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The *Drosophila* GW protein, a posttranscriptional gene regulator that influences progression through mitosis

by

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Abstract

Regulation of mRNA translation and stability can occur in cytoplasmic compartments known as mRNA processing bodies or P bodies. These compartments contain factors that function in multiple mRNA regulatory pathways and are thought to be centres for coordinating the action of these pathways. One class of proteins that resides in P bodies belongs to the conserved family of GW proteins. Members from this family have been identified only in metazoan genomes and include the prototype human GW182 protein and two additional human paralogues and *Caenorhabditis elegans* Ain-1 and Ain-2 proteins. In this study, the single *Drosophila melanogaster* gene encoding a GW protein was characterized. The similarity in structure and function of this gene that were observed in this study, with human orthologues suggest that *Drosophila* is an appropriate experimentally tractable organism for further advancements in understanding the functions of the human orthologues. This study also contributed evidence supporting the involvement of *Drosophila* GW in the RNA interference pathway through a physical association with Argonaute 2, an important effector in this pathway.

A *Drosophila* strain carrying a mutation in the *gw* gene showed multiple mitotic defects in homozygous mutant embryos. The mutant strain was named *gawky* because of the uncoordinated chromatin movements that were observed in live mutant embryos undergoing mitosis. This observation suggests that *Drosophila* GW may control the stability and/or translation of mRNAs encoding cell cycle regulators.

The endoribonuclease RNase MRP was chosen as a potential mRNA regulator that may be affected in the gw^1 mutant strain. In *Saccharomyces cerevisiae,* RNase MRP degrades the mRNA of the major mitotic cyclin Clb2 and localizes to a P body-like structure. Human RNase MRP also influences the levels of cyclin B mRNA. MRP RNA,

the non-coding RNA component of the RNase MRP enzyme has not been previously studied in *Drosophila*. In this study, expression of *Drosophila* MRP RNA was verified. MRP RNA was also localized to a subpopulation of structures containing *Drosophila* GW during mitosis, suggesting that these two components may functionally interact in regulating mitosis.

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List of Symbols, Abbreviations

aa	amino acids
AED	after egg deposition
Ago	Argonaute
ARE	AU rich element
CB	chromatoid body
Ci	Curie
cdk	cyclin dependent knase
clb2	cyclin b-like mRNA 2
Cnn	centrosomin
DIG	digoxigenin
DGRC	*Drosophila* Genomics Resource Center
dGW	*Drosophila* GW
EtBr	Ethidium Bromide
esiRNA	endogenous short interfering RNA
FISH	fluorescent in situ hybridization
g	grams
GFP	green fluorescent protein
Glo	Glorund
GMC	ganglion mother cell
hour(s)	h(s)
hnRNP	heterogeneous ribonucleoprotein
HRP	horseradish peroxidase
IF	immunofluorescence
l	litre
MBT	mid blastula transition
m	meter
min	minute(s)
miRNA	micro RNA
mRNP	mRNA and protein complex
NMD	nonsense-mediated mRNA decay

MBP	myelin basic protein
MBT	mid-blastula transition
NC	nuclear cycles
NLS	nuclear localization signal
nt	nucleotide
Nos	Nanos
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBs	P bodies
Pcm	Pacman
P-Tyr	phosphotyrosine
piRNA	PIWI interacting RNA
s	seconds
RISC	RNA-induced silencing complex
RFP	red fluorescent protein
RNA*i*	RNA interference
RNP	ribonucleoprotein
RRM	RNA recognition motif
rRNA	ribosomal RNA
siRNA	short interfering RNA
SDS	sodium dodecyl sulfate
SGs	stress granules
Smg	Smaug
SSC	sodium chloride sodium citrate
U	units
UBA	ubiquitin associated domain
UTP	uridine triphosphate
UTR	untranslated region
V	volts
WB	Western Blot

CHAPTER 1: INTRODUCTION

1.1 mRNA Localization and Stability Influence a Variety of Cellular Processes

Expression of genes can be regulated at each of the multiple steps leading to synthesis of final gene products within a cell. These steps include the events leading up to production of RNA such as transcriptional control and processing of primary transcripts as well as studies of the functions of final gene products, protein and non-coding RNA. Historically, the study of cytoplasmic mRNA regulation has lagged behind studies of other stages of gene regulation. Recent advances in this area have been made possible by the emergence of new technologies such as improved methods for intracellular transcript localization and technologies that emerged from genome sequencing projects. One of these technologies was microarray analysis, which facilitated the observation of a lack of correlation between relative levels of some mRNAs and the proteins they encode (Gygi et al., 1999; Ideker et al., 2001) [reviewed in (Keene and Lager, 2005)].

Regulation of mRNA expression has recently been studied from two perspectives. In a broader view, influences on cellular and developmental processes have been examined. As a result of these efforts, cellular processes such as cell cycle progression, synaptic plasticity of neurons and cell motility are now known to be governed by mRNA regulation. A more detailed perspective of studies of molecular functions of mRNA regulation revealed structural roles for localized mRNA. For example, evidence suggests that localized mRNA nucleates cellular compartments [section1.1.4a, reviewed in, (Condeelis and Singer, 2005)] (Lecuyer et al., 2007) and may play a role in transport of a protein encoded by another gene (Jenny et al., 2006). Localization of mRNA is also important in regulating its own activity. Cytoplasmic compartments known as mRNA processing bodies or P bodies (PBs) have recently been discovered, where the stability and translation of mRNA is regulated (Eulalio et al., 2007a; Parker and Sheth, 2007).

1.1.1 The prevalence of localized mRNAs

The first transcript that was observed to be enriched in a distinct subcellular location was the one encoding myelin basic protein in mammalian oligodendrocytes (Colman et al., 1982). Soon after this discovery, several localized transcripts were identified in various

cells and organisms such as oocytes of *Drosophila* (Frigerio et al., 1986) and *Xenopus* (Rebagliati et al., 1985) and in lamellipodia of migrating chicken fibroblasts (Lawrence and Singer, 1986), suggesting that mRNA localization may not be a rare event. Recent systematic screens have significantly expanded the number of known localized transcripts and have identified additional cellular compartments to which they are targeted. For example, in neurons approximately 400 mRNAs are targeted to dendrites (Eberwine et al., 2001) and in yeast 22 bud-associated transcripts have been identified (Shepard et al., 2003). In *Drosophila,* a genome-wide screen of embryonic mRNA localization revealed that transcripts of 71% of the 3370 genes analyzed showed a variety of subcellular localization patterns (Lecuyer et al., 2007). The prevalence of localized mRNAs described in these studies indicates its potential importance in cellular functions. While the reasons for localization of many of these transcripts remain unknown, these observations indicate that further investigation of mRNA localization may lead to significant advancements in understanding basic cellular functions.

1.1.2 Mechanisms of mRNA localization

Localization of mRNAs is generally determined by recognition of cis-acting targeting elements within the mRNA by trans-acting factors. The cis-acting elements are generally located in the 3' UTR (untranslated region) of the mRNA, a region that would not interfere with protein-coding. Trans-acting factors in the cell couple the transcripts with cellular localization machinery [reviewed in (St Johnston, 2005)]. A number of mechanisms have been described for mRNA localization. To provide a brief overview, the three most common and best understood mechanisms will be described. A comprehensive description of this topic can be found in the following reviews (Condeelis and Singer, 2005; Kloc et al., 2002; Lipshitz and Smibert, 2000; St Johnston, 2005).

1.12a Transport along microtubules or microfilaments

A mechanism that directly targets mRNA to a specific region of a cell is active transport along microfilaments or microtubules. Transport of β-actin mRNA to lamellipodia of migrating chicken embryo fibroblasts is a well documented example of transport along actin filaments driven by a myosin motor (Latham et al., 2001; Sundell and Singer,

1991). A cis-targeting sequence known as zipcode resides in the 3'UTR of β-actin mRNA (Sundell and Singer, 1991). This sequence is recognized by the trans-acting zipcode binding protein 1 (ZBP1), which couples the β-actin mRNA to the actin cytoskeleton (Farina et al., 2003; Ross et al., 1997). Transport of the mRNA encoding myelin basic protein (MBP) along processes of oligodendrocytes is an example of an mRNA that is transported along microtubules driven by the kinesin motor (Carson et al., 1997). The 3'UTR of the MBP transcript contains a cis-targeting sequence known as the RNA trafficking sequence (Ainger et al., 1997). This sequence is recognized by two factors classified as heterogeneous ribonucleoproteins (hnRNPs): hnRNPA2 (Hoek et al., 1998) and CBF-A (Raju et al., 2008). The precise roles of each of these proteins are not yet known; however, defects in transport when either protein is depleted indicate that they are both required for MBP mRNA transport (Hoek et al., 1998; Raju et al., 2008).

1.1.2b General degradation combined with localized stability

This mechanism results in local enrichment of mRNAs that are initially uniformly distributed throughout a cell. Molecules that are not in the appropriate location are simply degraded while appropriately localized molecules are protected from degradation. In the *Drosophila* embryo this mechanism accounts for localization of *Hsp83* mRNA to the posterior pole. Separate non-overlapping elements in the 3'UTR function in this process. One of these elements targets *Hsp83* mRNA for degradation and the other for localized protection (Bashirullah et al., 1999). Recently, an additional region in *Hsp83* mRNA was identified that promotes its degradation. Unlike most mRNA regulatory elements which reside in the 3'UTR [reviewed in (St Johnston, 2005)], this region was identified in *Hsp83* coding sequences. It consists of six elements known as Smaug (Smg) response elements that are recognized by the RNA binding protein Smg (Semotok et al., 2008). Binding of Smg to this region enhances *Hsp83* mRNA degradation by recruiting proteins from the general mRNA decay pathway (Semotok et al., 2005).

1.1.2c Diffusion and anchoring

Transcripts that move through the cytoplasm by passive diffusion are localized by being trapped at the appropriate location. Diffusion and anchoring were proposed as a

mechanism to localize several *Drosophila* mRNAs (*nanos*, *cyclin B* and *germ cell-less*) to the posterior of the oocyte (Jongens et al., 1992; Raff et al., 1990; Wang et al., 1994). This localization mechanism was initially inferred by several lines of indirect evidence. First, direct transport is not possible at this late time in oogenesis because the cytoskeleton is not polarized along the anterior-posterior axis. Second, localization of these mRNAs depends on the prior assembly of polar granules, the site at which they are enriched [reviewed in (St Johnston, 2005)].

The most convincing experimental evidence for this mechanism was provided by the results of live imaging of fluorescently tagged, endogenously expressed *nanos* mRNA. In this study, accumulation of *nanos* mRNA at the posterior of the embryo was not abolished by inhibitors of microtubules. However, *nanos* mRNA localization did not rely exclusively on passive diffusion, since intense cytoplasmic streaming that occurs in the oocyte and depends on microtubules enhanced *nanos* mRNA localization. Localization of some *nanos* mRNA to the posterior of the oocyte in the presence of microtubule destabilizing drugs that abolished this streaming suggests that it can occur independently of microtubules. The possibility of localization by transport along actin filaments was excluded because it was judged to be inconsistent with the long range of *nanos* mRNA transport (Gavis and Lehmann, 1992). Together these results point to diffusion as the major mechanism of nanos mRNA localization. Entrapment of *nanos* mRNA at the oocyte cortex was shown to require an intact actin cytoskeleton, since *nanos* mRNA dissociated from the cortex when actin filaments were destabilized (Forrest and Gavis, 2003).

It is likely that other mRNAs such as *cyclin B* and *germ cell-less* that localize to the same place in the *Drosophila* oocyte at the same time are localized by the same mechanism [reviewed in(Kloc and Etkin, 2005)]. It is worth noting that *nanos* mRNA is not localized exclusively by diffusion and anchoring. Degradation of un-localized nanos mRNA was reported in an earlier study (Bashirullah et al., 1999). The asymmetric distribution of some transcripts may therefore depend on more than one localization mechanism.

1.1.3 Molecular functions of localized mRNAs

1.1.3a mRNAs determine the subcellular localization of proteins

Transcript localization is important for sorting proteins they encode into various independently functioning domains within a cell. Trafficking transcripts rather than proteins has several advantages. First, the energy required for molecular transport is reduced since each transcript can give rise to many localized protein molecules (Miyashiro et al., 1994). Second, trafficking translationally repressed transcripts prevents deleterious effects of mislocalized proteins. For example, MBP which is synthesized in oligodendrocytes, is a component of the myelin sheath that surrounds neuronal axons. Its high affinity for membranes would result in inappropriate interactions of the protein with membranes on its route to cellular processes where it is required. Transport of the transcript results in proper protein localization while avoiding deleterious membrane interactions [reviewed in (St Johnston, 1995)]. Finally, independent regulation of translation would allow independent modulation of protein levels in different cellular locations. This may explain how an individual neuron is able to respond independently to activity at each of many synapses [reviewed in (Steward and Banker, 1992)].

1.1.3b Localized mRNA can alter the fate of dividing cells

Localized mRNA can alter future expression patterns in the progeny of dividing cells by delivering a particular mRNA or sets of mRNAs to one daughter cell. This mechanism was shown to alter cell fates in several organisms. In *Caenorhabditis elegans*, germ cell progenitors arise by segregating germ line determinants into a subset of cells during early embryogenesis. These germ line determinants, known as P granules, contain developmentally regulated mRNAs and proteins that are predicted to bind RNA or regulate translation [reviewed in (Strome, 2005)]. Partitioning of P granules into a cell is correlated with its determination as a germ cell. After fertilization, uniformly distributed P granules move to the posterior pole of the embryo and are inherited by one daughter cell, the germ cell progenitor. Partitioning into only one daughter cell, a germ cell progenitor, continues for three more cell divisions. In subsequent divisions of germ cell

progenitors, P granules are evenly partitioned between daughter cells and only germ cells are produced (Strome and Wood, 1982).

Unequal mRNA partitioning also occurs during differentiation of the *Drosophila* nervous system. When an embryonic neuroblast divides, it produces another neuroblast and a ganglion mother cell (GMC) [reviewed in(Gonczy, 2008)]. The fate of the GMC is determined by partitioning the mRNA encoding Prospero protein into the GMC (Li et al., 1997). Prospero is a transcription factor that is required to activate genes for GMC identity and to repress genes expressed in neuroblasts (Doe et al., 1991; Vaessin et al., 1991).

Regulation of mating type switching in *Saccharomyces cerevisiae* occurs by a similar mechanism. During cell division, the transcript encoding the transcriptional repressor Ash1p is partitioned into the daughter cell. There it inhibits mating type switching, allowing only the mother cell to switch mating type. The presence of this mechanism in *Saccharomyces cerevisiae* suggests that mRNA localization may have evolved early in eukaryotes (Long et al., 1997).

1.1.3c mRNA can nucleate the assembly of subcellular complexes

Studies of the morphology and movements of fibroblasts prompted the idea that localized mRNAs can nucleate the assembly of subcellular complexes. These studies showed a requirement for localized actin mRNA in maintaining a stable lamellipodium at one side of the migrating cell (Kislauskis et al., 1997; Shestakova et al., 2001) [reviewed in (Condeelis and Singer, 2005)].

Additional evidence for this idea emerged form a recent genome-wide screen of mRNA localization in the *Drosophila* embryo. For a number of genes, the mRNA and the protein it encodes were observed to colocalize. Surprisingly, observation of mRNAs and their corresponding colocalizing proteins in different ages of embryos revealed that in some cases, localization of the mRNA preceded the protein. Anillin, an actin-binding protein, is a striking example of this observation. Anillin mRNA forms a pattern that resembles the pattern made by actin filaments; however, this pattern precedes actin filament formation. This observation suggests that the anillin mRNA determines the sites of actin filament formation (Lecuyer et al., 2007).

1.1.3d A novel function for mRNA in protein transport

The results of a recent study of mutants in the *Drosophila oskar* gene show that the *oskar* transcript is required for proper localization of Staufen, a protein encoded by another gene. In this study, an allele that eliminated *oskar* mRNA displayed an earlier developmental defect than the protein null allele. Classical *oskar* mutants express significant levels of *oskar* mRNA but lack both *oskar* proteins. These mutants produce embryos without germ cells that do not develop posterior structures (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992). In contrast, mutant *oskar* alleles that express little or no mRNA show an earlier defect in failure to complete oogenesis. Further, these alleles were rescued with the 3'UTR of *oskar* mRNA so that they resembled classical *oskar* mutants. The *oskar* 3' UTR was found to be required for transport of Staufen protein from nurse cells into the oocyte (Jenny et al., 2006). Staufen is an RNA binding protein that is required for localization of *oskar* mRNA to the posterior of the embryo [reviewed in (Lipshitz and Smibert, 2000)]. The results of this study challenge the common assumption that loss of function of protein coding genes is represented by loss of protein expression.

1.1.4 Cellular processes influenced by localized mRNA

1.1.4a Cell polarity

Cell polarity is required for a variety of specialized cellular functions. Epithelial cells are a common type of polarized cell that forms a lining for the inner or outer surfaces of organs. Formation of distinct plasma membrane domains is a characteristic of polarity in epithelial cells. Generally, polarized epithelial cells form two domains, the basolateral domain that contacts other cells or the extracellular matrix and an apical domain. The apical domain often forms microvillae which expand the area of this domain. In the intestine, epithelial cells form a lining composed of a single layer of cells joined together by junctions in the basolateral domain. The microvillae at the apical domain increase efficiency of nutrient absorption by providing a large surface area (Lodish H., 2008). It is clear how maintenance of polarity is essential for normal cellular functions however, current evidence indicates that impairment of polarity can also lead to tumourgenesis.

The idea that mRNA localization can play a role in determining cell polarity originated from observation of β-actin mRNA in migrating fibroblasts. The first report of localized β-actin mRNA was in migrating chicken embryo fibroblasts in which it localized at the leading edge together with β-actin protein (Lawrence and Singer, 1986). Since then, β-actin mRNA has been observed at the leading edge of other motile cells and in apical structures of other polarized cells. These include actin filaments in microvilli of epithelial cells (Cheng and Bjerknes, 1989) and stereocilia of auditory hair cells (Pickles, 1993) [reviewed in (Condeelis and Singer, 2005)]. β-actin mRNA may be localized at these sites to produce high levels of actin protein where it is needed. It has been proposed that migrating cells require high levels of actin protein so that it can be polymerized at a sufficient rate to enable protrusion of the membrane at the leading edge. Alternatively, β-actin mRNA may nucleate formation of compartments to which it localizes such as microvilli of epithelial cells, stereocilia of auditory cells and the leading edges of fibroblasts [reviewed in (Condeelis and Singer, 2005)]. Localized β-actin mRNA may therefore contribute to establishing or maintaining cell polarity by nucleating the assembly of stable asymmetrically distributed compartments in polarized cells.

Evidence for the idea that localized β-actin mRNA can nucleate subcellular compartments emerged from studies of the morphology and movements of fibroblasts. Localization of β-actin mRNA to a stable lamellipod at one side of the cell is a morphological characteristic of motile cells with intrinsic polarity (Kislauskis et al., 1994). Intrinsic polarity is in turn required for directional motility in cells. When β-actin mRNA was delocalized, cells were still capable of extending lamellipods and were motile. However, the direction of both lamellipod extensions and movement was variable (Kislauskis et al., 1997; Shestakova et al., 2001). These results show that localized mRNA determines the location of a stable asymmetric structure that confers functional polarity to a motile cell (Condeelis and Singer, 2005).

