes 1, 3, 5 and 7 are control extracts, while lanes 2, 4, 6 and 8 are extracts from cells treated with Bsg25D double stranded RNA. The arrowheads demark Bsg25D isoforms expected to be generated through alternative splicing. Additional cross-reactive bands observed on these gels are indicated by (~). The loading control is α-Tubulin. **(B)** Immunofluorescence analyses of Drosophila embryos using Bsg25D antisera B. The Green represents Bsg25D protein localization while Blue represents DNA (DAPI). **(C)** Bsg25D localization in S2 cells. The same homemade antibodies were tested on S2 *Drosophila* cells. The first row shows the negative control with no antibody to test for a background signal. The second row shows Bsg25D protein localization (Green) in speckles (foci) around the DNA in the nucleus (Blue).

Embryos from WT flies, collected between 0-4 hours, were harvested and fixed in methanol prior to performing an IF with the Bsg25D antiserum B. This immunostaining revealed an enrichment of Bsg25D reactivity in the posterior end of the embryo and pole cells, consistent with the *Bsg25D* mRNA localization pattern (**Figure 14 B**). In follow up studies, this antibody has also been found to reveal Bsg25D protein localization to structures of the mitotic apparatus (Iampietro et al., 2014; Kowanda et al., 2016).

IF was also conducted on S2 cells, in which Bsg25D protein was found to localize to cytoplasmic foci (**Figure 14 C**). This localization is reminiscent to that of Nlp-myc epitope-tagged proteins, which produce intracellular assemblies around centrioles (Casenghi et al., 2003). However, co-labeling of centriolar markers would be required to confirm whether this is indeed the case for Bsg25D in S2 cells.

10.4 Mutagenesis of Bsg25D through P-element Excision

We next sought to characterize a *Bsg25D* loss of function model for *in vivo* studies in *Drosophila*. After testing the publicly available mutant stock $Bsg25D^{G13518}$ containing a P-element insertion in the 5'UTR, we found that the localization pattern of *Bsg25D* mRNA was similar to OreR wildtype (WT) flies revealed by FISH experiments. If the P-element interfered with transcriptional regulation of *Bsg25D*, subsequent crosses to deficiency lines were expected to result in a change in expression levels when observed through FISH mRNA localization patterns. This however was not the case when embryos of $Bsg25D^{G13518}$/Df(2l)exel16011 were hybridized with a *Bsg25D* probe to study localization pattern or defects (data not shown). In order to generate a mutant stock bearing a deletion of the *Bsg25D* coding sequence, we sought to induce remobilization of the P-element found within the $Bsg25D^{G13518}$ stock by crossing these flies to the Delta 2-3 stock, which carries a P-element insertion with a variant transposase that can potently induce the remobilization of other P-elements (Robertson et al., 1988). Using this strategy, we would thus aim to induce

the imprecise excision of the Bsg25D^{G13518} P-element in order to cause a truncation within the *Bsg25D* gene body. To achieve this goal, we followed the crossing scheme outlined in **Figure 15**, in which the Delta 2-3 P-element was combined with Bsg25D^{G13518} for one generation to create 'mosaic' progeny, of which the males were selected for a second cross to eliminate the Delta 2-3 element from the final stock. Our cross yielded 33 of such mosaic male flies (**Figure 15**). The second cross with mosaic males and subsequent crosses carried out to balance the mutation created through P-element excision and generate stable lines. The final outcome of these crosses was the establishment of 11 balanced excision lines in which the Bsg25D^{G13518} P-element was remobilized and which may harbor Bsg25D genome deletions.