In addition to β-actin mRNA, many other mRNAs are currently known to localize to the leading edge of migrating cells. These include mRNAs encoding all seven proteins of the actin-related protein (Arp) 2/3 complex, a nucleator of actin polymerization (Mingle et al., 2005). A recent screen of mRNAs enriched in the leading edge of

migrating mouse fibroblasts identified another 50 mRNAs localized to this compartment. Most of them encode proteins that function in membrane traffic, signaling, microtubule-based transport and RNA metabolism (Mili et al., 2008). Importantly, analysis of requirements for localization of a few of these mRNAs revealed a requirement for the tumour suppressor, adenomatous polyposis coli. This discovery suggests that the tumour suppression function of this protein may be mediated through its ability to localize multiple transcripts to the leading edge of the migrating cells (Mili et al., 2008).

Another correlation between loss of cell polarity due to mislocalized transcripts and tumourgenesis comes from studies of β-actin mRNA in migrating cells. β-actin mRNA localization was compared in two breast adenocarcinoma cells showing different metastatic potential (Shestakova et al., 1999; Wang et al., 2002). Cells with localized β-actin mRNA showed lower metastatic potential. These cells were intrinsically polarized, displaying both polarized morphology and directional movement. In contrast, cells with delocalized β-actin mRNA showed a higher metastatic potential. They were not intrinsically polarized but acquired polarity in response to chemotactic signals from blood vessels. This allowed them to direct their motility towards these signals and invade tissues secreting these signals (Condeelis and Segall, 2003; Wyckoff et al., 2000). Establishing causal relationships between observations that link mRNA localization with tumougenesis and identifying molecular defects that lead to mRNA mislocalization may lead to novel approaches to the treatment of some cancers.

Cell polarity generated by asymmetric distribution of mRNAs is a means for establishing a body plan in several developing organisms [reviewed in(Kloc and Etkin, 2005; St Johnston, 1995)]. This process has been well documented in the early development of *Drosophila*. Patterning of the *Drosophila* embryo begins in the oocyte where localized translation of a few mRNAs targeted to specific regions direct patterning of the future embryo [reviewed in (Johnstone and Lasko, 2001; Riechmann and Ephrussi, 2001)]. The anterior-posterior axis is defined by localization of *bicoid* and *oskar* mRNAs to opposite ends of the oocyte. Both of these mRNAs are produced by nurse cells and transported into the oocyte. *Bicoid* mRNA accumulates at the anterior cortex and *oskar* mRNA is transported to the posterior of the oocyte where Oskar protein accumulates. Oskar protein in turn directs assembly of the pole plasm which contains other mRNAs

and proteins that specify formation of the abdomen and germ cells (Breitwieser et al., 1996; Ephrussi et al., 1991). *Nanos* mRNA is one of these pole plasm components (Ephrussi et al., 1991). The embryonic anterior-posterior axis is specified by two opposing morphogen gradients formed by Bicoid and Nanos proteins. These proteins are translated in the early embryo from mRNAs localized at opposite ends, *bicoid* at the anterior and *nanos* at the posterior. (Bergsten and Gavis, 1999; Gavis and Lehmann, 1994) [reviewed in (Riechmann and Ephrussi, 2001; St Johnston and Nusslein-Volhard, 1992)]

The dorso-ventral axis of the embryo is established in the oocyte by Gurken protein translated from localized *gurken* mRNA [reviewed in (St Johnston, 2005) (Riechmann and Ephrussi, 2001)]. Gurken protein is a member of the TGF-α family of proteins and specifies the polarity of the oocyte by localized signaling to surrounding follicle cells (Neuman-Silberberg and Schupbach, 1993). Unlike *bicoid*, *oskar* and *nanos* mRNAs that are transcribed in nurse cells, *gurken* mRNA is transcribed in the nucleus of the oocyte (Saunders and Cohen, 1999). *Gurken* mRNA is localized by an unusual mechanism that depends indirectly on localization of the oocyte nucleus. g*urken* mRNA and the nucleus are both localized to the anterio-dorsal corner of the oocyte where the nucleus directs formation of a population of microtubules. *gurken* mRNA, driven by dynein, is transported along these microtubules to the anterio-dorsal cortex of the oocyte (Duncan and Warrior, 2002; Januschke et al., 2002; MacDougall et al., 2003).

1.1.4b Synaptic plasticity

Localized mRNAs are important for modulating the strength of a neuronal synapse in response to stimulation. This modulation is referred to as synaptic plasticity. The discovery of polysomes localized to dendrites, where neuronal cells receive impulses, prompted the idea that translation of localized transcripts was a molecular response to an impulse that could modulate synaptic plasticity (Steward and Levy, 1982). Targeting of several mRNAs to dendrites in response to synaptic stimulation supports this idea. Synaptic stimulation induces transcription and transport to dendrites of mRNAs encoding the activity-regulated cytoskeleton-associated (Arc) protein (Link et al., 1995; Lyford et

al., 1995), brain-derived neurotrophic factor (BDNF) and the tyrosine-related kinase B (TrkB) receptor (Tongiorgi et al., 2004; Tongiorgi et al., 1997).

Neurons are capable of forming multiple synapses that can be independently stimulated. Localized translation of transcripts targeted specifically to stimulated synapses could provide a mechanism for autonomous control of individual synapses. Results of a study showing distinct mRNA populations in different branches of the same neuron (Miyashiro et al., 1994) are consistent with this idea. A subsequent study that showed targeting of Arc mRNA and accumulation of Arc protein specifically to a stimulated dentritic area (Steward et al., 1998; Steward and Worley, 2001) provides further support for this idea.

Studies of visual cortex development in rats show that localized mRNAs play a role in its maturation and in development of synaptic plasticity. There is a critical period in postnatal development of the visual cortex that requires visual stimulation. A large body of evidence points to a role for BDNF in visual cortex development [reviewed in (Tongiorgi et al., 2006)]. Observations of changes in distribution of BDNF mRNA suggested a role for localized BDNF mRNAs in this process. The distribution of BDNF mRNA changes in response to visual experience during this critical period of development. Localization to dendrites is abolished in the dark and resumes within two hours of exposure to light (Capsoni et al., 1999). Protein expression levels in dendrites are positively correlated with mRNA levels (Tropea et al., 2001). Together, these results indicate that the spatial and temporal distribution of BDNF, which functions in visual cortex development, is regulated posttranscriptionally.

1.1.4c Cell cycle regulation

The effect of mRNA localization on cell cycle progression was demonstrated in studies of cyclin B mRNA regulation. In *Xenopus*, cyclin B1 mRNA and protein are concentrated on mitotic spindles, suggesting that cyclin B1 mRNA is targeted to where cyclin B1 protein is needed to regulate cell cycle progression. This idea is supported by observing a strain with a mutant CPEB (cytoplasmic polyadenylation element binding protein). CPEB interacts with microtubules and is involved in the localization of cyclin B1 mRNA to the mitotic apparatus. The mutant strain expressed a form of CPEB which

does not interact with the mitotic apparatus. In this strain, spindle localization of cyclin B1 mRNA was abolished and cell division was inhibited, while little effect on overall cyclin B1 protein levels was observed (Groisman et al., 2000). Reports of spindle-localized cyclin B mRNA or protein in other species suggest that targeting cyclin B protein and mRNA to the mitotic spindle is likely to be part of a conserved mechanism of cell cycle regulation. Spindle-localized cyclin B mRNA was reported in *Drosophila* embryos (Lecuyer et al., 2007), while spindle-localized cyclin B protein was reported in *Drosophila* embryos (Huang and Raff, 1999) and in human cells (Hagting et al., 1998).

Recent genome-wide screens identified additional mRNAs localized to the mitotic apparatus (Blower et al., 2007; Lecuyer et al., 2007). Gene ontology (GO) terms of some of these transcripts include roles in cell division or cell division-related processes such as cytoskeleton organization. The significance of their localization to components of the mitotic apparatus and how this localization influences cell cycle regulation are not yet known for many of these mRNAs and need to be addressed in further studies.

1.1.5 Some localized mRNAs are components of microscopically visible granules

Granules consisting of mRNAs and proteins have been associated with a variety of cellular processes. For example, granules containing mRNAs that determine organism development have been described in several species. These include polar granules in *Drosophila* oocytes that specify abdomen formation and germ cell differentiation (Breitwieser et al., 1996; Ephrussi et al., 1991), as well as P granules in *Caenorhabditis elegans* embryos that specify germ cell differentiation [reviewed in (Strome, 2005)]. Cells that have been subjected to various types of stress such as heat shock, oxidative conditions or hypoxia form another type of granule known as stress granules (SGs). These granules contain mRNAs whose stability and translation are thought to be altered to promote cell survival under stressful conditions [reviewed in (Anderson and Kedersha, 2006)]. Finally, PBs are a type of granule in which mRNAs that are translationally repressed or destined for degradation accumulate.

1.2 The Composition and Function of P bodies

1.2.1 Protein components of P bodies

The function of PBs in regulating mRNA translation and stability was initially deduced from analysis of their protein components. PBs contain proteins that function in most eukaryotic mRNA decay and translational silencing pathways (Table 1) [reviewed in (Eulalio et al., 2007a; Parker and Sheth, 2007)]. These can be classified into three functional groups. In the first group are core PB components common to most PBs. Members of this group include factors that function in general mRNA degradation and translational suppression. These include components of decapping and deadenylation complexes and the 5' to 3' exonuclease, Xrn1 [(Bashkirov et al., 1997; Cougot et al., 2004; Ingelfinger et al., 2002; Sheth and Parker, 2003; van Dijk et al., 2002) Table 1A]. Notably, components of the 3' to 5' mRNA decay pathway have not been detected in PBs (Brengues et al., 2005; Sheth and Parker, 2003). Components of this pathway localize to the exosome, which forms a distinct class of cytoplasmic foci (Graham et al., 2006; Lin et al., 2007). A second class of PB-associated proteins consists of components of pathways that select specific mRNAs for degradation, such as nonsense-mediated decay (NMD) (Sheth and Parker, 2006; Unterholzner and Izaurralde, 2004) and AU rich element (ARE)-mediated decay (Fenger-Gron et al., 2005). Finally, components of the translational machinery, the cap-binding proteins eIF4E and eIF4E-T, localize to PBs (Table 1B). The presence of some components of translational machinery combined with the absence of ribosome components implicates PBs in the role of storage of translationally inactive mRNA (Andrei et al., 2005; Ferraiuolo et al., 2005; Kedersha et al., 2005).

Metazoan PBs contain several proteins that have not been identified in genomes of unicellular eukaryotes such as *Saccharomyces cerevisiae*. These include members of the GW182 protein family (GW proteins), which are known to function in the RNA*i* (RNA interference) pathway (Behm-Ansmant et al., 2006; Ding et al., 2005; Eystathioy et al., 2002; Eystathioy et al., 2003; Schneider et al., 2006). Metazoans also encode the additional PB-associated decapping activators, RAP55 (or LSm14) (Barbee et al., 2006; Squirrell et al., 2006; Yang et al., 2006) and Ge-1 (or Hedls) [(Yu et al., 2005). Table1B].

The more complex metazoan PB composition may be associated with an increased complexity in their function in metazoans [reviewed in (Eulalio et al., 2007a)].

The significance of the presence of several PB proteins that have been recently localized to PBs has not been investigated. These include Staufen and Mex3, proteins involved in mRNA transport (Table1B). Their presence suggests that translationally suppressed mRNAs can be transported in PBs. The presence of these and other proteins such as centrosomin (Table 1B) suggests that PBs may participate in a range of processes that have not yet been characterized. Table 1 lists most of the currently known components of human, *Drosophila*, *C. elegans* and *Saccharomyces cerevisiae* PBs and their functions. Although the list is fairly comprehensive, it may not be complete, since the number of PB components has been rapidly increasing and may not include the most recently identified ones.

Table 1.1 Protein Components of P bodies

A) Core components from general mRNA decay pathways

Name	Function	Organism	References
XRN1	5' to 3' exonuclease	Human Mouse *S. cerevisiae* *Drosophila*	(Ingelfinger et al., 2002; Lykke-Andersen, 2002; van Dijk et al., 2002) (Bashkirov et al., 1997) (Sheth and Parker, 2003) (Grima et al., 2008; Schneider et al., 2006; Zabolotskaya et al., 2008) (Lin et al., 2008)
DCP1 **DCAP-1**	decapping enzyme regulatory subunit	Human *C. elegans* *Drosophila* *S. cerevisiae*	(Ingelfinger et al., 2002) (Squirrell et al., 2006) (Behm-Ansmant et al., 2006) (Lin et al., 2008) (Sheth and Parker, 2003)
DCP2 **DCAP2**	decapping enzyme catalytic subunit	Human *C. elegans* *Drosophila* *S. cerevisiae*	(Ingelfinger et al., 2002) (Ding et al., 2005) (Behm-Ansmant et al., 2006) (Lin et al., 2008) (Sheth and Parker, 2003)
Rck/p54 **CGH-1** **ME31B** **Dhh1**	DEAD box helicase, translational repressor, decapping activator	Human *C. elegans* *Drosophila* *S. cerevisiae*	(Cougot et al., 2004) (Strome, 2005) (Eulalio et al., 2007b; Lin et al., 2008) (Barbee et al., 2006; Lin et al., 2006) (Sheth and Parker, 2003)
Hpat **Pat1**	decapping activator	*Drosophila* *S. cerevisiae*	(Eulalio et al., 2007b) (Sheth and Parker, 2003)
LSm1-7	decapping activator	Human *Drosophila* *S. cerevisiae*	(Ingelfinger et al., 2002) (Schneider et al., 2006) (Sheth and Parker, 2003)

Table 1.1 Protein Components of P bodies

A) Core components (continued)

Name	Function	Organism	References
Edc3	decapping activator	Human *Drosophila* *S. cerevisiae*	(Fenger-Gron et al., 2005) (Eulalio et al., 2007b) (Kshirsagar and Parker, 2004)
RAP55 **TRAL** **CAR-1** **Scd6p**	translational repressor	Human *Drosophila* *C. elegans* *S. cerevisiae*	(Yang et al., 2006) (Eulalio et al., 2007b) Squirrell, Eggers et al. 2006) (Muhlrad and Parker, 2005)
Ge1/Hedls	decapping activator	Human *Drosophila*	(Fenger-Gron et al., 2005; Yu et al., 2005) (Behm-Ansmant et al., 2006)
Cr4/Pop2/ Not1-5p	deadenylase	Human *S. cerevisiae*	(Cougot et al., 2004) (Sheth and Parker, 2003)

B) Additional components

Name	Function	Organism	References
Argonautes	RNA*i*	Human *C. elegans* *Drosophila*	(Liu et al., 2005b; Sen and Blau, 2005) (Ding et al., 2005) (Behm-Ansmant et al., 2006)
GW182 family	RNA*i*	Human *C. elegans* *Drosophila*	(Eystathioy et al., 2003) (Meister et al., 2005) (Ding et al., 2005) (Behm-Ansmant et al., 2006; Schneider et al., 2006)
MOV10	RNA*i*	Human	(Meister et al., 2005)
Spb1	decapping activator	*S. cerevisiae*	(Segal et al., 2006)
CPEB cytoplasmic polyadenylation element binding	translational suppressor	Human	(Wilczynska et al., 2005)
eIF4E	cap binding translation regulator	Human	(Andrei et al., 2005) (Ferraiuolo et al., 2005)
eIF4E-T **Cup**	translation repressor	Human *Drosophila*	(Andrei et al., 2005) (Ferraiuolo et al., 2005) Simmonds (unpublished)

B) Additional components (continued)

Name	Function	Organism	References
Gemin5	eIF4E binding translation repressor	Human	(Fierro-Monti et al., 2006)
Staufen	dsRNA binding; mRNA localization, transport	*Drosophila*	Eulalio, Izaurralde (unpublished)*
Rbp1	RNA binding; mitochondrial porin mRNA decay	*S. cerevisiae*	(Jang et al., 2006)
eRF1	translation termination	*S. cerevisiae*	(Buchan et al., 2008)
eRF3	translation termination	*S. cerevisiae*	(Buchan et al., 2008)
TTP	ARE-binding	Human	(Fenger-Gron et al., 2005) (Kedersha et al., 2005) (Stoecklin et al., 2006)
Upf1	NMD factor	Human: accumulates when SMG7 is overexpressed *S. cerevisiae*: accumulates in xrn1, dcp1, dcp2, upf1, upf2 deletion strains	(Unterholzner and Izaurralde, 2004) (Sheth and Parker, 2006)
Upf2	NMD factor	*S. cerevisiae*: accumulates in xrn1, dcp1, dcp2, upf1, upf2 deletion strains	(Sheth and Parker, 2006)
Upf3	NMD factor	*S. cerevisiae*: accumulates in xrn1, dcp1, dcp2, upf1, upf2 deletion strains	(Sheth and Parker, 2006)

Table 1.1 Protein Components of P bodies

(B) Additional components (continued)

Name	Function	Organism	References
SMG-7	NMD factor	human: accumulates when overexpressed	(Unterholzner and Izaurralde, 2004)
SMG-5	NMD factor	human: accumulates when SMG-7 is overexpressed	(Unterholzner and Izaurralde, 2004)
Mex-3	nuclear shuttle	human	(Buchet-Poyau et al., 2007)
Glorund	translation suppressor	*Drosophila*	Simmonds (unpublished)
Cup	translation suppressor	*Drosophila*	Simmonds (unpublished)
Smaug	translation suppressor mRNA degradation	*Drosophila*	Simmonds (unpublished) (Eulalio et al., 2007b)
Centrosomin	microtubule nucleation	*Drosophila*	Simmonds (unpublished)
Kinesin KLp61F	microtubule motor	*Drosophila*	Simmonds (unpublished)
PABP	polyA binding	*Drosophila*	Simmonds (unpublished)

Adapted from: *(Eulalio et al., 2007a; Parker and Sheth, 2007)*

1.2.2 P bodies are sites of mRNA storage and degradation

Localization of proteins that regulate mRNA metabolism to PBs indicates that they could function either as sites for sequestering these proteins away from mRNA or as sites where they are engaged in mRNA storage or degradation. Localizing mRNA to these structures would distinguish between these possibilities. The results of several studies demonstrated targeting of specific mRNAs to PBs by several pathways including NMD (Sheth and Parker, 2006), RNA*i* (Liu et al., 2005b; Pillai et al., 2005) and under conditions of general repression of translation triggered by glucose deprivation (Teixeira et al., 2005). These results indicate that PBs are sites where mRNA can be stored or degraded.

1.2.3 P bodies are dynamic structures

1.2.3a P body assembly depends on non-translating mRNA

PBs are dynamic structures whose size and number can vary. In human cells PBs are typically 100-300 nm in diameter (Yang et al., 2004). The combined results of several studies indicate that assembly and disassembly of PBs depends on mRNA trafficking into and out of PBs. Treatments that prevent trafficking of mRNA into PBs by reducing total mRNA levels such as exposure to ribonuclease A (Schneider et al., 2006; Sen and Blau, 2005; Teixeira et al., 2005) or by interfering with transcription by exposure to actinomycin D (Cougot et al., 2004; Teixeira et al., 2005) result in PB disassembly. Conversely, interfering with mRNA degradation results in an increase in the size and number of PBs. Inhibiting mRNA degradation by depleting the exonuclease XRN1 (Cougot et al., 2004) or by inserting a nuclease-resistant poly(G) tract in a reporter mRNA (Sheth and Parker, 2003; Teixeira et al., 2005) results in accumulation of mRNA intermediates and an increase in the size of PBs. Notably, PBs assemble when mRNA degradation is blocked at late stages of the process. Blocking early degradation by preventing deadenylation by CCR4 (Andrei et al., 2005; Sheth and Parker, 2003)] results in PB dispersal. This observation indicates that mRNAs are targeted to PBs after deadenylation.

Studies of effects of changes in growth (Teixeira et al., 2005; Yang et al., 2004) or induction of stress on PB abundance revealed additional information about the

relationship between translation and PB formation. Stress inducers such as glucose deprivation, osmotic stress, exposure to ultraviolet light and oxidative stress increase PB formation (Kedersha et al., 2005; Teixeira et al., 2005). Some of these stress inducers (glucose deprivation and osmotic stress) are known to inhibit translation initiation, prompting the investigation of the relationship between levels of cellular translation on PB assembly. Inhibiting translation initiation with mutant translation initiation factors eIF4E or Prt1p in *Saccharomyces cerevisiae* increased PB size (Teixeira et al., 2005). Conversely, trapping mRNA in the process of translation on ribosomes by cycloheximide treatment resulted in dissociation of PBs (Andrei et al., 2005; Cougot et al., 2004; Teixeira et al., 2005). These results indicate that PB assembly depends on levels of non-translating mRNA.

Observations of mRNA trafficking in response to stress showed that mRNAs are not necessarily degraded in PBs but can be released and return to translation. In mammalian cells, an mRNA targeted to PBs by miRNA-mediated repression could exit PBs and re-localize to polysomes in response to amino acid deprivation (Bhattacharyya et al., 2006). Tracking a reporter mRNA in *Saccharomyces cerevisiae* showed that stress had the opposite effect. The reporter was released from polysomes and targeted to PBs in response to glucose deprivation. Re-addition of glucose resulted in reassembly of polysomes and relocation of the transcript to polysomes (Brengues and Parker, 2007). This opposite effect of stress on targeting mRNAs to PBs or releasing them from PBs suggests that PBs may play a role in altering the cellular translational profile in response to stress.

1.2.3b Proteins transit in and out of P bodies

Like mRNAs, proteins also transit in and out of PBs. Differences in rates of exchange of various PB-localized proteins with the cytoplasmic pool have provided further insights into the functions of these proteins. Some proteins transit through PBs so rapidly that they can only be detected in PBs under certain conditions. For example, some components of the *Saccharomyces cerevisiae* NMD pathway (Upf1p, Upf2p and Upf3p) can be detected only when levels of general mRNA decay factors are reduced [Table 1 (Sheth and Parker, 2006)]. Quantitative measurements by fluorescent recovery after

photobleaching of several other PB components showed a range of exchange rates and recovery levels. Fluorescent fusions of LSm6, eIF4E, eIF4E-T recovered rapidly, with 50% of the original intensity recovered in 5 to 8 seconds (Andrei et al., 2005). Exchange rates of Tristetraprolin (TTP) and DCP1a were also quite rapid showing ~90% and ~ 60% recovery, respectively, of the original intensity after 30 seconds. In contrast, GW182 recovered an insignificant amount of the original signal after 30 seconds (Kedersha et al., 2005). The slow exchange rate of GW182 is consistent with its proposed role as a scaffolding protein (Yang et al., 2004).

It has been proposed that rapidly transiting proteins associate with mRNAs before they accumulate in PBs [reviewed in (Parker and Sheth, 2007)]. These mRNA and protein (mRNP) complexes would deliver mRNAs to a more stable complex containing components such as GW182 (Kedersha et al., 2005). Experimental evidence suggests that TTP could form such an mRNP complex. TTP binds both ARE-containing mRNAs and general mRNA decay enzymes and promotes mRNA decay (Lykke-Andersen and Wagner, 2005), acting as an adapter linking specific mRNAs to the mRNA decay machinery. Upf1p, a rapidly transiting component of the NMD pathway, could act in a similar way. Upf1p recognizes mRNAs whose translation has been prematurely terminated (Muhlrad and Parker, 1994) and targets them to PBs (Sheth and Parker, 2006). It is not clear if the other rapidly transiting NMD components are part of this complex or if they transit in and out of PBs independently.

1.2.4 P bodies are sites of functional interactions between multiple mRNA regulatory pathways

A single PB typically contains factors that function in more than one mRNA decay pathway. Localization of components from those mRNA regulatory pathways that recruit specific transcripts to PBs, such as NMD or RNA*i*, is generally based on a colocalization with a core PB component such as a decapping factor that serves as a marker to distinguish PBs from other related foci such as SGs. For example, localization of Argonaute proteins, components of the RNA*i* pathway to PBs, was based on co-localization with decapping enzymes DCP1 or DCP2 (Sen and Blau, 2005). Similarly NMD factors Upf1p, Ufp2p, Upf3p were colocalized with Dcp2p in *Saccharomyces*

cerevisiae (Sheth and Parker, 2006) and UPF1, SMG5 and SMG7 with LSm4 in human cells (Unterholzner and Izaurralde, 2004).

Localization of factors from more than one mRNA regulatory pathway to PBs suggests that functional interactions between these pathways are important in mRNA regulation. Several studies have addressed this question directly, asking if components of one pathway affect the function of another. In several cases this was shown to be the case. For example, depletion of XRN1 and DCP2 inhibited degradation of a reporter transcript targeted for NMD (Unterholzner and Izaurralde, 2004). In the case of ARE-mediated decay, multiple components of general mRNA decay were shown to be functionally important. Several decapping factors were found to interact with TTP, an activator of ARE-mediated decay, and this interaction enhanced decapping of transcripts containing AREs (Fenger-Gron et al., 2005). Further, depletion of XRN1 and LSm1 inhibited ARE-mediated decay (Stoecklin et al., 2006). RNA*i*-mediated gene silencing also depends on components of general mRNA decay. Degradation of target mRNAs depends on XRN1 (Orban and Izaurralde, 2005) and a number of decapping and deadenylation factors (Barbee et al., 2006; Behm-Ansmant et al., 2006; Chu and Rana, 2006; Rehwinkel et al., 2005). In general, it appears mRNA regulatory pathways such as NMD, ARE-mediated decay and RNA*i* identify specific transcripts and direct them to the general mRNA decay machinery [reviewed in (Parker and Sheth, 2007)]. Aggregation of components from different mRNA regulatory pathways in a single particle such as a PB could facilitate their interactions.

1.3 RNA Granules and Other P body-like Structures

A variety of RNA granules in the cells of several organisms have been characterized. These include granules that are restricted to specific cell types such as previously described granules in the pole plasm of *Drosophila* oocytes and P granules in germ cells of *C. elegans* (section 1.13b and 1.14 a). Other granules such as stress granules are more widely distributed among various cell types. Recent analysis of the composition of these granules has revealed the presence of proteins involved in regulating translation that are also found in PBs. (Table 2) [reviewed in (Anderson and Kedersha, 2006)]. This suggests that PBs may be one of many classes of RNA granules that regulate mRNA.

Examination of the literature on PBs shows that the identity of some cytoplasmic foci containing PB components is not clear. Data from these studies show that foci containing PB components do not always colocalize. While some studies show complete colocalization of foci (Behm-Ansmant et al., 2006; Liu et al., 2005a; Liu et al., 2005b; Sen and Blau, 2005), others show partial colocalization (Barbee et al., 2006; Eystathioy et al., 2003; Ingelfinger et al., 2002; Schneider et al., 2006). In some cases the degree of colocalization of these foci increases when cells are stressed (Garneau et al., 2007; Kedersha et al., 2005) or when a component is overexpressed (Barbee et al., 2006). These observations suggest that there may be multiple PB-like structures with overlapping protein composition within a cell (Barbee et al., 2006; Buchan et al., 2008). Some of these foci could be functionally related and change in composition by fusing with each other or by exchange of components between foci. Alternatively, some of these foci may be functionally distinct, consisting of components of non-overlapping mRNA regulatory pathways.

1.3.1 Stress granules are functionally linked to P Bodies

Under conditions of environmental stress, such as heat shock, oxidative conditions or changes in nutrient availability, translation of a subset of cellular mRNAs is arrested. This results in reprogramming of cellular metabolism as to allow the cell to overcome damage due to stress [reviewed in(Anderson and Kedersha, 2008)]. Translation arrest by inactivation of the translation factor eIF2α (Buchan et al., 2008; Kedersha et al., 1999; Krishnamoorthy et al., 2001) is correlated with the formation of a class of cytoplasmic foci called SGs [reviewed in [(Anderson and Kedersha, 2008)]. Components of translation initiation complexes consisting of a subset of translation initiation factors and the small ribosomal subunit are characteristic SG components (Table 2) [reviewed in (Anderson and Kedersha, 2008; Buchan et al., 2008)].

Based on these observations, SGs were initially viewed as sites of accumulation of stalled translation initiation complexes (Kedersha et al., 1999). This idea initially led to the proposal that SGs were storage sites for translationally repressed mRNA. However, observation of rapid transit of proteins and mRNA between SGs and the cytoplasm (Kedersha et al., 2005; Mollet et al., 2008) was inconsistent with this idea and led to the

proposal of the mRNA triage theory. This theory proposes that SGs are triage sites where mRNAs that have exited translation accumulate. Once in SGs mRNAs are sorted and subsequently redirected to other compartments for storage, degradation or re-initiation of translation (Kedersha et al., 2005) [reviewed in (Anderson and Kedersha, 2008)].

The function of SGs remains controversial because of an alternative theory proposed in a recent study. In this study, the formation of PBs was consistently observed before the formation of SGs. This observation led to the proposal that SGs are derived from PBs. According to this theory, mRNAs that exit PBs accumulate in SGs where they are stalled in the process of reentering translation (Buchan et al., 2008). The inconsistency between these theories is due to the difference in the order of appearance of SGs and PBs in different experiments using different methods to induce SGs. For example, in human cells, arsenite-induced stress granule formation results in appearance of PBs first (Buchan et al., 2008), while heat shock results in the appearance of SGs first (Kedersha et al., 2005). A modified triage theory could account for all of these observations. In this theory, stalled translation initiation complexes could enter SGs after exit from polysomes or PBs.

Additional evidence initially drawn from studies of mammalian cells indicates a close functional relationship between SGs and PBs. First, both compartments contain common components. These include proteins such as XRN1, eIF4E, TTP and BRF-1 [reviewed in (Anderson and Kedersha, 2008)]. Also, a single reporter transcript was detected in both PBs and SGs (Kedersha et al., 2005). The presence of common components suggests that these components are transferred between SGs and PBs. This idea is supported by observations of a close and sometimes overlapping spatial relationship between these two compartments. In stressed cells PBs and SGs frequently form adjacent to each other. Occasionally, they were observed fusing with each other or PBs were engulfed by SGs (Kedersha et al., 2005; Wilczynska et al., 2005). Similar spatial relationships between PBs, SGs and their components were subsequently observed in *Saccharomyces cerevisiae* where SGs were discovered at a later time (Buchan et al., 2008). Together these observations indicate that SGs and PBs interact with each other; however, the functional significance of these interactions is not yet understood.

1.3.2 RNA granules restricted to specific cells

Over the last few years an increasing number of RNA granules has been identified in several specialized cell types in several species. Like PBs and SGs, these granules consist of mRNA and conserved proteins that function in mRNA regulation. RNA granules have been identified in both male and female germ cells. In oocytes of some organisms, localized pools of maternally transcribed and translationally repressed mRNAs are produced. These mRNAs are translated in specific spatial and temporal patterns that are required for formation of the oocytes and early patterning of the embryo [reviewed in (Kloc and Etkin, 2005)]. A pool of translationally repressed mRNAs also accumulates during mammalian spermatogenesis. These mRNAs, which encode proteins that are required for sperm cell development, are produced during a period of intense post-meiotic transcription. Translation and stability of these mRNAs are regulated in a single large granule known as the chromatoid body [reviewed in (Kotaja and Sassone-Corsi, 2007). RNA granules have also been studied in neurons. In neurons, long distances between the cell body and distal parts of the cell such as dendrites require mechanisms for localized gene regulation. This is accomplished by transport of translationally repressed mRNAs and their subsequent local activation [reviewed in (Kosik and Krichevsky, 2002)].

1.3.2a Germ cell determinants

Germ cell determinants are ribonucleoprotein (RNP) particles that specify germ cell differentiation. They have been described in oocytes or in early embryos in several species. In *Caenorhabditis elegans,* P granules are determinants of germ cell specification. Their composition has been characterized mainly in embryos. Although the identity of many protein components of these granules has been known for some time [reviewed in (Strome, 2005)], their similarity to PBs was not recognized until recently when several PB components were identified. These include both components of the decapping complex DCP1 and DCP2 and the translational suppressor CAR-1 (Noble et al., 2008; Squirrell et al., 2006).

In *Drosophila,* granules in the oocyte that contain *oskar* mRNA specify germ cell fate and abdomen formation. They contain several proteins that function in mRNA

transport and regulation of translation. These include mRNA transport factors Staufen (St Johnston et al., 1991) and Exuperantia and the translational repressors YPS and ORB (cytoplasmic poly (A) element binding protein homologue) (Lin et al., 2006; Mansfield et al., 2002). Me31B, a translational repressor and decapping activator, transiently localizes with these granules during their transport, until they reach the posterior of the oocytes (Lin et al., 2006). Recently, one of the two components of the conserved decapping enzyme DCP1 was localized to these granules. The decapping enzyme consists of two components DCP1 and DCP2. DCP1 regulates the enzymatic activity of DCP2 (Coller and Parker, 2004). Interestingly, *Drosophila* DCP1 carries out an additional function. It is also required for localization of these granules to the posterior of the oocyte (Lin et al., 2006). The presence of DCP1 in these granules raises the question of why a decapping regulator is there at a time before mRNA degradation is known to occur. Degradation of some transcripts in these granules begins in early embryogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1993). One possibility is that early recruitment of DCP1 may facilitate the rapid assembly of the degradation machinery at a later time when it is needed (Lin et al., 2006). Indeed, foci that contain additional components of the degradation machinery were recently identified in embryos at a time mRNA degradation is known to occur. These foci have a typical PB-like composition, containing the catalytic decapping component DCP2 and the 5' to 3' exonuclease Pacman (Pcm) (Lin et al., 2008). These observations suggest potential relationships between related RNA granules during development. Granules formed in the oocyte containing translationally repressed mRNAs may acquire additional components at a later time to degrade these mRNAs when they are no longer needed.

1.3.2b Germ cell granules

Germ cell granules are restricted to male or female germ cells although they do not necessarily determine germ cell fate. In *Caenorhabditis elegans*, two types of germ cell granules were recently identified. The first class, germ line related PBs (grP bodies), is similar to P granules. These granules contain translationally repressed mRNAs and some of the same proteins as P granules. The second class is DCAP-2 enriched bodies (dcP bodies). Not much is yet known about dcP bodies except that they contain DCP2, lack P

granule and grP body components CAR-1 and CGH-1, and they form later in oogenesis (Noble et al., 2008). The functional relationships between these granules are not yet understood.

The chromatoid body (CB) is a structure that was first discovered over 100 years ago in mammalian sperm cell progenitors. Early morphological studies of the CB described it as a fibrous-granular network in meiotic spematocytes which becomes compacted into a single filamentous perinuclear granule in the cytoplasm of postmeiotic spermatids [reviewed in (Kotaja and Sassone-Corsi, 2007)]. The function of the CB remained elusive for some time. The discovery of the mouse VASA homologue (MVH), which plays a role in regulating maternal mRNA translation (Styhler et al., 1998), a DEAD-box RNA helicase and other proteins involved in RNA metabolism in the CB suggested a role for this structure in RNA metabolism [reviewed in(Parvinen, 2005)]. A role for the CB in RNA*i* was not determined until after molecular characterization of the players in this pathway. Identification of MIWI, Ago2, Ago3, dicer and miRNAs in the CB provided a functional link to RNA*i*. Components of general mRNA decay, Dcp1a and Xrn1 and the RNA binding protein GW182 were also identified in the CB (Kotaja et al., 2006). The presence of components of both of these mRNA regulatory pathways in the CB indicates a strong similarity between the CB and PBs (Table2).

1.3.2c RNA granules in neurons

A recent comprehensive study of neuronal granules in *Drosophila* showed that they contain numerous known PB components. These include several components of the general mRNA decay pathway, Xrn1 and Dcp1, the RNA*i* component Ago2 and Upf, a component of the NMD pathway (Barbee et al., 2006). They also share a number of components with maternal RNA granules including Staufen and FMRP [reviewed in (Barbee et al., 2006)] and SGs (G3BP and eIF2: Table 2). The functional significance of proteins with diverse functions in mRNA regulation in these granules is not yet understood. However, not all of these PB components colocalized. Rather, different classes of granules composed of subsets of these components were identified. Observations of overexpression of some of these components suggest that there may be a functional relationship between them. Overexpression of STAU or a GFP fusion of

dFMR1 resulted in an increase in the degree of colocalization of these two proteins with a concurrent increase in granule size and decrease in granule number. These observations suggest that fusion may occur between different classes of these granules, indicating a functional relationship between them (Barbee et al., 2006) that is not yet understood.

Neuronal granules have also been characterized in mammalian cells. A number of similarities can be noted between human and *Drosophila* neuronal RNA granules. First, some components of mammalian neuronal granules are homologous to components of *Drosophila* neuronal granules (Table 2). Second, different types of granules with different protein compositions were observed (Macchi et al., 2003; Shiina et al., 2005). Finally, distinct classes of human neuronal granules may also be capable of interacting with each other. For example, overexpression of Staufen (Kiebler et al., 1999) or a novel translational repressor RNG105 (Shiina et al., 2005) results in an increase in granule size and a reduction in granule number, indicating potential fusion of granules. Also, transfer of Staufen from neuronal granules to SGs was observed in response to stress (Thomas et al., 2005). Similarities in their composition and in their dynamic properties suggest functional homology between neuronal granules in humans and *Drosophila.*

1.3.3 TAM bodies and cell cycle regulation

A novel specialized PB-like structure was recently identified in *Saccharomyces cerevisiae* that is present exclusively during mitosis (Gill et al., 2006). This structure contains the PB component Xrn1p (5' to 3' exonuclease) and RNase MRP (mitochondrial RNA processing), an essential and highly conserved ribonucleoprotein complex found only in eukaryotes. RNase MRP has three known functions: it processes an RNA transcript to produce primers for mitochondrial DNA replication, it plays a role in ribosome biogenesis by processing ribosomal RNA and it was recently shown to cleave the *Saccharomyces cerevisiae* B-type cyclin (*CLB2*) mRNA(Gill et al., 2004) [reviewed in (Martin and Li, 2007)] . Colocalization of Xrn1p with RNase MRP to a PB-like structure suggests that these two ribonucleases act together to regulate mitosis by degrading mRNAs encoding mitotic regulators. These structures are referred to as TAM bodies for temporal asymmetric MRP bodies (Gill et al., 2006).