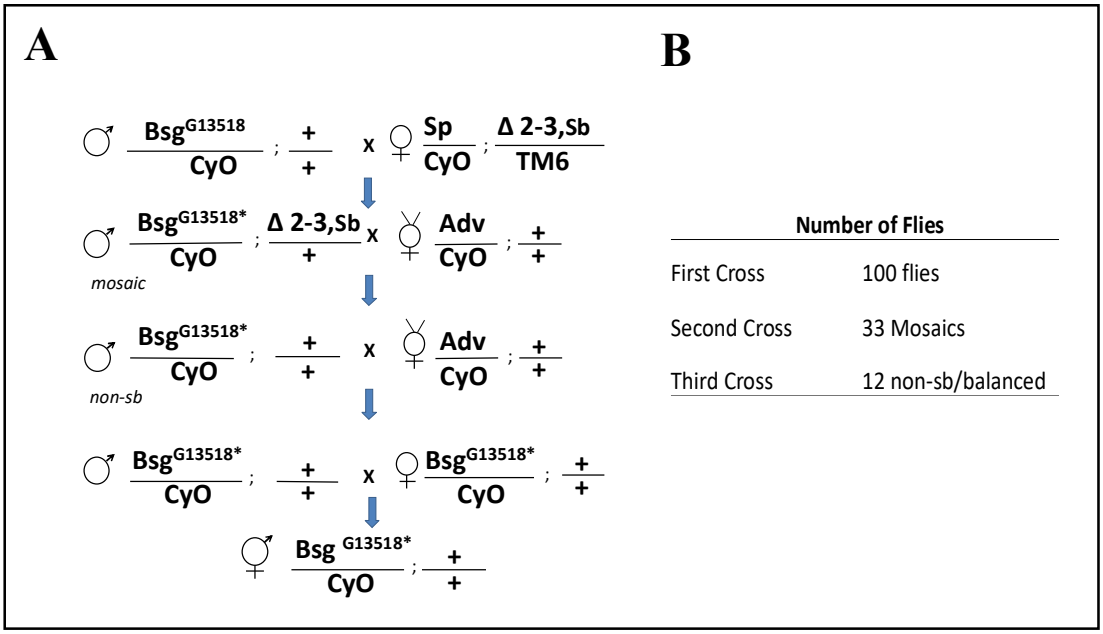


Figure 15: Fly Cross for generating *Bsg25D* loss-of-function mutants

Transposon excision strategy crossing flies expressing Delta 2-3 transposase with white eyes to the initial fly stock of Bsg25D^{G13518}/CyO with red eyes, containing P-element within the 5'UTR. The initial cross was started with 100 virgin female flies in a bottle incubated for a week at 25 degree Celsius. The progeny (F1) was screened for mosaic eyes, white eyes with swirls of pink/red indicative of an active integrase. There were a total of 33 flies that demonstrated this phenotype. Subsequent crosses were performed to first remove the active transposase to eliminate potential reintegration of the P-element. Once removed, the stock needed to be balanced to ensure the stability of the new mutation (Bsg25D*/CyO) so that it is not lost through recombination. The final step is to cross flies that are balanced to each other (brothers and sisters) to establish a balanced mutant stock. We were able to obtain 12 non-stubble/balanced Bsg25D*/CyO potential mutants for screening.

10.5 Bsg25D Transgenic Flies via microinjection of pUAST-attB GFP-Bsg constructs

RNA trafficking is dictated by *Cis*-Regulatory Motifs (CRMs) that can reside throughout the mRNA molecule, but are often located in the 3'UTR (Tekotte and Davis, 2002). In order to identify the region that is necessary for *Bsg25D* mRNA targeting, we initiated a structure-function analysis using the GAL4-UAS system, which allows us to inducibly express transgenes in a tissue specific fashion in *Drosophila*. We generated transgenic flies encoding chimeric RNAs in which the GFP sequence is fused in 5' to different segments of the Bsg25D sequence [Coding + 3'UTR (Full length), Coding , 3'UTR] (**Figure 16A**). These GFP fusion cassettes were cloned into the pUAST-attB transgenesis vector, which allows targeted insertion of transgenes into a precise genome attP target site. We performed microinjections of the fusion constructs of GFP-Bsg25D full, GFP-coding and GFP-3'UTR, respectively into the attP site located on chromosome 3 at position 65B2 (i.e. Bloomington stock #24871). The experiment was performed twice, as the first round of injections were not successful leading to 90% lethality. The second round of injections were much better, giving rise to a 34% viability rate.