Table 1.2 Components of RNA Granules

Type of Granule	Name of granule and organism	Proteins shared with PBs	Proteins not shared with PBs	References
Stress granules	Mammals	XRN1, CPEB, Staufen FAST, TIAR, TIA-1, TTP, RAP55, RCK/p54,A GO2 eIF4E	40S ribosome eIF2, eIF3, eIF4G, FMRP, G3BP, HuR, PABPC1, SMN1, Ataxin-2	(Anderson and Kedersha, 2006; Anderson and Kedersha, 2008) *
	S. cerevisiae	eEF4GI, eEF4GII, eEF4E	Pab1, Pub1, Ngr1, PbP1(Ataxin-2)	(Buchan et al., 2008)
Germ cell determin-ants	*Drosophila* maternal RNA granules	DCP1, Staufen, ORB (CPEB), Me31B, dFMR1	YPS, EXU,VASA, Buno, AUB, Maelstrom, ribosomes	(Lin et al., 2008) (Lin et al., 2006) (Costa et al., 2005; Findley et al., 2003; Johnstone and Lasko, 2001; Nakamura et al., 2001)
	C. elegans P granules	CAR-1, DCP-1, DCP-2 CGH-1	PGL-1,3 GLH-1,2,3,4 GLD-1,2.3 PIE-1, MEX-1,3 POS-1, OMA-1,2 SPN-4	(Boag et al., 2005; Lall et al., 2005; Navarro et al., 2001; Squirrell et al., 2006; Strome, 2005)
Germ cell granules	*C. elegans* grP bodies	CAR-1, DCP -1, DCP-2 CGH-1	PUF5, GLD-1 MEX-3, 5	(Noble et al., 2008)
	C. elegans dcP bodies	DCP-2	unknown	(Noble et al., 2008)
	Chromatoid body Mammalian sperm	XRN1, DCP1a, GW182, Ago3, Ago2, MIWI, Dicer	MVH(VASA)	(Kotaja et al., 2006)

*A comprehensive list of mammalian stress granule components can be found in (Anderson and Kedersha, 2008)

Table 1.2 Components of RNA Granules (continued)

Name	Organism	Proteins shared with PBs	Proteins not shared with PBs	References
Neuronal granules	*Drosophila*	Staufen, Me31B, TRAL, PCM, DCP1, UPF1, Ago2, EIF4E, CUP	dFMR1,YPS,ZBP 1 PUM, NOS, BTZ	(Barbee et al., 2006)
	mammals	staufen,eIF4E, PABP, RNG105	ribosomes, eIF2,G3BP, HuR/D IMP-1(ZBP1), eIF1α, Barentz	(Atlas et al., 2004; Krichevsky and Kosik, 2001; Macchi et al., 2003; Shiina et al., 2005; Smart et al., 2003)
TAM body	*S. cerevisiae*	XRN1, RNase MRP		(Gill et al., 2006)

1.4 A model for mRNA trafficking in cellular compartments

The results of studies of mRNA distribution among polysomes and various RNA granules such as SGs and PBs and can be combined to create a model that represents the movements of mRNAs among these compartments (Fig.1). This model illustrates the relationships between the major pathways known to regulate mRNA expression. mRNA movements can be summarized in four major steps.

Step 1. Transcripts exiting the nucleus can be translationally repressed or ready for translation. Transcripts, whose products are not immediately required, such as those found in PBs or other RNA granules, are transported to their appropriate destinations in a translationally repressed form. Transcripts whose products are required immediately such as those encoding housekeeping genes, are targeted to ribosomes [reviewed in (Parker and Sheth, 2007)].

***Step 2. Transcripts that exit translation can be targeted to PBs or SGs*.** Studies in human cells and in *Saccharomyces cerevisiae* show that in response to stress, SGs can be observed either before (Kedersha et al., 2005) or after PB formation (Buchan et al., 2008), depending on the stress-inducing treatment. Also, SGs and PBs can form independently of each other (Kedersha et al., 2005; Serman et al., 2007).

Step 3. Transcripts can be exchanged between PBs and SGs. This was inferred from observations of a close physical association of PBs and SGs, the presence of a single transcript in both compartments (Kedersha et al., 2005; Wilczynska et al., 2005) and formation of hybrid compartments containing PB and SG components that were initially in separate foci (Buchan et al., 2008) [section 1.3.1]. This idea is further supported by the results of a recent study showing transfer of the translational repressor RCK/P54 (presumably in association with mRNA) from PBs to SGs after induction of stress (Mollet et al., 2008).

Step 4. Repressed transcripts can return to translation from both PBs and SGs.
As described in section 1.2.3*a*, reporter transcripts targeted to PBs can relocate to polysomes under certain conditions. In *Saccharomyces cerevisiae* addition of glucose resulted in relocation of a transcript from PBs to polysomes (Brengues and Parker, 2007).

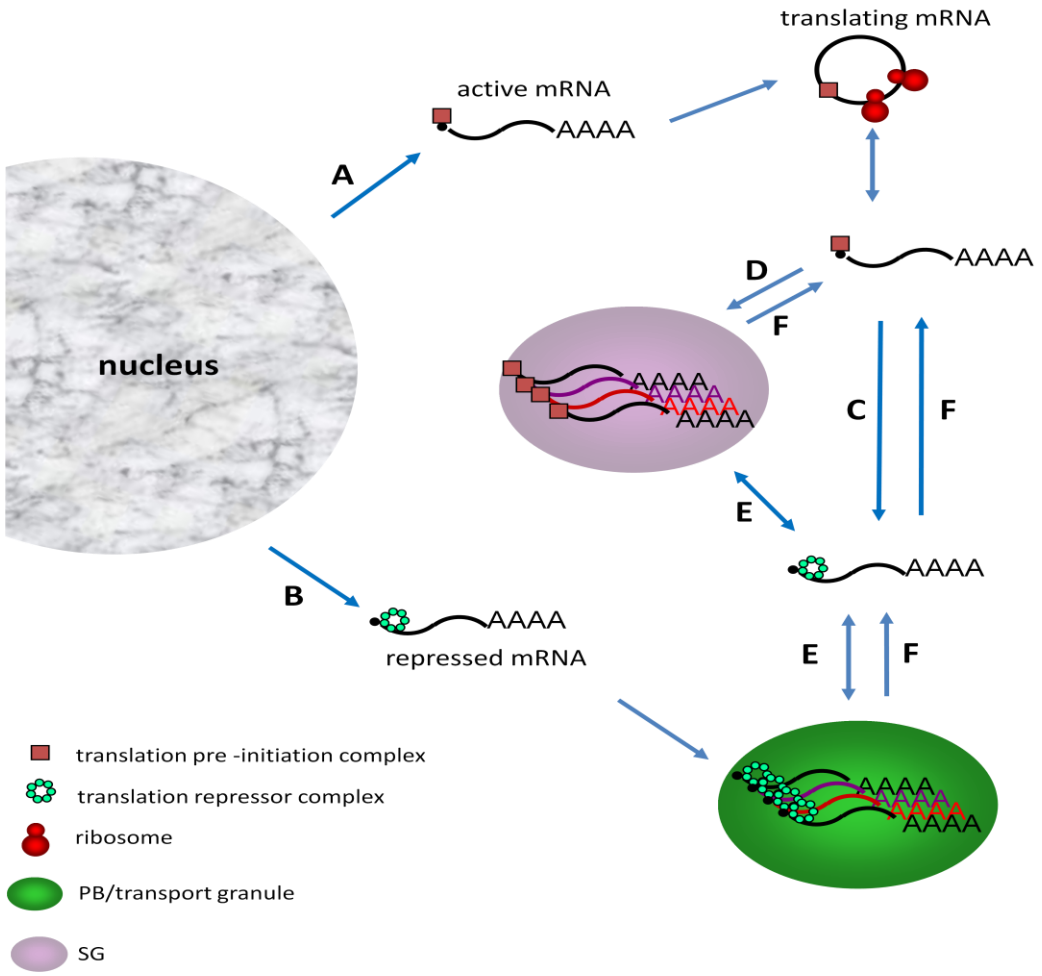


Figure 1. A model for mRNA trafficking during interphase. *Step 1*. Upon export from the nucleus, some mRNAs are destined for immediate translation such as those encoding housekeeping genes (A). Other mRNAs are translationally repressed and form PBs/transport granules where their translation and stability will be regulated spatially and temporally (B). *Step 2*. Active mRNAs are translated until they are selected for repression or decay. mRNAs targeted by regulatory pathways such as RNA*i*, NMD, or ARE-mediated decay and accumulate in PBs (C). Under conditions of stress when the cellular metabolism needs to be temporarily reprogrammed, translation of some mRNAs is stalled and these mRNAs, still associated with translation pre-initiation complexes, are sorted into stress granules (D). *Step 3*. mRNAs can be move between PBs and SGs (E). *Step 4*. When their activities are needed, repressed mRNAs can exit PBs/transport granules or SGs to become active (F). *Modified from Parker & Sheth 2007.*

In mammalian cells, a transcript relocated from PBs to polysomes; however, this response was induced by starvation (Bhattacharyya et al., 2006). The movement of transcripts between polysomes and SGs was also documented. This movement was inferred by observing the effects of treatment with drugs that alter polysome and stress granule stability. Treatment with cycloheximide, which stabilizes polysomes, inhibits stress granule assembly while drug combinations that promote polysome assembly after induction of stress granule assembly result in subsequent gradual stress granule dispersal (Kedersha et al., 2000; Mollet et al., 2008).

This model represents current knowledge of mRNA trafficking during interphase; however, it needs to be further developed to describe mRNA trafficking during mitosis.

1.5 Project Objectives

The *Drosophila* GW protein (dGW) is a member of the GW182 family of proteins. This is a novel a class of conserved proteins with unique features whose significance are still poorly understood. These include several regions of the protein that are predicted to be unstructured and interspersed glycine/tryptophan repeats. Understanding the significance of these features and how they function with the structured domains provide an opportunity for discovery of novel mechanisms of protein function.

The GW182 family of proteins are components of PBs which are believed to believed to be sites that regulate mRNA stability and translation. Using a small experimentally tractable organism, such as *Drosophila,* provides opportunities to observe the effects of this protein within a variety of metazoan tissues. This could in turn lead to identification of additional cellular processes that may be influenced by posttranscriptional gene regulation.

The human genome encodes three paralogues belonging to the GW182 protein family while the *Drosophila* genome encodes only one. My central hypothesis is**: dGW may be structurally and functionally homologous to the human GW182 protein; however, as the single GW protein in the *Drosophila* genome it may perform additional functions that are carried out by the other two human GW182 paralogues, which have been less extensively characterized.**

Objective 1: To determine if dGW is a component of structures similar to PBs.

Specific goals:

1. To determine if dGW has a similar cellular distribution as GW182.
2. If dGW accumulates in cyctoplasmic foci, like GW182, are these foci similar to PBs?

Objective 2: To identify cellular processes influenced by dGW

Specific goal:

To characterize the phenotype of the *gw*^{1} mutant strain, a *Drosophila* strain carrying a mutation in dGW.

Objective 3: To identify identify regulatory pathways that may function with dGW in cellular processes identified in Objective 2, and mRNA targets that are regulated by these processes.

Specific goals:

1. To characterize defects in the process identified in Objective 2, by observing the effects of these defects on specific molecular markers. Based on these observations, candidate mRNA targets and regulatory pathway could be identified.
2. To perform tests of physical and functional associations between components of potential mRNA regulatory pathways affected by the *gw*^{1} mutation and dGW. These would include colocalization, physical interactions and genetic interactions of dGW with components of candidate mRNA regulatory pathways.

CHAPTER 2 MATERIALS AND METHODS

2.1 Reagents Used

2.1.1 Chemicals

[α-^{32}P]-UTP (800Ci/mmol 20mCi/ml)	Perkin Elmer
Halocarbon oil 700	Sigma
Formamide, redistilled	Invitrogen
PicoGreen	Invitrogen
DABCO [1,4-Diazabicyclo[2.2.2]octane]	Sigma
Mowiol 40-88	Calbiochem
Saponin	Sigma
methyl 4-hydroxybenzoate	Sigma

2.1.2 Multicomponent systems

Centricon-10	Millipore
complete EDTA-free protease inhibitior	Roche
Gateway® BP Clonase® Enzyme Mix	Invitrogen
Gateway® LR Clonase® Enzyme Mix	Invitrogen
GeneJet Plamid Miniprep Kit	Fermentas
GenElute™ Minus EtBr Spin Column	Sigma
HiTrap *N*-hydroxysuccinimide–activated column	GE Healthcare
Immunopure Gentle Elution Buffer	Pierce
MEGAscript High Yield Transcription Kit	Ambion
Ni-NTA Agarose	Qiagen
PCR Supermix	Invitrogen
Protein A sepharose	Zymed
Qiagen Plasmid Mini and Midi Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
RNA Gel Loading Buffer	Eppendorf
Restore Western Blot Stripping Buffer	Thermo Scientific
Super Signal West Pico Chemiluminescent Substrate	Pierce
Zero Blunt PCR Cloning Kit	Invitrogen
10X DIG labeling mix	Roche

2.1.3 DNA/RNA ladders

λ DNA/HindIII Fragments	Invitrogen
1 Kb Plus DNA Ladder	Invitrogen
Millennium™ Markers-Formamide	Ambion

2.1.4 Enzymes

T4 DNA ligase	Roche
Shrimp alkaline phosphatase	Fermentas
restriction endonucleases	Invitrogen or NEB
Pfu Turbo DNA polymerase	Stratagene

2.1.5 Antibodies

Table 2.1 Primary Antibodies

Name/Target	Dilution	Source
anti-dGW	IF 1:6,000 WB 1:1,000	this study
rabbit anti-centrosomin	IF 1:100	T. Kaufman (Indiana University
mouse anti–β-tubulin (E7)	WB 1μg/ml	Developmental Studies Hybridoma Bank.
mouse anti-phosphotyrosine	IF 1:1,000	Cell Signaling
rabbit anti-fibrillarin	IF 1:1,000	Abcam
mouse anti-cytochrome C (clone 7H8.2C12)	IF 1:100	PharMingen
sheep anti-DIG	FISH 1:200	Roche
mouse anti-FLAG	WB 1:1,000	Sigma

WB: western blot
IF: immunofluorescence

Table 2.2 Secondary Antibodies

Conjugate	Dilution	Source
AlexaFluor- 488, 546, or 555	1:2,000	Invitrogen
HRP	1:50,000	Jackson ImmunoResearch Laboratories

2.1.6 Other reagents

Trans-Blot Transfer Memebrane (0.2 μm nitrocellulose)	Bio Rad
Bright Star Plus (+ charged nylon membrane)	Ambion
Hartley Guinea Pigs	Charles River Laboratories
Lab-Tek Chambered #1 Borosilicate Coverglass System	Nalge Nunc International

2.17 Oligonucleotides

Table 2.3

Name	Sequence	Application
dgw 5' pGS	CGCA**GACGTC**TTATGCGTGAAGCCC	subcloning *dgw* ORF into expression vectors
dgw 3' +AatII	TGCG**GACGTC**GACATATACATACATATGTATG	subcloning *dgw* ORF into expression vector
5′ PCM	**CACC**ATGGGCGTTCCCAAGTTCTTTC	subcloning *pcm* ORF into Gateway vector
3′ PCM	AGTTGGATGCGGGGAGTCGGG	subcloning *pcm* ORF into Gateway vector
5′ LSm4	**CACC**ATGCTGCCACTTTC	subcloning *dlsm4* ORF into Gateway vector
3′ LSm4	CGATCCGAAGAACTATTTCCTATT	subcloning *dlsm4* ORF into Gateway vector
5′*gw*, RNAi	TA A T A C G A C T C AC T A T A G G G A A G A T C A A T T A C C A G T T C C A	synthesis of double-stranded RNA for knockdown of *dgw* expression
3′*gw*, RNAi	T A A T A C G A CT C A C T A T A G G G A C A T A T A C A T A C A T A T G T A T G	synthesis of double-stranded RNA for knockdown of *dgw* expression
5′ outside (intron 6)	T G T A A C A G G C A G A A G G A A G C G T T T C C G AC C A T	Genotype verification of single embryos
3′outside (exon 9)	G G C A G T C A A T C C T G G C G G G G G A C C T CG A G A C G	Genotype verification of single embryos
5′ inside (intron 6)	C C A T C T G T C C G T A T G A A C T T C G A G	Genotype verification of single embryos
3′ inside (exon 9)	T C C G A A G T CGC G G T A C A T T G T T G A	Genotype verification of single embryos
MRP fwd	GCCGGTTTGAGTCTTCC	amplifying *dmrp* for synthesis of RNA probe

Table 2.3 cont.

Name	Sequence	Application
MRP rev +T7	**TAATACGACTCACTATAGGG** A AAAAAGGGAGTGCGCCG	amplifying *dmrp* for synthesis of RNA probe
GFP	GCGATCACATGGTCCTGC	verifying structure of C-terminal GFP fusion constructs
RFP	GCTGCGCGGCACCAACTTCC	verifying structure of C-terminal RFP fusion constructs
RFPr	GGACAGCTTCAAGTAGTCGG	verifying structure of N-terminal RFP fusion constructs
T3 promoter	AATTAACCCTCACTAAAG GG	amplifying CG7939 for synthesis of RpL32 RNA probe
T7 promoter	TAATACGACTCACTATAGGG	amplifying CG7939 for synthesis of RpL32 RNA probe

Restriction endonuclease recognition sites
Gateway *attB* sequence
T7 promoter

2.2 Plasmids

2.2.1 Plasmid DNA preparation

Plasmid DNA was prepared according to the instructions provided by the manufacturers of plasmid DNA isolation kits (section 2.1 Multicomponent Systems). BAC DNA was isolated according to a modified Qiagen Plasmid Midi Kit protocol. This protocol called "Isolation of BAC DNA using the Qiagen Plasmid Midi Kit (QP01 Apr-04)" http://www1.qiagen.com/literature/Default.aspx?Term=BAC+DNA+isolation&Language=EN&LiteratureType=4%3b8%3b9%3b10&ProductCategory=0 follows the Plasmid Maxi Prep protocol except the plasmid DNA is purified on a Qiagen-tip-100.

Plasmids used in this study are listed in Table 2.4 below.

Table 2.4 Plasmid Vectors

Name	Vector	Source
pENTR/D	Gateway entry	Invitrogen
pARW	N-terminal RFP expression; actin promoter	Drosophila Gateway Collection
pAWR	C-terminal RFP expression; actin promoter	Drosophila Gateway Collection
pHFW	Gateway N-terminal FLAG expression hsp70 promoter	Drosophila Gateway Collection
pP[GSry^{+}, hsEGFP]	*Drosophila* modular expression vector; actin promoter	(Schotta and Reuter, 2000)
pGS5-ry	modified from pP[GSry^{+}, hsEGFP] ; actin promoter	this study
pzero blunt	Cloning blunt PCR products	Invitrogen
pRSETA	N-terminal His, Xpress-tagged protein expression in bacteria	Invitrogen

Table 2.5 Plasmids with Inserts

Name	Inserted gene	Vector	Source
LD47780 cDNA	*dgw* (CG31992)	pOT2	DGRC
pZBgw	*dgw* (CG31992)	zero blunt	this study
pGFP*dgw*	*dgw* (CG31992)	pP[GSry^{+}, hsEGFP]	this study
pENTR-*dgw*	*dgw* (CG31992)	pENTR/D	this study
LD22664 cDNA	*pcm* CG3291	pOT2	DGRC*
pENTR-*pcm*	*pcm* CG3291	pENTR/D	this study
p*pcm*-RFP	*pcm* CG3291	pAWR	this study
RE35747 cDNA	*lsm4* (CG33677)	pFLC-1	DGRC
pENTR-*dlsm4*	*lsm4* (CG33677	pENTR/D	this study
pRFP-*dlsm4*	*lsm4* (CG33677	pARW	this study
pRFP-*dago2*	*dago2*	pARW	Justin Pare
phsFLAG-*dago2*	*dago2*	pHFW	this study
pENTR-GW182	Human GW182	pENTR	E. Chan
pGFP-GW182	Human GW182	pARW	this study
pDONR-GW2FL	Human TNRC6B	pDONR207	E. Chan
pGFP-TNRC6B	Human TNRC6B	pARW	this study
pENTR-GW3FL	Human TNRC6C	pENTR	E. Chan
pGFP-TNRC6C	Human TNRC6C	pARW	this study
pd*gw*A	dGW aa 1-1061	pRSETA	this study
pCoHygro	hygromycin resistance	pUC	Invitrogen
RH03940 cDNA	RpL32 (CG7939)	pFLC-1	DGRC
pBAC11E7	genomic DNA containing MRP gene	pBACe3.6	DGRC

*DGRC: *Drosophila* Genomics Resource Center

2.3 *Drosophila* Culture and Handling for Observation

2.3.1 *Drosophila* culture

Drosophila melanogaster strains (Table 2.6) were maintained at 18 or 25^{o}C according to standard maintenance procedures on semi-solid medium consisting of 1.2% brewer's yeast, 1.2% agar, 8% cornmeal 7.5% v/v blackstrap molasses supplemented with 0.25 g of methyl 4-hydroxybenzoate (Sigma, dissolved in 1.5 ml of 95% ethanol) as a preservative.

Table 2.6 *Drosophila* Strains

Strain name	Genotype /description	Source
***gw*^{1} mutant**	*gw*^{1}*/ciD ;* identified during an ethylmethylsulfonate mutagenesis screen for recessive lethal loci located on chromosome four; mapped to the 102C region.	Dr. J. Lock University of Alberta
***w*^{1118}**	Carries a mutant eye color gene, otherwise normal; isogenic for chromosomes 1, 2 and 3.	Bloomington stock #5905
Third chromosome GFP balancer	w+;Sb^{1}/TM3 P (w^{+mC}=ActGFP) JMR2,Ser^{1} Lab strain #155	Bloomington stock #4534
***dmrp* mutant**	Carries P[EPgy2]CG10365[EY08633] in *dmrp*	Bloomington stock #17481
histone-GFP	Carries the histone2AvD-GFP fusion on chromosome 3.	Clarkson and Saint, 1999.
histone-GFP, *gw*^{1}	histone-GFP/Sb; *gw*^{1}/CiD; used for characterizing *gw*^{1} mutant phenotype	made for this study
lethal P element insertion in CG10365	P[SUPor-P]CG10365[KG00107]	Bloomington stock #13040
***dmrp*/GFP**	P[EPgy2]CG10365[EY08633] w+;Sb^{1}/TM3 P (w^{+mC}=ActGFP) JMR2,Ser^{1} strain used for characterizing the *dmrp* mutant phenotype	made for this study

2.3.2 Observations of growth and development of *Drosophila* larvae

Embryos were collected on 60 x 15 mm apple juice-agar plates [17.5% (w/v), agar 20% (w/v) sugar, 50% (v/v) apple juice]. After hatching, larvae were maintained at 25^{o}C on the same plates supplemented with yeast paste, for the period of observation. Mouth hooks were dissected and mounted on slides in mounting medium (5g Mowiol 40-88 , 20 ml PBS, 10 ml glycerol, 2.5% DABCO [1,4-Diazabicyclo[2.2.2]octane]).

2.4 Preparation of anti-dGW antibody

2.4.1 Raising anti-dGW antibody

The 5′ XhoI fragment of p*ZB**gw*** encoding the first 1,061 amino acids of dGW was subcloned into pRSETA (Invitrogen) to make pGWA. The N-terminal dGW fragment was expressed in the BL21(DE3) bacterial strain according to the procedure described in the manual, http://tools.invitrogen.com/content/sfs/manuals/prset_man.pdf. The recombinant protein was first purified on Ni-NTA (nitrilotriacetic acid) agarose (QIAGEN) and re-purified by SDS-PAGE and electroelution from polyacrylamide according to the procedures in (Harlow, 1988), with the following modifications. The volume of elution buffer was reduced to 2 ml/g of wet gel. Instead of dialysis, the eluate was concentrated to 1 mg/ml using an Amicon Ultra-4 Centrifugal Filter Unit. The buffer was replaced twice with PBS and once with PBT [(PBS 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20)]. PBT prevented precipitation of protein. Hartley guinea pigs (Charles River Laboratories) were injected 4 times with 80 μg of protein per injection.

2.4.2 Affinity purification of anti-dGW antibody

The immunogen for raising the anti-dGW antibody, a recombinanat protein consisting of the first 1,060 amino acids of dGW, was coupled to a 1-ml HiTrap *N*-hydroxysuccinimide-activated high performance column (GE Healthcare).

100 μg of serum protein was bound to this column and then eluted using Immunopure gentle elution buffer (Pierce Chemical Co.). The concentration of the eluted antibody was increased to 15 μg/μl in a solution of 50 mM Tris pH 8.0 and 50% glycerol, using a Centricon-10 ultrafiltration unit (Millipore). Anti-dGW serum recognized cytoplasmic foci colocalizing with GFP-dGW in stably transformed S2 cells fixed with 2% PFA (Fehon et al., 1990), whereas no specific signal was seen with the preimmune serum. *Drosophila* embryos were fixed as described in (Hughes and Krause, 1999) and rehydrated in 1× PBS. The following primary antibodies were used: mouse anti-α-tubulin (1:100; Sigma-Aldrich), rabbit anti-centrosomin (1:100; a gift from T. Kaufman, Indiana University, Bloomington, IN), mouse anti-phosphotyrosine (1:1,000; Cell Signaling), rabbit anti-fibrillarin (1:1,000; Abcam) and mouse anti-cytochrome c clone 7H8.2C12 (1: 100 Pharmingen). All secondary antibodies were conjugated to AlexaFluor 488, 546, or 647 (Invitrogen) and used at 1:2,000. DNA was stained using PicoGreen (1:1,000; Invitrogen).

2.5 Molecular Biological Methods

2.5.1 DNA sequencing

DNA sequences were determined using the Applied Biosystems Big Dye Terminator Cycle Sequencing materials and methods.

2.5.2 Preparation of protein extracts from *Drosophila* tissues

Protein extracts were prepared in 2.5x SDS gel sample buffer (157 mM Tris, 0.025% bromophenol blue, 5% SDS, 25% glycerol, and 50 mM DTT), immediately heated to 98°C and centrifuged for 5 min at 12,000 *g*. For each extract derived from multiple individuals the equivalent of 200 μg wet mass was loaded. For analysis of individuals, each adult or embryo was solublized in 8 μl of 2.5x SDS gel sample buffer. Early developmental extracts contained 5 embryos in 25 μl of sample buffer

2.5.3 Protein separation by polyacrylamide gel electrophoresis

Proteins were separated by discontinuous SDS (sodium dodecyl sulfate) PAGE [polyacrylamide gel electrophoresis (Laemmli, 1970)]. Proteins were first compressed on stacking gels consisting of 3.5% acrylamide (30:1 acrylamide: N,N′ -methylene-bis-acrylamide), 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.05 % ammonium persulfate, 0.001% TEMED. Proteins were separated on resolving gels consisting of 6% acrylamide (30:1 acrylamide: N,N′ -methylene-bis-acrylamide), 375 mM Tris pH 8.8, 0.05% ammonium persulfate, 0.001 % TEMED. Gels were cast and run using the Bio-Rad Mini-Protean 3 system. Gels were run at 150-200 V in buffer consisting of 25 mM Tris, pH 8.8, 0.2 M glycine and 0.1% SDS.

2.5.4 Western blot analysis

Samples were loaded onto a 6% discontinuous SDS polyacrylamide gel. After fractionation, proteins were transferred to a nitrocellulose membrane (BioRad) using a BioRad Mini Trans-Blot Cell in transfer buffer (96 mM Tris, 80 mM glycine, 40% methanol) at 90 V for 1 h. Non-specific protein binding to membranes was blocked in PBT with 0.1% powdered milk. dGW was detected by incubating with anti-dGW serum (1:1,000). Protein loading was standardized using 1 μg/ml of E7 anti-β-tubulin monoclonal antibody (Developmental Studies Hybridoma Bank). Primary antibodies were detected by incubating with horseradish peroxidase (HRP)-conjugated anti-guinea pig antibody (1:50,000; Jackson ImmunoResearch Laboratories), followed by incubation with Super Signal West Pico Chemiluminescent Substrate (Pierce). Blots were exposed to X-ray film and signals were quantitated by densitometry. For re-probing, blots were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) according to the manufacturer's protocol.

2.5.5 Preparation of RNA antisense probes

Each probe was transcribed from a DNA template using T7 or T3 polymerase. dMRP RNA probes were transcribed from PCR-amplified genomic dMRP RNA cloned in BAC11E7 (the *Drosophila melanogaster* BAC library genebank accession AC008201.8). T7 promoter sequences were appended to amplified dMRP RNA by including these

sequences in the reverse primer. The template for RpL32 (CG7939) was amplified from the cDNA RH03940 using T3 and T7 promoter primers from corresponding phage promoter sequences in the polylinker regions of the vector (pFLC-I). The RpL32 antisense RNA probe was transcribed using T3 polymerase. For Northern blot analysis, probes were labeled with ^{32}P in a 20 μl transcription reaction containing T7/T3 Buffer (Invitrogen), 0.5 mM each of ATP, CTP, and GTP, 12 μM UTP, 20 U SUPERase-In (Ambion), 50 U T7 polymerase (Invitrogen), 100 ng of DNA template and 50 μCi of [α-^{32}P]-UTP (800 Ci/mmol, 20 mCi/ml Perkin Elmer). For fluorescent *in situ* hybridization, probes were labeled with the steroid digoxigenin (DIG) conjugated to UTP in a 20 μl transcription reaction containing T7 Buffer (Invitrogen), 100 ng of template, 2 ul of 10X DIG labeling mix (10 mM each of ATP, CTP, and GTP 6.5 mM UTP, 3.5 mM DIG-UTP; Roche), 20 U RNase OUT (Invitrogen) and 100 U T7 polymerase (Invitrogen). Labeling reactions were assembled at room temperature and incubated for 2 h at 37^{o}C. Labeled probes were purified using mini Quick Spin RNA Columns (Roche) according to the manufacturer's protocol.

2.5.6 Northern blot analysis

Total RNA was extracted using Trizol (Invitrogen) from adults and staged collections throughout development. Equal amounts of denatured RNA (1-2 μg) were loaded in RNA gel loading buffer (Eppendorf); 2.5 to 5 μl of buffer for each 1.0 μl of RNA. Redistilled formamide (Invitrogen) was added to samples to a minimum of 60% final concentration to allow fractionation on native 2% agarose gels (Masek et al., 2005). RNA was capillary-transferred to a Brightstar-Plus Membrane (Ambion) according to the Qiagen Bench Guide pages 57-66 (http://www1.qiagen.com/literature/BenchGuide/pdf/1017778_BenchGuide.pdf) and UV cross-linked with a 120 mJ burst for 30 s. The membrane was pre-hybridized in 5-10 ml hybridization buffer (3 M urea, 5X SSC, 0.1% (w/v) N-laurylsarcosine, 0.02% SDS, 0.5% BSA, 0.1 mg/ml sonicated salmon sperm DNA) in a glass hybridization tube for 1 h at 60°C. Hybridization buffer was replaced with 5 ml of fresh buffer containing the appropriate antisense RNA probe labeled with [α-^{32}P]-UTP (see preparation of antisense RNA probes) and the membrane was hybridized overnight at 60°C. The entire probe

labeling reaction was added to the hybridization solution. Membrane was washed for 15 min at increasing wash stringencies until the background signal was removed. The low stringency wash was at room temperature using 2X SSC, 0.1% SDS. The high stringency wash was at 65 °C using 0.1X SSC, 0.1% SDS. For both MRP and RpL32 probes, final exposures with lowest background were reported after the highest stringency washes. Images of blots were acquired on a Storm 840 phosphorimager (Molecular Dynamics). For re-probing, membranes were stripped in 0.1% SSC, 0.5% SDS for 30 min to 1 h at 95^{o}C. Memebranes were exposed after stripping to ensure removal of probe.

2.6 Expression of Tagged P Body Components

2.6.1 Construction of tagged P body components

N-terminal fusions of human GW proteins and *dgw* were constructed to avoid interfering with the C-terminal RRM domain. Choice of terminus for appending tags for other *Drosophila* genes was based on previously reported fusions of homologous of these genes in other species. RFP was appended to the N-terminus of *dlsm4* like the N-terminal fusion of human LSm4 (Ingelfinger et al., 2002). RFP was appended to the C-terminus of *pcm* like the C-terminal fusion of *Saccharomyces cerevisiae* XRN-1 (Sheth and Parker, 2003). FLAG was appended to the N-terminus of *dAgo2* like the N-terminal fusions of human Ago2 (Liu et al., 2005b; Sen and Blau, 2005). Construction of fusions was verified by sequencing across the junction of the tag and the *Drosophila* open reading frame (ORF).

The *dgw* ORF and 3′ UTR were amplified from the cDNA LD47780 with primers dgw 5′ pGS, and dgw 3'+ *Aat*II to append flanking AatII sites and cloned into pZero Blunt (Invitrogen) to make pZBgw. A GFP-dGW fusion was constructed by subcloning *gw* from pZBgw into the *Aat*II site of pP(GS5-ry), a modified pP(GS[ry^{+}, hsEGFP]) expression vector (Schotta and Reuter, 2000) to make pGFP*dgw*. The vector was modified by removing the rosy (ry^{+}) eye colour gene (a 7291 nucleotide HindIII fragment), which was not needed for transfection of S2 cells. This resulted in a smaller vector that could be transfected more efficiently.

Gateway technology was used to construct remaining fusions of PB components (Hartley et al., 2000). Briefly, ORFs of *dlsm4* and *pacman* were inserted into a Gateway entry vector by site-specific recombination. ORFs were PCR-amplified, appending the *attB* sequence to the 5'end of the ORF. Enzymes that mediate site-specific recombination recognize the *attB* sequence on the PCR product and *attP* sequences on the vector. The recombination reaction, using Gateway® BP Clonase® Enzyme Mix (Invitrogen), directionally inserts the ORF into the entry vector to create an entry clone. Expression constructs were made by a second site-specific recombination reaction between the entry vector and the appropriate expression vector obtained from the *Drosophila* Gateway Vector Collection (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html) using Gateway® LR Clonase® Enzyme Mix. This produced an expression construct in-frame with the desired tag. *dlsm4* was recombined into pARW to make an N-terminal RFP fusion and *pacman* was recombined into pAWR to make a C-terminal RFP fusion. The d*ago2* ORF from the REO4347 cDNA (Hammond et al., 2001) was cloned into pAWR by Justin Pare, University of Alberta (Schneider et al., 2006). The human GW genes cloned in Gateway entry vectors were a gift from Edward Chan (Department of Oral Biology, University of Florida). These were recombined into pARW to make N-terminal RFP fusions.

2.6.2 Detection of tagged P body components in *Drosophila* cells

Tagged P body components were expressed in *Drosophila* S2 cells grown in HyQ-CCM3 serum-free medium (Hyclone). To observe fluorescent fusions of PB components in live cells, approximately 10^6 cells in 35 mm cell culture dishes were transfected with 1.6 μg of each plasmid and 7 μl of Cellfectin (Invitrogen) according to the manufacturer's protocol for suspension cells. Cells were left as a loosely attached monolayer in dish and medium was changed by aspirating. Transiently transfected cells were observed 24-48 hs after transfection. To observe live cells, cells were transferred to chambered coverglasses (Lab-Tek Chambered #1 borosilicate Coverglass System; Nalge Nunc).

Cells stably expressing GFP-dGW were transfected with 0.1 μg of pCoHygro (Invitrogen) and 1.6 μg of pGFP*dgw*. Stably transformed cells were selected using 300 μg/ml of hygromycin B. To induce GFP-dGW expression, cells were heat shocked for 1-

2 h at 38°C, incubated 1-2 h at 25°C to recover and heat shocked again for 1-2 h at 38°C. GFP-dGW was visible 6 h after the first heat shock. Images of GFP-dGW localization were acquired approximately 24 h after the first heat shock.

To detect human GW182 expression, endogenous dGW was knocked down by RNA*i*. To make double-stranded *dgw* RNA, a template encompassing the 3′ UTR of *dgw* was amplified using primers 5′*gw*, RNAi and 3′*gw*, RNAi. Double-stranded (ds) RNA was synthesized from this template using the MEGAscript High Yield Transcription Kit (Ambion). 15 μg of dsRNA was added directly to approximately 10^6 S2 cells in a 35mm cell culture dish containing 1 ml of medium. After 1 h, an additional 2 ml of medium was added. After 48 h cells were transiently transfected with GFP-GW182.

To detect a physical interaction between dGW and *dAgo2*, S2 extracts from cells transfected with FLAG-Ago2 were immunoprecipitated with anti-dGW antibody. Approximately 5 x 10^7 cells were transiently transfected with 8 μg of hsFLAG-Ago2. After 24 h cells were heat shocked at 38 °C for 30 min to induce FLAG-Ago2 expression, allowed to recover for 1 h and lysed in 4 ml of RIPA buffer (1% sodium deoxycholate, 1% NP-40, 0.2% SDS 150 mM NaCl, 50 mM Tris pH 7.4, supplemented with complete EDTA-free protease inhibitior [Roche]). The extract was divided into 2 parts. To one part, 5 μl of anti-dGW antibody was added and to the other part, 5 μl of pre-immune serum was added. Extracts were incubated on a rocker for 30 min at room temperature. 40 μl of a 1:1 suspension of protein A sepharose beads (Zymed) were added and incubated for 2 h at 4°C. Beads were washed 4 times with RIPA buffer, then once with PBS. Bound proteins were removed from beads by boiling for 5 min in 2x sample buffer (125 mM Tris, 0.02% bromophenol blue, 4% SDS, 20% glycerol, and 40 mM DTT).

After fractionating dGW-associated proteins on a 6% polyacrylamide gel, dAgo2 was identified by Western blotting. The blot was probed with anti-FLAG antibody diluted 1:1,000 (Sigma).

2.7 Genotype Verification of Single *Drosophila* Embryos

The gw^1/gw^1 genotype of individual embryos was confirmed by amplification of genomic DNA by PCR. Embryos were lined up on a glass slide, hand-dechorionated, covered with Halocarbon oil 700 (Sigma) and photographed. Oil was blotted away from each embryo

prior to transfer to a PCR tube. Primers [5′ outside (intron 6) and 3′ outside (exon 9)] and PCR Supermix (Invitrogen) in a volume of 50 µl were added to each tube. After PCR amplification, 3 µl of the reaction was further amplified in a second nested PCR reaction with 5′ inside (intron 6), and 3′ inside (exon 9) primers. The stop mutation (TGG to TGA) in *gw*^{1} disrupts an NcoI recognition sequence. The genotype of each embryo was initially characterized by digesting PCR products purified using the QIAquick PCR Purification Kit (QIAGEN) with NcoI. DNA that failed to be digested with NcoI was identified as homozygous mutant, DNA that was completely digested was normal and DNA that was partially digested was heterozygous mutant. Genotypes were confirmed by DNA sequencing.

2.8 Subcellular Localization in Fixed Cells and Tissues

2.8.1 Embryo fixation

Embryos were fixed according to the procedure described in (Hughes and Krause, 1999). Briefly, staged collections of embryos were dechorionated in a 1:1 mixture of bleach and water and fixed by shaking for 20 min in a mixture of 8 ml heptane, 2.5 ml of PBS (8mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) and 250 µl of 40% paraformaldehyde. After fixation embryos were transferred into methanol and stored at -20^{o}C.

2.8.2 Fluorescent antibody staining of *Drosophila* embryos

Fixed embryos were rehydrated in 1 ml of PBT for 15 min at room temperature, then washed once in fresh PBT. Non-specific protein binding was blocked by incubating in blocking buffer (PBT + 0.5% powdered milk) for 1h. Embryos were then resuspended in 1 ml of fresh blocking buffer containing primary antibody and incubated at 4^{o}C overnight. Embryos were washed 4 times with fresh blocking buffer for 15 min. Embryos were incubated for 2 h in the dark with secondary antibody conjugated to AlexaFluor 488, 546 or 555 (Invitrogen) diluted 1:2,000 and Pico-Green (Invitrogen) diluted 1:2,000 in blocking buffer. Embryos were then washed as for primary antibody incubation, except

PBS was used for the last wash because Tween produces a slight fluorescence. All incubations were performed at room temperature, unless specified, with gentle rocking.