To visualize the transgenic transcripts, we performed a FISH experiment using a *gfp* antisense RNA probe, which allowed us to discriminate between the endogenous *Bsg25D* mRNA and the *gfp-Bsg25D* chimeric mRNAs (**Figure 16B**). We expected to see that the observed localization pattern of *Bsg25D* was a result of a localization element found in the 3'UTR of Bsg25D, like in the case of bicoid (Ferrandon et al., 1997). The FISH experiment on embryos of the transgenic lines, however, was not able to confirm this hypothesis. The expression of the different transgenes appears to be localized to the pole plasm, like in wild type conditions. We see no visible difference in the localization pattern of the different transgenes.

Once we completed our FISH analysis on our transgenic embryos, we learned that the early embryonic patterns we wished to observe were not captured using the pUAST-attB

vector because the UAST promoter does not allow efficient expression in the germline. Instead, when crossed to the NGV Gal4 driver, the pUAST backbone drives zygotic gene expression that is more prominent in the posterior end of the cellular blastoderm embryo, which is where we observe the signal for all of our fusion mRNAs. pUASP transgenic flies were subsequently generated and used to demonstrate that *Bsg25D* mRNA localization elements are found both coding region and 3'UTR of this transcript (Kowanda et al., 2016).

Finally, the different GFP-Bsg25D fusion cassettes were cloned into the pAC5.1 expression vector, which I subsequently used to perform transient transfection studies in S2 cells to study how the encoded GFP protein variants are localized by performing IFs (**Figure 16 C**). The overexpressed proteins were detected with an anti-GFP antibody (Red) and the DNA was stained with DAPI (Blue). The first panel, the negative control, shows low level of background while the second panel represents the GFP control, where the empty vector harbours only the GFP sequence. GFP protein localizes strongly throughout the cytoplasm of the cells. GFP-Bsg25D fusion proteins expressed in S2 cells, however, appear to form distinct foci in all three fusions. mRNA localization pattern showed similar subcellular localization patterns to the protein. The only notable differences between the three fusion constructs was that cells transfected with GFP-Bsg25D 3'UTR and GFP-BSG25D Full show many more foci of GFP protein than the GFP-Bsg25D coding.