2.8.3 Fluorescent antibody staining of S2 cells

Cells were grown on coverslips in 35 mm culture dishes. Cells were washed once with PBS and fixed in 2% paraformaldehyde in PBS for 15 min at room temperature. After fixation, cells were washed 3 times with PBS and incubated with primary antibody in PSN (PBS, 0.1% saponin, 1% normal goat serum). Cells were washed three times with PBS, then incubated with secondary antibody (Alexa fluor; Invitrogen) for 1 h at room temperature in the dark. Cells were washed three times and mounted in mounting medium (section 2.3.2).

2.8.4 Fluorescent *in situ* hybridization (FISH)

DIG-labeled antisense RNA probes were hybridized to fixed embryos as described in (Hughes and Krause, 1999). Treatment of embryos with proteinase K prior to hybridization was not required for visualization of dMRP RNA but unexpectedly enhanced detection of nuclear dGW by fluorescent antibody staining. RNA probes were visualized by fluorescent antibody staining using a primary sheep anti-DIG antibody (1:200; Roche) and secondary anti-sheep alexa-conjugated fluorescent antibody (1:2000; Molecular Probes).

2.9 Imaging

All imaging was performed at 25°C. Confocal images were obtained using an Axiovert 200 (Carl Zeiss MicroImaging Inc) inverted microscope equipped either with a spinning disk confocal system (Ultraview ERS; PerkinElmer) mated with a camera (Orca AG; Hamamatsu) or a LSM 510 confocal scanner.

CHAPTER 3: *DROSOPHILA* GW IS REQUIRED FOR EARLY *DROSOPHILA* DEVELOPMENT

3.1 Overview

3.1.1 Discovery and characterization of human GW182

The human GW182 protein was identified as an autoantigen recognized by the serum of a patient with a motor and sensory neuropathy that localized to novel cytoplasmic foci (Eystathioy et al., 2002). The protein was named after the multiple glycine (G) / tryptophan (W) repeats it contains. A role for GW182 in mRNA regulation was initially proposed because of the presence of an RNA recognition motif (RRM) at its C terminus and because it was found to physically interact with a subset of mRNAs (Eystathioy et al., 2002). Consistent with this role, GW182 was subsequently found to colocalize with proteins involved in mRNA regulation, the decapping activators Dcp1 and LSm4 (Eystathioy et al., 2003). The addition of GW182 to a growing number of mRNA regulatory proteins that localize to cytoplasmic foci, such as the general mRNA decay factors Xrn1 (Bashkirov et al., 1997), Dcp2 and the Lsm1-7 complex (Ingelfinger et al., 2002; van Dijk et al., 2002), contributed to the development of the concept of the mRNA processing body or PB as a compartment for the regulation of mRNA (Eulalio et al., 2007a).

3.1.2 Proteins related to human GW182 form a conserved metazoan family

Proteins that are structurally related to GW182 (GW proteins) have been identified in metazoan but not yeast genomes. These include two additional human paralogues of GW182, TNRC6B and TNRC6C, as well as orthologues in *Drosophila* and *C. elegans* (Ding and Han, 2007; Ding et al., 2005; Meister et al., 2005; Schneider et al., 2006). GW proteins from all three species share a region of amino acid sequence homology that has not yet been identified in other proteins (Ding and Han, 2007; Ding et al., 2005). This domain is named the GAGH domain after GW proteins of all three species (GW182 (human)/ AIN-1 (*C. elegans*)/ Gawky (*Drosophila*)/ homologue). The human GW proteins and Gawky also share an RRM domain and glutamime-rich regions, which are

not present in the *Caenorhabditis elegans* GW proteins. It is possible that in *Caenorhabditis elegans* there is another protein that contains these motifs and acts in conjunction with Ain-1 and Ain-2 to perform the function of single human or *Drosophila* GW proteins. The ubiquitin-associated domain (UBA) is found in Gawky and one human GW protein, TNRC6C. Some sequence motifs have been identified in only one GW protein. These include a potential nuclear localization signal (NLS), which has been identified only in GW182, and a serine-rich region which is present only in Gawky (Fig.2).

3.1.2 Functions of GW proteins

The results of some studies suggest that GW proteins play a role in PB assembly. siRNA-mediated depletion of GW182 (Yang et al., 2004) or expression of an N-terminal fragment of GW182 resulted in dispersal of PBs (Jakymiw et al., 2005). The disruption of PBs in the absence of functional GW182 could be caused by two possible mechanisms. First, GW182 could function as a matrix or scaffold for PB assembly (Yang et al., 2004) so that in its absence, PB components would be unable to aggregate into larger structures. Second, GW182 could target mRNA for translational suppression or degradation so that GW182 depletion reduces the levels of non-translating mRNA.To elucidate the mechanism of PB assembly, one study observed the effects of increasing levels of non-translating mRNA by treatment with arsenite, an inhibitor of translation initiation (Serman et al., 2007). After dispersing PBs by siRNA-mediated depletion of GW182, Dcp1-containing PBs lacking GW182 were reformed upon treating cells with arsenite. These results suggest that GW182 may influence PB formation by targeting mRNA for translational suppression or degradation and may not be required as a scaffold for PB assembly. These results do not, however, exclude the possibility that GW182 may enhance formation of some types of PBs by acting as a scaffold. Moreover, the presence of PBs in HEp-2 cells (Eystathioy et al., 2002; Eystathioy et al., 2003), which do not express detectable levels of GW182 (Bloch et al., 2006), further supports the idea that GW182 may not be required for formation of all PBs. Formation of PBs in these cells may be enhanced by the other human GW proteins.

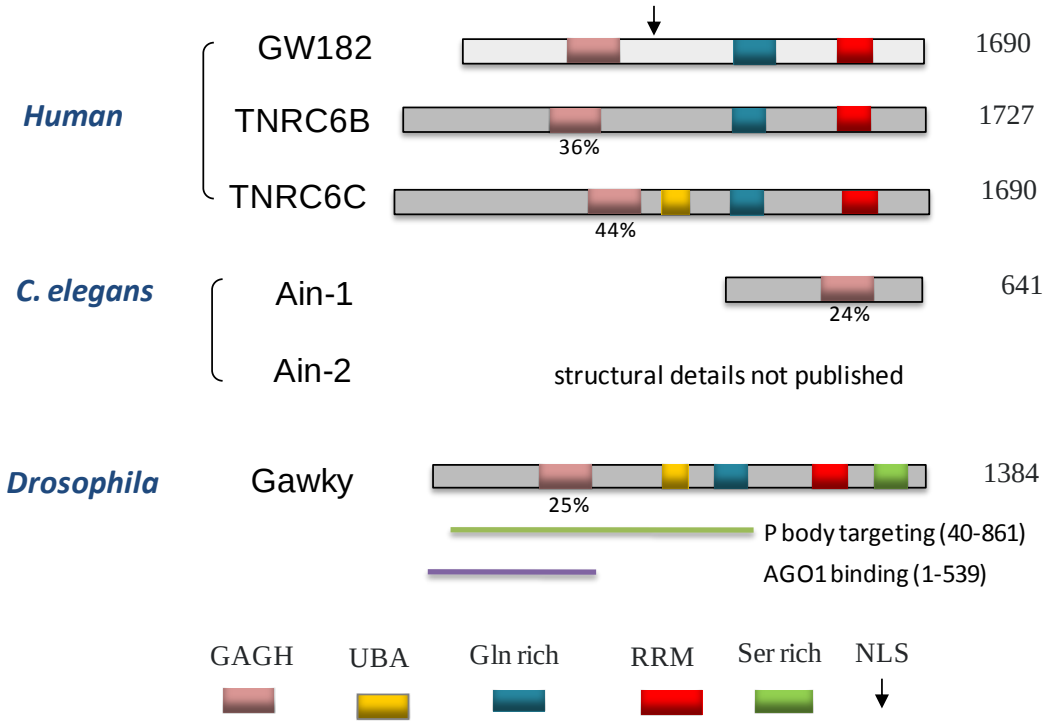


Figure 2. A comparison of the GW protein family. Human, *Caenorhabditis elegans* and *Drosophila* GW proteins contain a conserved GAGH domain that is unique to this family of proteins. Numbers indicate the percent of amino acids in GAGH domains identical with GW182 (Ding, Spencer et al. 2005). An RRM and a glutamine-rich region are found in all GW proteins except Ain-1. A potential NLS is present in GW182. A UBA domain is present on *Drosophila* GW and TNRC6C. *Drosophila* GW contains a C-terminal serine-rich region that is not present in other GW proteins. Coloured lines represent functions mapped to N-terminal regions of *Drosophila* GW. A region including the UBA and glutamine-rich domains is required for P body targeting, and a smaller N-terminal region interacts with Ago1 (Behm-Ansmant, Rehwinkel et al. 2006).

A relationship between GW proteins and the RNA*i* pathway was initially established on the basis of interaction of GW proteins with Argonaute proteins. Argonaute proteins are components of the RNA-induced silencing complex (RISC) that associates with specific mRNAs targeted for repression or degradation (Meister and Tuschl, 2004). GW proteins encoded in human, *Drosophila* and *Caenorhabditis elegans* genomes physically and/or genetically interact with Argonaut proteins (Behm-Ansmant et al., 2006; Ding et al., 2005; Liu et al., 2005a; Schneider et al., 2006). Further, Argonaute proteins accumulate in cytoplasmic foci that contain GW (Ding et al., 2005; Liu et al., 2005a; Schneider et al., 2006; Sen and Blau, 2005).

Most organisms encode several Argonaute paralogues that function in different RNA*i*-mediated gene silencing mechanisms. Targeting specific transcripts for RNA*i*-mediated gene silencing requires complementary base pairing with small 21-25 nucleotide-long guide RNAs. Targeted transcripts are subject to two possible outcomes: repression or degradation. The outcome is determined by the degree of complementarity between the guide RNA and the target mRNA. Guide RNAs with partial complementarity to the target transcript, such as animal microRNAs (miRNA)s, direct translational suppression, while those with perfect or near perfect complementarity, such as small interfering RNAs (siRNA)s, direct degradation [reviewed in (Meister et al., 2005; Pillai et al., 2005)]. Different RNA*i*-mediated silencing pathways are mediated by different Argonaute proteins. In human and *Drosophila* genomes Argonaute(Ago)2 efficiently cleaves the target mRNA while Ago1 functions in translational repression (Behm-Ansmant et al., 2006; Hammond et al., 2001; Liu et al., 2004; Meister et al., 2004; Okamura et al., 2004). Other *Drosophila* Argonaute paralogues, Piwi, Aubergine and Ago3 bind repeat-associated siRNAs that silence selfish genetic elements such as transposons in the germ line (Brennecke et al., 2007; Saito et al., 2006; Siomi et al., 2008; Vagin et al., 2006).

The combined results of a number of recent studies indicate that GW proteins play a role in gene silencing by both miRNA and siRNA. Depleting GW182 or expressing a dominant-negative form of GW182 resulted in failure in siRNA-mediated gene silencing (Jakymiw et al., 2005; Lian et al., 2007). Depleting the GW182 paralogue TNRC6B had a similar outcome (Meister et al., 2005). The results of studies that

compared the influence of GW proteins on silencing by siRNA versus miRNA showed that depletion of human GW182 or *Drosophila* GW impaired silencing by miRNA to a greater degree than by siRNA (Chu and Rana, 2006; Liu et al., 2005a; Rehwinkel et al., 2005).

There are no published reports demonstrating a role for GW proteins in known mRNA regulatory pathways other than RNA*i*. One study showed that *Drosophila* GW can cause mRNA degradation through a direct interaction with mRNA. This was documented by observing degradation of a reporter mRNA that was artificially tethered to dGW (Behm-Ansmant et al., 2006). This suggests that GW proteins may promote mRNA degradation by a mechanism that has not yet been identified and may not involve RNA*i*. There is evidence suggesting that GW proteins may not be required for some mRNA regulatory pathways. Depletion of *Drosophila* GW by siRNA does not interfere with NMD (Rehwinkel et al., 2005) and depletion of human GW182 by siRNA does not interfere with ARE-(AU-rich element)-mediated decay (Stoecklin et al., 2006).

Functional analysis of some individual domains of GW proteins has been reported although a comprehensive structure/function analysis is still lacking. Functions have been mapped to two broad regions of dGW (Fig. 2). Targeting to PBs was mapped to an N-terminal fragment including the UBA and Q-rich domains, while a smaller overlapping N-terminal fragment containing a region enriched in GW repeats was required for physical interaction with Ago1 (Behm-Ansmant et al., 2006).

A role for regions enriched in GW repeats in interacting with Argonaute proteins was identified in a protein unrelated to GW proteins. A GW-rich region was identified in a subunit of *Arabidopsis* RNA polymerase IV. RNA polymerase IV functions in RNA-directed DNA methylation as part of a nuclear gene silencing pathway. This subunit binds to Ago4 through a GW-rich region. This binding activity can be substituted by a GW-rich region from GW182 (El-Shami et al., 2007). Consequently, regions enriched in GW repeats may function by interacting with Argonaute proteins, at least in the RNA*i* pathway.

3.1.3 Objectives

The *Drosophila* genome encodes a single gene with a high degree of sequence similarity to human GW182 [CG31992; GenBank/EMBL/DDBJ accession no. AE003843] (Fig. 2). The first objective was to characterize the temporal and spatial expression of this gene. The second objective was to determine the degree of functional similarity it shared with GW182. The final objective was to characterize the phenotype of a *Drosophila* strain with a mutation in CG31992.

3.2 Results

3.2.1 *Drosophila* GW is expressed throughout development.

To examine dGW protein expression, a polyclonal antibody was raised against the N-terminal 1061 out of 1384 amino acids of the protein, up to but not including the RRM domain. Multiple protein species were detected by Western blot analysis, using this antibody. The number of these species varied between different protein extracts. These multiple species may be degradation products of full length dGW. The largest species, which migrates at approximately 160 kD in SDS PAGE, is within 10% of the predicted mass of 143 kD and likely represents full length dGW (Fig. 3A and B). To observe the temporal expression pattern of dGW throughout the *Drosophila* life cycle, protein levels at different stages of development were analyzed by Western blot. dGW was expressed throughout development, with higher levels present during embryonic development (Fig. 3B). Elevated dGW expression levels during early development suggest an important role for this protein at this time.

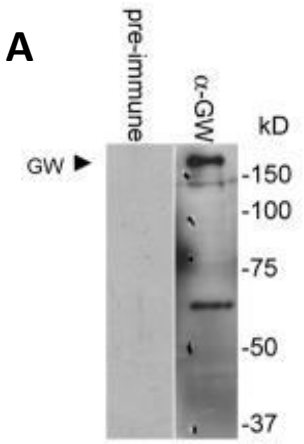
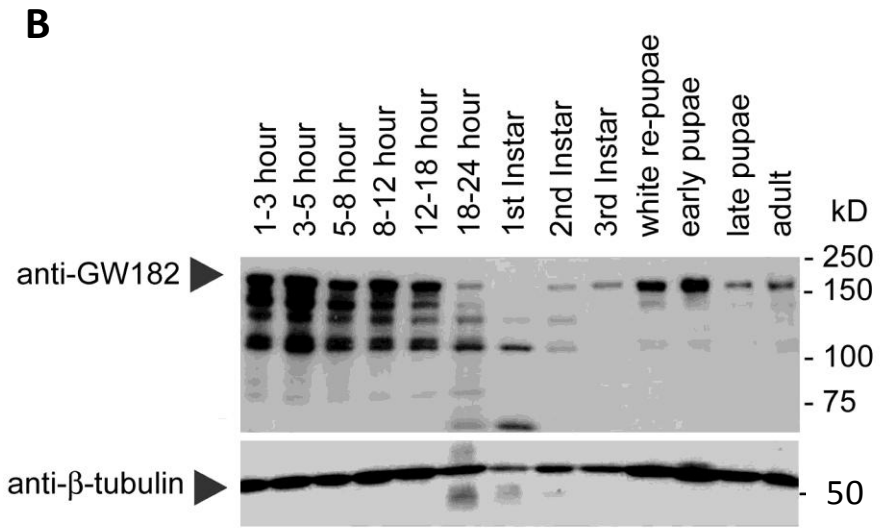


Figure. 3. dGW is expressed throughout development. Western blots showing: (A) recognition of a 160 kD protein by polyclonal anti-dGW antibody in an extract from S2 cells, (B) variable levels of dGW expression throughout development. Highest levels of dGW protein are seen up to 18 h during embryonic development.



3.2.2 *Drosophila* GW localizes to cytoplasmic foci homologous to human P bodies.

To determine if dGW is homologous to human GW182, its subcellular localization was observed in S2 cells. Like GW182, a GFP fusion of dGW localized to cytoplasmic foci resembling PBs, while expression of GFP was diffuse throughout the cell (Fig. 4 A and B), indicating that formation of foci was not due to aggregation of GFP. Cytoplasmic GFP-dGW foci were also detected by anti-dGW antibody, indicating the antibody is suitable for detecting the endogenous protein by immunofluorescence (Fig. 4 C and D).

The localization of GFP-dGW was compared with *Drosophila* homologues of other PB components to determine if the composition of these foci resembles PBs (Fig. 5). Fluorescent fusions of Pacmnan, the *Drosophila* homologue of the 5' to 3' exonuclease Xrn-1 (Grima et al., 2008; Newbury and Woollard, 2004; Zabolotskaya et al., 2008), and Ago2 (Hammond et al., 2001) also formed cytoplasmic foci. The subcellular distribution of CG33677 was also observed. CG33677 is annotated in FlyBase as an orthologue of LSm4 on the basis of sequence similarity to LSm4 genes in other species; however, the product of this gene has not been previously characterized. LSm4 is a component of two heptameric complexes with overlapping components that function in RNA processing. The LSm1-7 complex functions as a decapping co-activator and the LSm2-8 complex is involved in mRNA splicing (He and Parker, 2000; Salgado-Garrido et al., 1999). CG33677 formed cytoplasmic foci, but the majority of the protein localized to the nucleus. This distribution of *Drosophila* LSm4 (dLSm4) corresponds to the distribution of LSm4 previously observed in human cells (Eystathioy et al., 2003; Ingelfinger et al., 2002). As a component of both the LSm1-7 and LSm2-8 complexes, the nuclear fraction participates in mRNA splicing, and the cytoplasmic component is involved in activation of mRNA decapping (Parker and Song, 2004). Formation of cytoplasmic foci by *Drosophila* homologues of proteins that have been identified as PB components in other species is consistent with the idea that these cytoplasmic foci are *Drosophila* PBs.

dGW localized to some but not all foci containing PB components (Fig. 5). This is consistent with some published observations (Eystathioy et al., 2003; Ingelfinger et al., 2002) and suggests that dGW may be homologous to GW182.

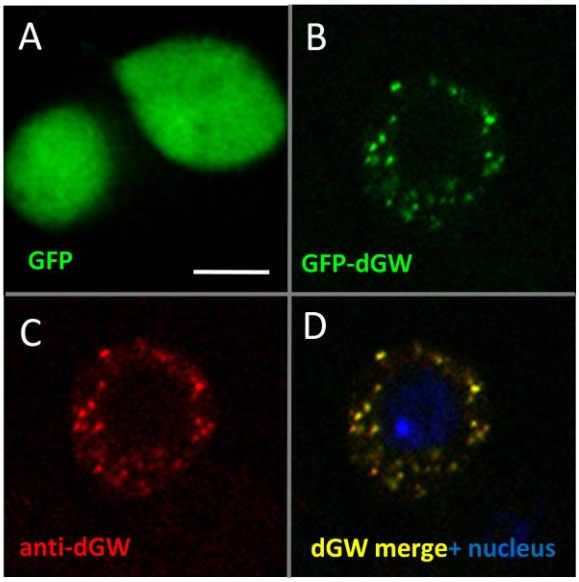


Figure 4. dGW localizes to cytoplasmic foci in S2 cells. (A) A cell line stably transformed with GFP under the control of a heat shock promoter shows diffuse GFP distribution throughout the cell. (B) A GFP fusion of dGW, expressed in a stably transformed S2 cell line under the control of a heat shock promoter, forms cytoplasmic foci. This suggests that cytoplasmic foci are not the result of aggregation of the GFP tag. (C) GFP-dGW is detected with anti-dGW antibody. (D) Cytoplasmic foci containing GFP-dGW are also recognized by anti-dGW antibody indicating the antibody is suitable for immunofluorescence. Bar is 5μm.

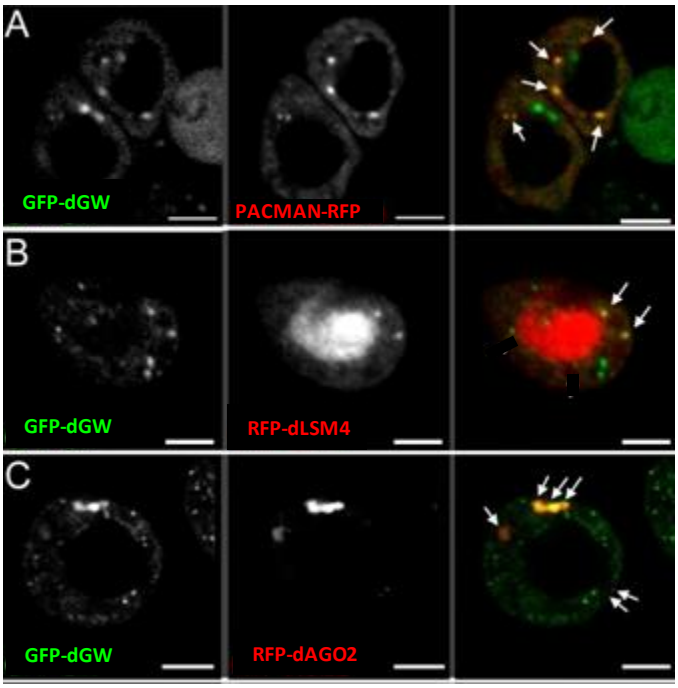


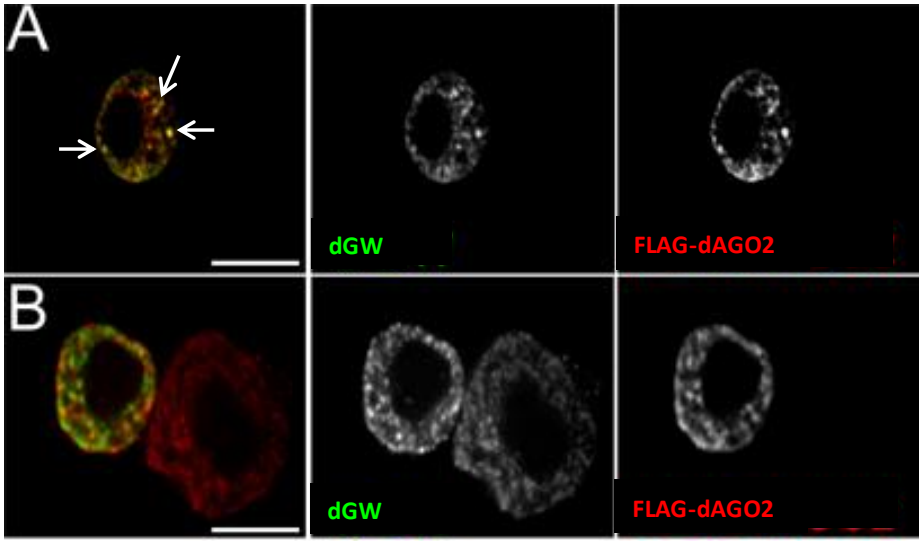
Figure 5. dGW localizes to cytoplasmic foci similar to human PBs. S2 cells stably transformed with GFP-dGW were transfected with expression vectors carrying the following PB components: (A) pcm-RFP, (B) RFP-dLSM4 or (C) RFP-dAgo2. Some, but not all, foci containing dGW colocalized with other PB components. Arrows indicate foci containing both dGW and another PB component. Bars are 5 μm.

However, some published studies showed complete colocalization of PB components (Liu, Rivas et al. 2005; Liu, Valencia-Sanchez et al. 2005; Sen and Blau 2005; Behm-Ansmant, Rehwinkel et al. 2006). To further support the idea that incomplete localization of PB components in this study was not due to mislocalization of fluorescently tagged proteins, localization of endogenous dGW with dAgo2 tagged with the small FLAG epitope was compared. Like results obtained with fluorescent fusions, colocalization of these proteins was seen in some but not all foci, with variable degrees of colocalization in different cells (Fig. 6A, B). To determine if the observed colocalization of FLAG-dAgo2 with dGW represented a physical interaction between these proteins, dGW was immunoprecipitated with anti-dGW antibody from cells transfected with FLAG-dAgo2. A protein corresponding to the size of dAGO2 detected with anti-FLAG antibody co-immunoprecipitated with anti-dGW antibody but not with pre-immune serum. (Fig.6C). This result is consistent with published reports of a physical interaction between human homologues of these proteins (Liu et al., 2005a). Incomplete colocalization of cytoplasmic foci containing various PB components seen in this study therefore indicates the presence of different classes of foci with distinct compositions.

A more direct approach was taken to further investigate the functional homology between dGW and human GW182 family proteins. Localization of GFP-dGW and RFP-fusions of human GW182 and its paralogues TNRC6B and TNRC6C was compared. The high degree of colocalization of dGW seen with all three human GW182 paralogues is consistent with a functional homology between *Drosophila* and human GW182 family proteins (Fig. 7).

3.2.3 dGW is required for early *Drosophila* development

A mutation in the *Drosophila gw* gene (CG31992), designated gw^{1}, was isolated in a screen for recessive lethal mutations on the *Drosophila* fourth chromosome. The mutant allele has a nonsense mutation that encodes a truncated protein lacking the RRM domain (Fig. 8 A). The location of this gene on chromosome four and a lack of early developmental markers on this chromosome required an alternative approach to identify mutant embryos. The gw^{1}mutation resulted in the loss of an NcoI site which facilitated



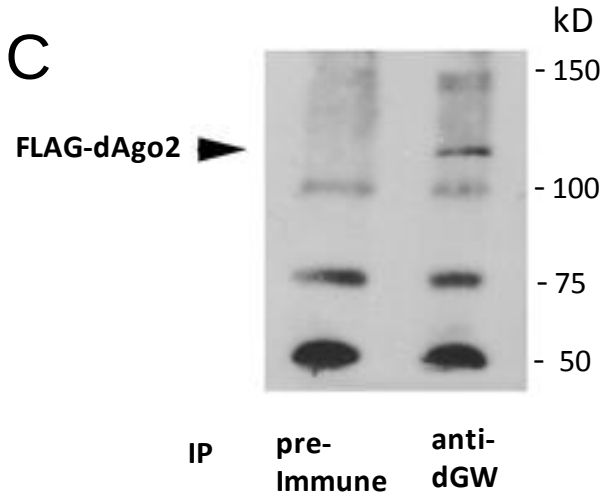


Figure 6. FLAG-dAgo2 colocalizes and associates with endogenous dGW.

(A) FLAG-dAgo2 colocalizes with endogenous dGW. Little or no colocalization is seen in (B). (C) Extracts of S2 cells transfected with FLAG-dAgo2 immunoprecipitated with pre-immune serum or anti-dGW antibody and Western blotted using anti-FLAG antibody. Arrowhead points to the band representing FLAG-dAgo2. dAgo2 is 137 kD (FlyBase). Bars are 5 μm.

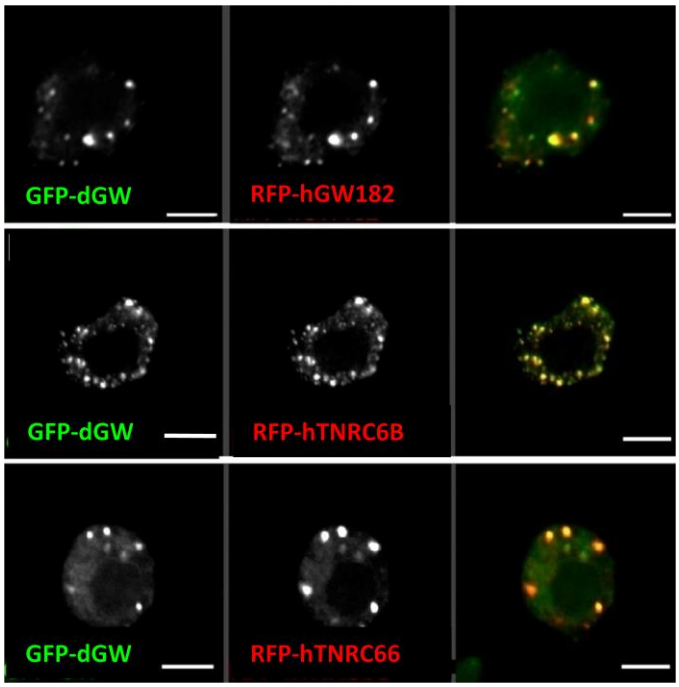


Figure 7. dGW colocalizes with all three human GW182 paralogues.

RFP fusions of the three major human GW182 family proteins (GW182, TNRC6B and TNRC6C) transfected into *Drosophila* S2 cells were found in the same structures as dGW. The expression of human GW182 could not be detected without a coincident RNA*i* knockdown of endogenous dGW. Bars are 5 μm.

genotyping of individuals. Genomic DNA of the region flanking the mutation was PCR-amplified from individual embryos and digested with NcoI. DNA samples that failed to be digested by NcoI were sequenced to confirm the presence of the mutation (Fig. 8 B, C). Mutant embryos could also be identified by detecting the truncated dGW protein by Western blot analysis of proteins from single embryos (Fig. 8 D).

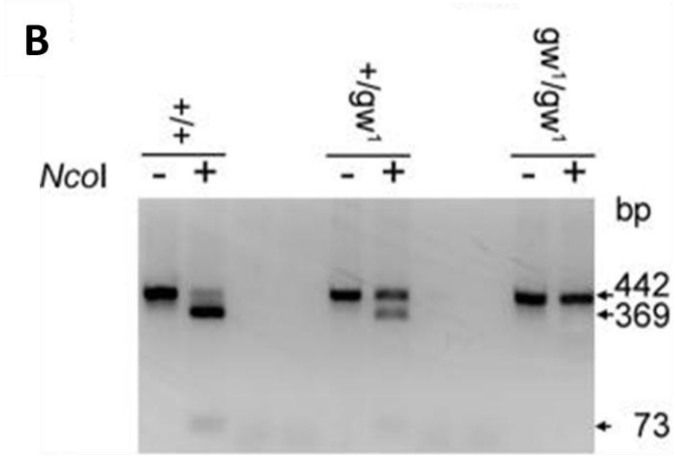
Disordered internal structures were seen in DIC images of some embryos produced by heterozygous gw^1/ci^D parents at 90–130 min after egg deposition (AED; Fig 9). It was impossible to determine the frequency of these embryos because many of them were fragmented during fixation. Genotype analysis of these embryos by PCR and NcoI digestion showed that the embryos with disordered internal structures were homozygous gw^1 mutant, while embryos that developed normally had at least one gw^+ allele (n = 200). These results indicate a lack of dominant interfering effects caused by expression of the truncated gw^1 mutant protein. This conclusion is supported by the viability of embryos produced by gw^1 /ci^D parents. Approximately 75% of these embryos hatched (Table 3).

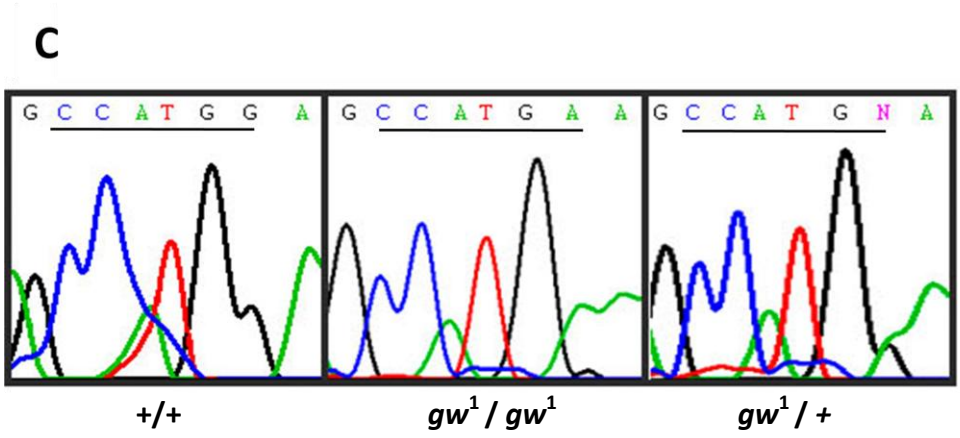
More detailed observation of the internal morphology of these embryos at this time showed that normal embryos were cellularizing. DNA staining showed that their nuclei were at the cortex, while plasma membranes, marked by anti-phosphotyrosine antibody, partly enveloped the nuclei. dGW was distributed both diffusely and in foci that formed a band below the cortex and surrounded the nuclei. In the abnormal embryos, the DNA, dGW and membranes formed aggregates (Fig. 9).

Table 3. Viability of gw^1/ci^D Embryos

Parental Genotype	# Hatched	# Unhatched	Total	% Viability
$\mathbf{W^{11118}}$	955	21	976	98
$\boldsymbol{gw^1/ci^D}$	956	300	1256	76


A
W967 STOP
RRM


B
+/+
+/gw1
gw1/gw1
NcoI
- + - + - +
bp
442
369
73


C
G C C A T G G A
G C C A T G A A
G C C A T G N A
+/+
gw1 / gw1
gw1 / +

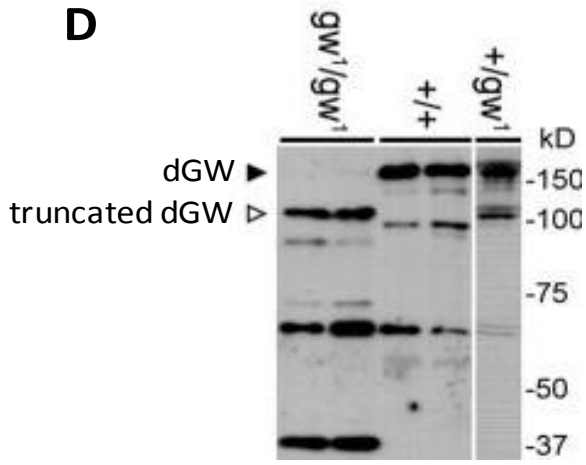


Figure 8. Identification of *gw*^{1} mutant individuals. (A) The *gw*^{1} allele has a mutation in the tryptophan codon at position 967 changing it to a stop codon. (B) The loss of an NcoI restriction site in the *gw*^{1} mutation facilitated genotyping of individual embryos. PCR-amplified genomic DNA from single embryos was digested with NcoI. (C) Genotypes were confirmed by sequencing PCR-amplified DNA. Normal and mutated *Nco*I sites are underlined. (D) Anti-dGW antibody recognizes a 100-kD truncated form of dGW in *gw*^{1}/*gw*^{1} embryos that is not present in wild-type embryos.

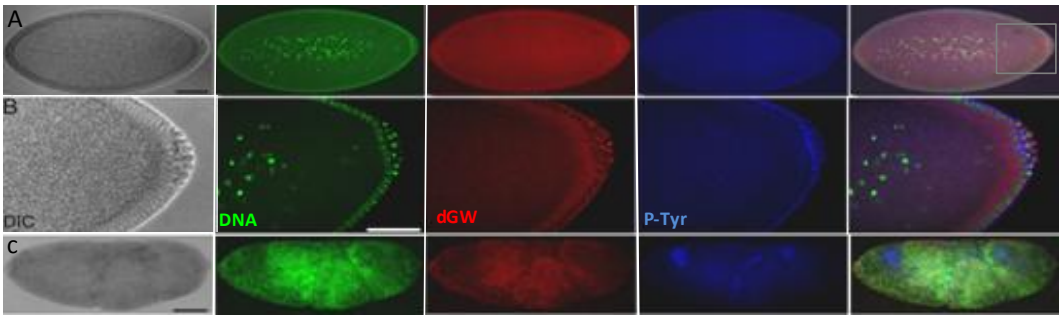


Figure 9. dGW localization in normal and homozygous *gw*^{1} mutant *Drosophila* embryos. (A–C) Embryos were fixed 90-130 min after egg deposition (AED). (A) In normal embryos undergoing cellularization (differential interference contrast [DIC]), dGW (red) localized to foci surrounding the cortical nuclei (DNA, green). The plasma membrane was stained with anti-phosphotyrosine (P-Tyr, blue) antibody. (B) The boxed area in A is shown magnified. dGW is seen in a band below the cortex and in foci surrounding the nuclei. (C) In homozygous *gw*^{1} mutant embryos, the DNA, anti-dGW, and anti-phosphotyrosine staining form disorganized aggregates. Bars are100 μm.

3.2.4 Homozygous *gw*^{1} mutants display defects in organization of nuclei at the cortex and multiple mitotic defects

During early *Drosophila* embryogenesis, nuclei undergo 13 synchronous nuclear divisions or nuclear cycles (NCs) without cytokinesis to produce a syncytial embryo. Nuclei migrate to the embryo cortex, forming a syncytial blastoderm during the 10th NC (Foe and Alberts, 1983). During the 14th NC, each nucleus becomes enclosed in a membrane, forming an individual cell. This is also the time of the mid-blastula transition (MBT) when the transfer from maternal to zygotic control of cellular processes is completed. After the MBT, the embryo begins to gastrulate [reviewed in (Cooperstock and Lipshitz, 1997)].

In homozygous *gw*^{1} blastoderm embryos, defects in spacing and morphology of cortical nuclei were observed. Unlike normal embryos which showed regular spacing of cortical nuclei, each with a pair of centrosomes, mutant embryos had fewer and irregularly spaced nuclei with abnormally positioned centrosomes (Fig. 10). To observe progression of these mitotic defects chromatin dynamics in live embryos expressing histone-GFP (Clarkson and Saint, 1999) were tracked. In normal embryos the blastoderm forms by synchronous movement of nuclei to the embryo cortex followed by several synchronous rounds of mitosis (Video 1). In homozygous *gw*^{1} embryos, movement of nuclei to the cortex was not synchronous and fewer nuclei reached the cortex. Consistent with the appearance of fixed mutant embryos (Fig.10), the chromatin at the cortex was unevenly spaced. Further, the chromatin underwent a much larger displacement with each NC indicating defective anchoring at the cortex. Finally, each nuclear division became progressively less coordinated, chromatin failed to segregate and large chromatin aggregates formed (Video 2). We named the mutant *gawky* (*gw*) because of the uncoordinated mitosis that was observed. Because the mutant phenotype develops shortly before the MBT, when transfer of cellular processes from maternal to zygotic control is complete, the mutant phenotype is likely to be caused by depletion of the maternal pool of normal dGW protein. A transient drop in dGW protein levels in normal embryos 50-60 min AED is consistent with this idea (Fig. 11).