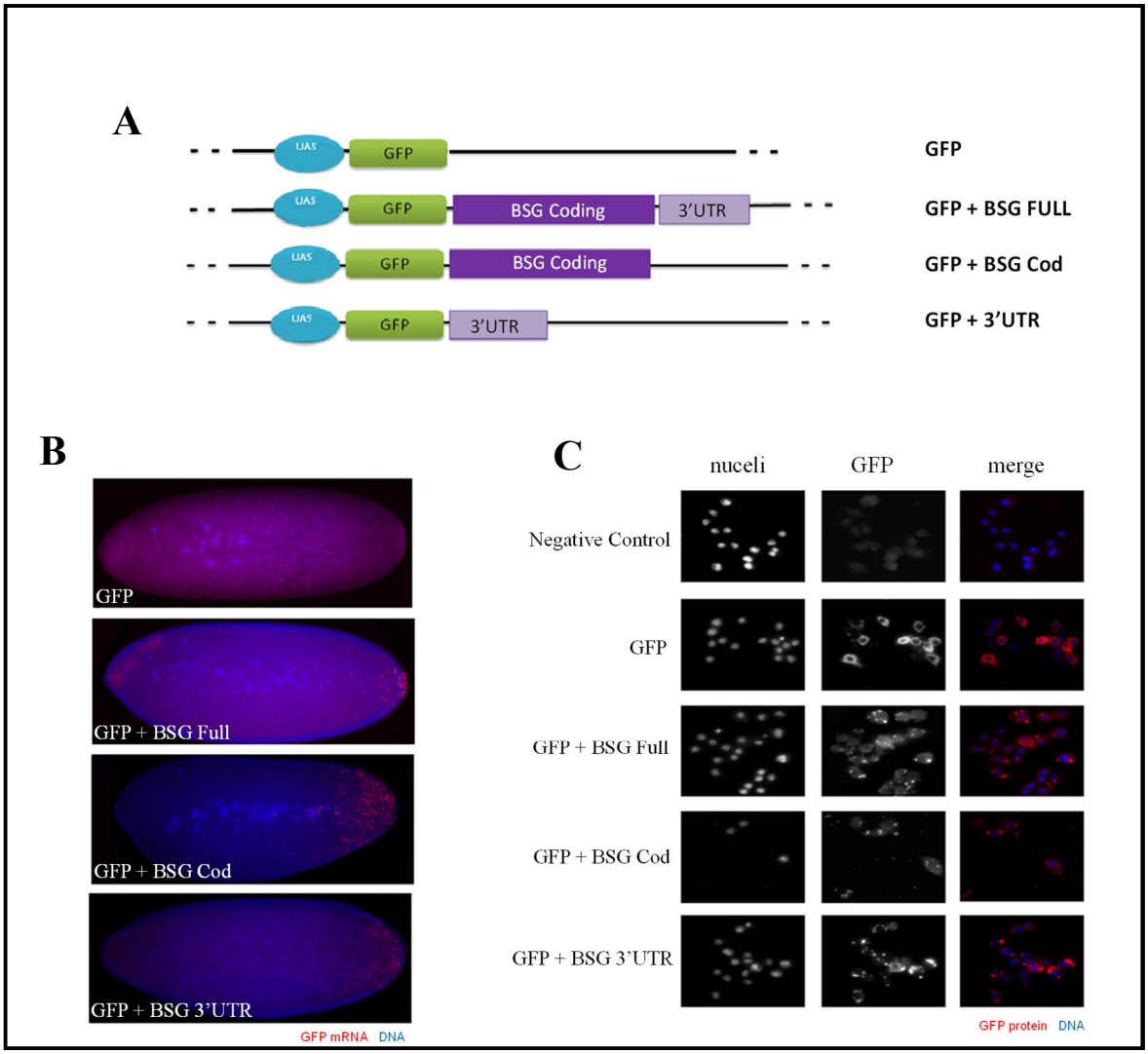

A
UAS
GFP
GFP
BSG Coding
3'UTR
GFP
GFP + BSG FULL
GFP + BSG Cod
GFP + 3'UTR
B
GFP
GFP + BSG Full
GFP + BSG Cod
GFP + BSG 3'UTR
GFP mRNA DNA
C
nuceli
GFP
merge
Negative Control
GFP
GFP + BSG Full
GFP + BSG Cod
GFP + BSG 3'UTR
GFP protein DNA

Figure 16: Bsg25D targeting is mediated by both coding and 3'UTR regions

(**A**) General strategy to map *Cis*-Regulatory Motifs. Constructs containing Bsg coding + 3'UTR (full), Bsg coding or just Bsg 3'UTR were fused to GFP and subcloned into either pUAST-attB vectors for targeted transgenesis or into pAC 5.1 expression vector for *in vitro* transient expression in S2 cells. (**B**) Identifying *cis*-regulatory motifs (CRMs) required for *Bsg25D* mRNA targeting subcellular localization using GFP-fusions in transgenic embryos. GFP mRNA (Red) localizes to the pole plasm in all three. (**C**) GFP protein localizes to the cytoplasm of the cells while each of the fusions, GFP-Bsg25D full length, coding and 3'UTR respectively, form small dots within the cytoplasm at opposing ends of the cells. The negative control is the non-transfected cells with GFP antibody, where the Red represents GFP protein (GFP antibody) and the Blue represents DNA (DAPI).

11- Discussion

11.1 Bsg25D mRNA localization is dynamic and diverse

Bsg25D mRNA showed three main types of localization during the early stages of development - pole plasm localization, followed by spindle pole localization during the formation of the syncytial blastoderm and finally, perinuclear localization after cellularization. Subcellular localization of *Bsg25D* mRNA is strong and apparent throughout all 5 stages of mitosis, starting with a hazy appearance around the nucleus, followed by a more distinct appearance as two points at opposing ends of the nucleus, localizing to the presumptive spindle poles. Analysis of tissue culture cells (S2 cells) also showed *Bsg25D* mRNA localization in the form of foci within the cytoplasm of the cells.

Interestingly, the GFP protein encoded by *GFP-Bsg3'UTR* mRNA exhibited a similar cytoplasmic speckle localization pattern to wild-type Bsg25D protein (**Figure 14**). This suggests that the Bsg3'UTR sequence itself is capable of imparting a distinctive Bsg25D-like localization pattern to GFP protein, which is normally diffusely localized throughout out the cell, in a manner that is independent of any protein domain of Bsg25D.

11.2 Bsg25D is an ortholog of human Ninein and Ninein-like protein

A protein BLAST search result indicates that Bsg25D is an ortholog of human Ninein and *Xenopus laevis* Ninein-like protein with E values of 3e-07 and 6e-06, respectively. Both proteins have been found to be important and play significant roles in microtubule stability, nucleation and anchoring at the centrosome in mammalian cells. Further analysis of interacting partners of Bsg25D revealed that Bsg25D is likely important in the regulation of cell division. A similar phenotype was reported by Casenghi *et al.* for Ninein or Nlp localization suggesting a potential link in overall function that needed to be further studied (Casenghi et al., 2003).

We hypothesized that Bsg25D may be part of a larger family of proteins and since the sequence is conserved within the different species of *Drosophila*, its potential evolutionary implications of resisting selective pressures might be very important.

Ninein is involved in the positioning and anchoring of microtubules thereby playing an important role in centrosomal function. Nlp is involved in centrosome function and is found amplified in 80% of lung and breast carcinomas (Casenghi et al., 2003; Jin et al., 2009; Shao et al., 2010). Since Bsg25D has sequence similarity to both Ninein and Nlp proteins this may indicate that it is involved in a similar processes or biological function. Consequently, Bsg25D was chosen for further analysis using several bioinformatic tools as well as biochemical techniques.

11.3 Characterization of mutants generated through P-element excision

We expected to see a difference in localization patterns and potentially mRNA levels in the mutant stock compared to wild-type. However, the publicly available P-element stock of Bsg25D (Bsg25D^{G135118}/CyO) showed no difference in localization of *Bsg25D* mRNA through FISH analysis, even when the stock was crossed to a deficiency line to eliminate the balancer chromosome.

The solution was therefore to create new mutants of the *Bsg25D* gene through the excision of the existing P-element (**Figure 15**). The aim of this approach was to generate at least one mutant fly line created through the imprecise excision of the P-element. The excision mutant would allow the characterization of the Bsg25D mutant phenotype. This resulting stock would serve as a starting point to perform rescue experiments with transgenes encoding wildtype or localization-defective versions of the mRNA in order to more clearly demonstrate the functional importance of mRNA localization for Bsg25D function. The process was labour intensive with several different rounds of crosses to first generate the

excision mutants by introducing an active transposase capable of excising the P-element, followed by several crosses to remove the transposase to prevent further transposition and to finally stabilize the new stock. Two important steps were taken to ensure that the mutation was not lost: first, only males of the desired genotype were selected and utilized for the crosses since recombination does not occur in males and second, several rounds of backcrossing between brothers and sisters were performed to stabilize the mutant line since putting them over a balancer chromosome (CyO) anchors the mutation with a dominant phenotype that is easily distinguishable, in this case wings that are bent upwards.

The excision strategy yielded 12 potential mutant lines that required further analysis and characterization. The eye-colour phenotype is used as an indicator of an excision event since the P-element contains a mini-white gene that confers a red eye colour. In a white eye colour background, the extent of excision also needed to be determined since the use of a transposase can give rise to precise and imprecise excisions. While looking for physical changes, the progeny showed no obvious phenotypic changes (wings, bristles, eye-shape, appendages etc.). In order to understand the extent to which the excision events occurred, further analysis of the 12 fly lines would have be necessary.

The goal of the crosses was to express a transposase that would re-mobilize the P-element from the stock we used, which we expected would take a portion of Bsg25D with it, thus generating a mutant. Generating a Bsg25D mutant stock was a first step to defining its function. We then wanted to express the GFP-fusion transgenes that were generated for rescue experiments. By rescuing with a version of the mRNA that doesn't localize, we wanted to more directly test whether mRNA localization was required for its function. Due to time constraints, we did not have the opportunity to entirely complete this goal.

11.4 Future Perspectives

The scope of this thesis was to understand the functional relevance of the localization pattern of *Bsg25D* and its role in cell division. Though I was unable to demonstrate everything I intended within the allotted time, I was able to set the project in the right direction by doing all the legwork necessary and troubleshoot some of our strategies that were not ideal. The project was taken over in collaboration with Paul Lasko's lab, through which Kowanda *et al.* (2016) confirmed that *Bsg25D* accumulates in the oocyte of stage 2-7 egg chambers, mainly in the anterior and posterior poles of the oocyte from stage 10 onward (Kowanda et al., 2016). They showed the encoded protein expression profile was the same as mRNA expression up to and including stage 10 (Kowanda et al., 2016). The coding region and its 3'UTR are involved in the localization of *Bsg25D* and the mislocalization of *Bsg25D* affects both microtubule polarity and gurken deployment in the developing oocyte (Kowanda et al., 2016). *Bsg25D* was found to bind microtubules and with dynein, it can move along microtubules toward their minus-ends *in vivo* (Kowanda et al., 2016). *Bsg25D* was confirmed to function *in vivo* to ensure accurate chromosome segregation during early embryonic nuclear divisions (Kowanda et al., 2016).

Around the same time, another lab published interesting findings corroborating most of the findings. In 2016, Zheng *et al.* demonstrated that Mammalian Nin has a centriole-targeting domain that is not conserved in *Drosophila* or in the mammalian paralog (Zheng et al., 2016). *Bsg25D* localizes to the daughter centrosome in neural and germline stem cells. However, it is not required for asymmetric division (Zheng et al., 2016). *Bsg25D* was also shown to localize to noncentrosomal MTOC in wing epithelia and muscle (Zheng et al., 2016). The loss of *Bsg25D* expression did not impact development, fertility, or locomotion thereby indicating that *Bsg25D* has a "supportive" role in centrosomal and microtubule organization and asymmetric stem cell division (Zheng et al., 2016).

If *Bsg25D* only plays a supportive role, it begs the question of which other mRNAs are working together with *Bsg25D* to ensure faithful division of cells and genomic stability. Very recently, Rosen *et al.* discovered that *Bsg25D* physically interacts and colocalizes with Ensconsin (Ens), a MT-associated protein (Rosen et al., 2019). *Bsg25D* loss in an Ens sensitized background creates positional defects of myonuclei in skeletal muscles, which are normally found to be evenly spaced within the cell (Rosen et al., 2019). Interestingly, overexpression of *Bsg25D* causes formation of ectopic MT organizing centers, disrupts perinuclear MT arrays, reduces muscle stiffness, and decreases larval crawling velocity (Rosen et al., 2019). They suggest *Bsg25D* positively regulates Ens, but in abundance, *Bsg25D* disrupts Ens localization which leads to deleterious outcomes in muscle function (Rosen et al., 2019).

12 – Conclusion

The association of ribosomes and microtubules has long been known and this led to the idea that localized translation of mRNAs along the mitotic apparatus may play a key role in the regulation of mitosis. However, the mechanism underlying the recruitment of these mRNAs to the mitotic apparatus remains poorly understood. Here, we have described an mRNA that has a diverse and dynamic localization pattern during *Drosophila* embryogenesis called *Bsg25D*. Its localization pattern suggests that it functions at the spindle pole and therefore is possibly involved in regulating mitosis. Bsg25D protein shares significant sequence similarity to Ninein and Nlp, proteins that have been implicated in the anchoring of MT minus-ends and involved in centrosomal MT nucleation, respectively. Furthermore, we have also demonstrated that *Bsg25D* is conserved across six other species within the *Drosophila* family both at the level of the mRNA sequence as well as the corresponding localization pattern. This suggests that *Bsg25D* has been evolutionarily conserved, possibly due to a crucial functional role. Finally, the phenotypic consequence of mutating *Bsg25D* was not determined since we were unable to generate *Bsg25D* loss-of-function mutants using the transposon excision strategy.

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