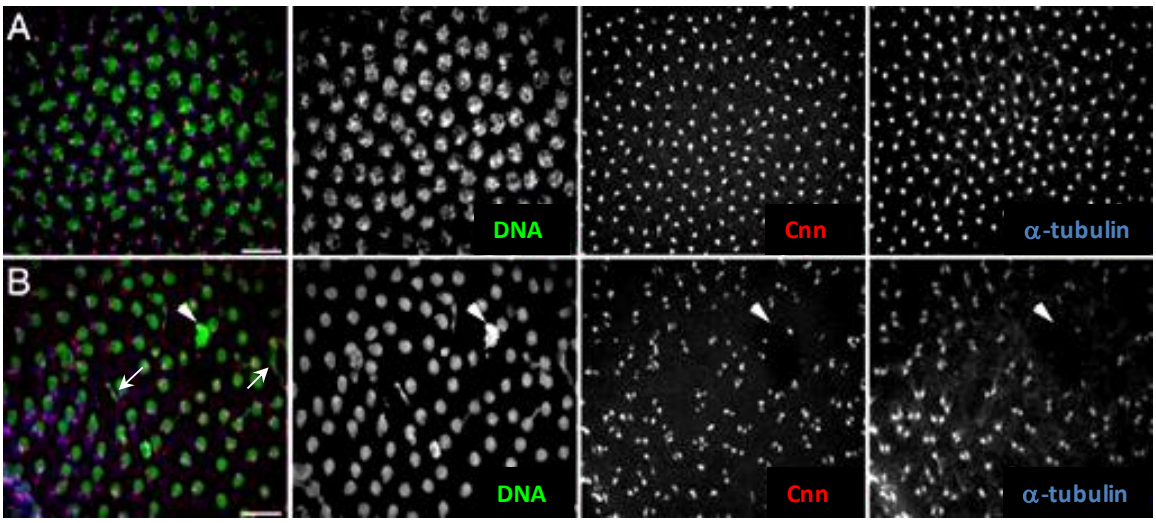


Figure 10. The lack of functional dGW protein leads to mitotic defects. (A) In wild-type embryos 90 min AED a regular array of nuclei (DNA, PicoGreen), each with a pair of centrosomes stained with anti-centrosomin (Cnn, red) antibody and anti-α-tubulin (blue), can been seen immediately below the embryo cortex. (B) In homozygous gw^{1} embryos of the same age, severe defects are observed after NC10. Fewer nuclei are seen, and the majority of them have improperly localized centrosomes. Defects in chromosome segregation (arrows) and large DNA aggregates are also seen (arrowheads). Images show a maximum projection of 125 slices that are 10 μm deep. Bars are 5 μm.

Video 1. A time-course video showing normal chromatin movements in an early *Drosophila* embryo. An embryo expressing histone-GFP was injected with guinea pig preimmune serum 1h AED. As expected in a normal embryo, nuclei form the blastoderm by synchronous movement to the cortex. This is followed by several synchronous rounds of mitosis. Anchoring of nuclei at the cortex during these divisions results in even chromatin spacing. The embyo gastrulates after the blastoderm is fully formed. Images were captured at 1 frame/10 s and are displayed at 20 frames/s. See DVD attached to back cover.

Video 2. **A time-course video showing abnormal chromatin movements in a homozygous *gw*^{1} mutant expressing histone-GFP.** The video begins at approximately 30 min AED. During blastoderm formation, fewer nuclei arrive at the cortex, particularly at the anterior and posterior ends. In subsequent NCs, a large displacement of chromatin is seen, indicating a failure in anchoring at the cortex. Chromatin divisions become less synchronous, and failure in separation becomes apparent as separating chromatin rejoins, forming paired structures. Finally, large chromatin aggregates form similar to those observed with DNA staining (Fig. 10 B). Images were captured at 1 frame/10 s and are displayed at 20 frames/s. See DVD attached to back cover.

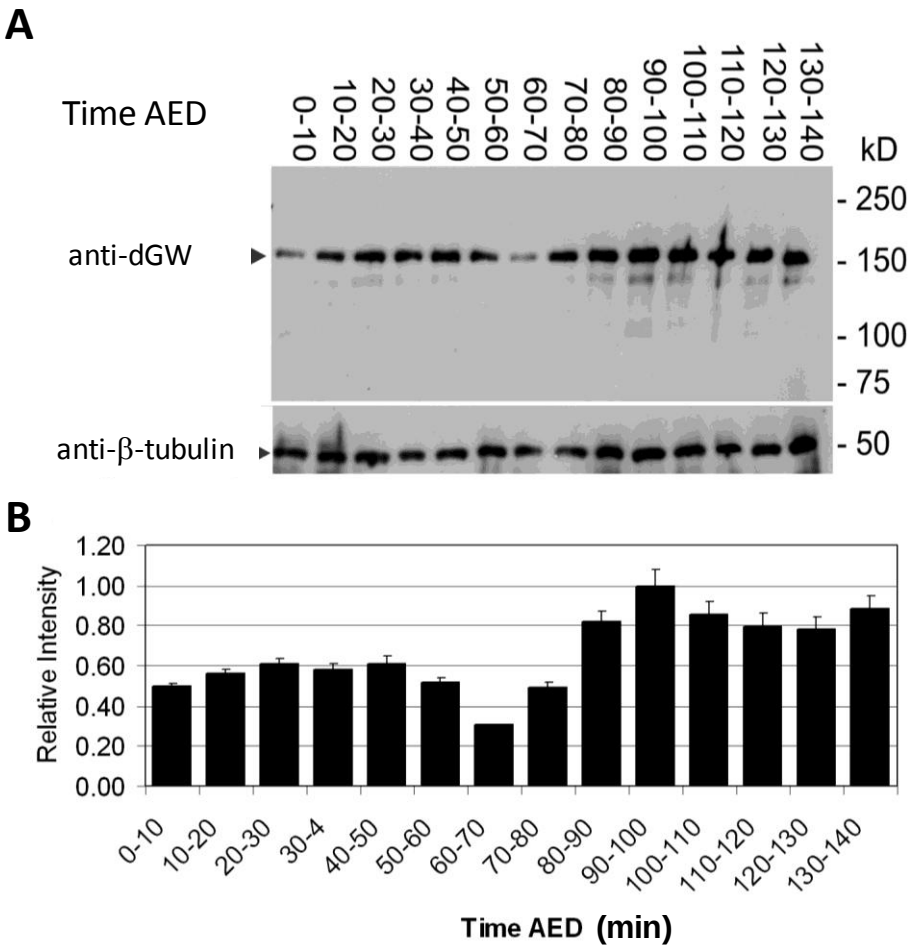


Figure 11. dGW expression levels drop transiently during early embryonic development. (A) dGW levels are reduced 60-70 min AED and rise again after 70 min AED. (B) Error bars represent the standard deviation of relative values from three separate experiments.

3.2.5 Injecting anti-dGW or anti-dAgo2 into embryos causes defects in nuclear division

To further investigate the effects of dGW on progression through mitosis, the protein was immuno-depleted from syncytial embryos by injecting them with affinity-purified anti-dGW antibody. This approach facilitated examination of the effects of dGW depletion on the distribution of GFP fusions of other proteins without creating additional gw^1 mutant strains expressing these fusions. Embryos expressing GFP with a NLS (NLS-GFP) were injected with pre-immune serum or anti-dGW (Fig. 12). In contrast to the normal size and number of nuclei in embryos injected with pre-immune serum (Fig. 12 A), in embryos injected with anti-dGW the diameters of cortical nuclei were enlarged approximately 8-10 times and fewer nuclei were seen at the cortex (Fig. 12B). These embryos also lacked pole cells. When anti-dGW was injected later, a graded effect was observed which was largest at the site of injection. Injecting anti-dGW antibody 1 h 40 min AED resulted in the formation of three regions of differently sized nuclei. The largest nuclei, closest to the injection site, were approximately 10 times the diameter of normal nuclei (Fig.12C). Injecting anti-dGW antibody 1 h 50 min AED resulted in the formation of regions with two different sizes of nuclei. Nuclei in the region closest to the injection site were enlarged, while nuclei in the adjacent regions were similar to normal size. The various sizes of nuclei likely formed as a result of a gradient of dGW activity caused by diffusion of anti-dGW antibody away from the injection site and impairment of nuclear division when dGW activity droped below a critical level. The effect of dAgo2 depletion was also tested because of previously observed colocalization of dAgo2 with dGW and detection of a physical interaction between them (Fig. 6). Embryos injected with anti-dAgo2 antibody resembled embryos injected with anti-dGW. Fewer nuclei were seen the cortex and nuclei were enlarged (Fig. 12F).

Anti-dGW was also injected into embryos expressing a GFP fusion with the actin-binding domain of moesin (Edwards et al., 1997) to observe changes in organization of the actin cytoskeleton that occur in *Drosophila* embryos during the cell cycle. In normal syncytial embryos, actin alternates between two patterns of organization.

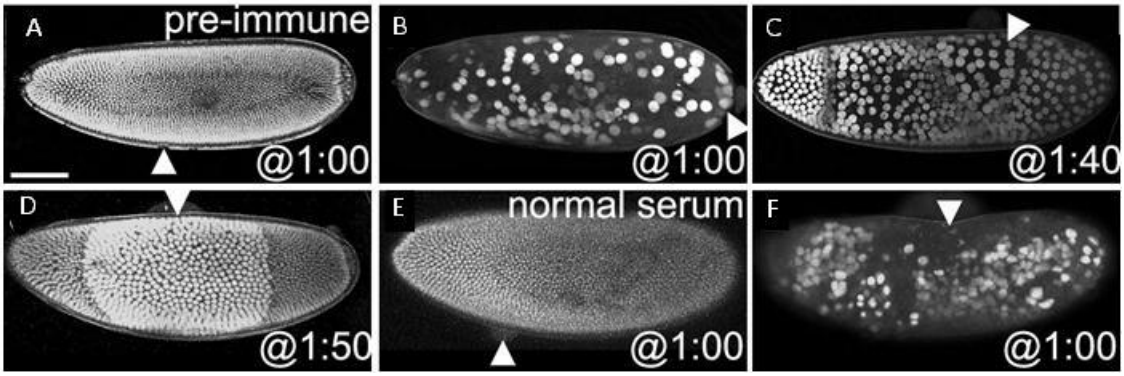


Figure 12. Loss of dGW results in enlarged nuclei. Embryos expressing an NLS-GFP fusion were injected with antibodies at the indicated times AED and photographed at 2 h 10 min AED. The arrowhead indicates the site of injection. (A) Normal blastoderm and pole cell nuclei are seen in an embryo after NC14 (2 h 10 min AED) injected with guinea pig pre-immune serum at 1 h AED. (B) Injecting anti-dGW antibody at 1 h AED produces embryos with fewer cortical nuclei that were enlarged and lacked pole cells. (C) Anti-dGW injection at 1 h 40 min AED shows stepwise nuclear enlargement that is greatest proximal to the injection site. (D) Injecting anti-dGW at 1 h 50 min produces a region of enlarged nuclei proximal to the injection site at 2 h 10 min AED. (E) Nuclei appear normal after injecting normal rabbit serum into embryos 1 h AED. (F) Injecting rabbit anti-dAgo2 antibody 1h AED into an embryo produces a similar phenotype to injecting with anti-dGW as seen in B. Bar is 100 μm.

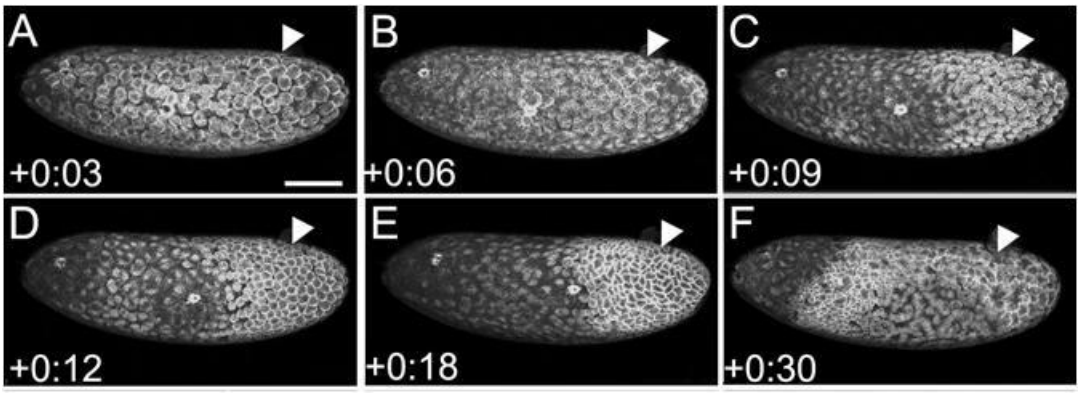


Figure 13. Loss of dGW alters cortical actin cytoskeleton dynamics. (A-F) An embryo expressing a GFP fusion with the actin-binding domain of moesin, injected with anti-dGW antibody at NC10. The injection site is marked by an arrowhead; time after injection (min) is indicated below each image. Rearrangements in the actin cytoskeleton are initially delayed and then inhibited close to the injection site. These effects occur at a later time distal to the injection site. (A) At 3 min after injection a normal honeycomb pattern of actin surrounds each mitotic nucleus throughout the embryo with a pseudocleavage furrow. (B) At 6 min after injection, the pseudocleavage furrows appear less distinct, as they begin to rearrange. (C) At 9 min after injection, actin cap formation is seen close to the injection site. (D) 12 min after injection, close to the injection site, pseudocleavage furrows form. (E) 18 min after injection, close to the injection site the pseudocleavage furrows remain; however, begin to aquire an abnormal elongated shape. Formation of actin caps, distal to the injection site, indicates progression through one more round of mitosis. (F) 30 min after injection, close to the injection, site no further rearrangement into the actin cap formation is seen, most of the actin cytoskeleton is in the pseudocleavage furrow formation, with structures nearest the injection site beginning to break down. Bar is 100 μm.

During mitosis, actin forms barriers between individual spindles known as pseudocleavage furrows. At the end of mitosis, actin rearranges and forms a cap above each interphase nucleus (Sullivan and Theurkauf, 1995; Warn, 1986; Warn et al., 1984). Injecting anti-dGW antibody into embryos expressing a GFP fusion with the actin-binding domain of moesin at NC10 resulted in impaired rearrangement of the actin cytoskeleton (Fig. 13). These effects occurred more rapidly closer to the injection site where the activity of dGW was reduced earlier than in the region distal to the injection site. 3 min after injection with anti-dGW, the pseudocleavage furrows are seen surrounding each mitotic nucleus (Fig. 13 A). 6 min after injection, the pseudocleavage furrows begin to rearrange (Fig.13 B) and actin caps can be seen close to the injection site (Fig. 13 C). 12 min after injection, close to the injection site, the actin cytoskeleton formed pseudocleavage furrows (Fig. 13 D). At 18 min after injection, these pseudocleavage furrows began to form an abnormal elongated pattern (Fig. 13 E). Close to the injection site, the pseudocleavage furrows did not undergo further normal rearrangement into actin caps; however, after 30 min they began to break down (Fig.13 E-F). Distal to the injection site, one more round of actin cap formation was seen (Fig. 13 E) and stabilized pseudocleavage furrows formed later (Fig. 13 E-F) indicating the occurance of one more round of mitosis. Stabilized pseudocleavage furrows could be the cause of enlarged nuclei seen in embryos injected with anti-dGW and anti-dAgo2 (Fig. 12). The pseudocleavage furrows would form barriers that would prevent chromosome separation. This would in turn result in polyploidy and therefore enlarged nuclei.

3.3 Discussion

3.3.1 Multiple dGW protein species are detected by Western blot analysis

Western blot analysis of dGW, using anti-dGW antibody revealed multiple protein species in some extracts (Fig. 3, 8D and 11). The appearance of several distinct dGW species rather than a smear suggests that they may be products of endopeptidases. Two observations are consistent with these multiple protein species being degradation products of dGW. First, there were no major protein species larger than normal dGW, or truncated dGW from extracts of single mutant embryos. If the anti-dGW cross-reacts

with proteins unrelated to dGW, then species larger than dGW could be observed. Second, the number of these species and their relative quantities varied between different protein extracts, suggesting that variations in sample preparation may be associated with varying degrees of degradation. Alternatively, these potential dGW degradation products could represent physiologically regulated proteolytic cleavage products. If GW proteins play a role in PB assembly, then their regulated proteolysis could be a mechanism for PB dispersal. This could also account for the variability in the number of protein species in different protein extracts. dGW could be protelytically cleaved to varying degrees before extraction, depending on the degree of PB disassembly that may be occurring in the cells at the time just prior to preparation of the extract.

3.3.2 *Drosophila* P bodies are similar to P bodies in other species

The results presented in this chapter show that in *Drosophila*, like in other organisms such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and humans, mRNA regulatory proteins accumulate in cytoplasmic foci (Ding et al., 2005; Eystathioy et al., 2002; Eystathioy et al., 2003; Sheth and Parker, 2003). These proteins include the general mRNA decay factors, Pacman, the *Drosophila* XRN-1 homologue (Till et al., 1998), and the previously uncharacterized *Drosophila* orthologue of LSm4. Like in other metazoan species these foci also contain dAgo2, a component of the RNA*i* pathway (Liu et al., 2005a; Sen and Blau, 2005), and a member of the GW182 protein family (Ding et al., 2005; Eystathioy et al., 2002; Eystathioy et al., 2003; Rehwinkel et al., 2005). The similarity in composition of these foci in *Drosophila* is consistent with the idea that that they are analogous to PBs previously described in other organisms.

Characterization of dGW suggests that it is structurally and functionally homologous to GW182 family proteins of other species. dGW shares several conserved sequence domains with other members of this protein family (Fig. 2) and localizes to similar cytoplasmic foci (Fig 5). Further, like in human cells the integrity of these foci depends on the presence of intact RNA. In both species, treatment of cells with RNase A resulted in dispersal of these foci (Schneider et al., 2006; Sen and Blau, 2005).This response to treatment with RNase A is evidence for these foci being composed of complexes consisting of RNA and proteins and is consistent with their proposed function

in regulating mRNA stability and translation. Importantly, a high degree of colocalization of all three human GW182 paralogues with dGW was observed (Fig.7). This suggests functional homology between GW182 family proteins in both species, because targeting human GW182 family proteins to *Drosophila* PBs would require interactions with other *Drosophila* PB components.

The composition of PBs characterized in this study is heterogeneous since PB components did not always colocalize. The degree of colocalization of PB components reported in the literature varied between different published reports. Several studies showed a high degree of colocalization between PB components (Behm-Ansmant et al., 2006; Liu et al., 2005a; Liu et al., 2005b; Sen and Blau, 2005). Consistent with our observations, several other studies observed heterogeneous PBs. Populations of heterogeneous PB-like foci were reported in recent studies of neurons (Barbee et al., 2006) and glial cells (Moser et al., 2007). These specialized cells would be engaged in different cellular processes than the often transformed human epithelial cells that show a high degree of colocalization of PB components (Liu et al., 2005a; Liu et al., 2005b; Sen and Blau, 2005). In some studies, the degree of colocalization of PB components was found to increase under certain conditions such as stress (Garneau et al., 2007; Kedersha et al., 2005) or when a PB component is overexpressed (Barbee et al., 2006; Ingelfinger et al., 2002). Based on these observations, it is likely that the composition of foci containing mRNA regulatory proteins would differ in cells that are engaged in different processes. The varying PB composition could reflect different requirements for mRNA regulation by different mRNA regulatory processes.

Different cellular processes have also been associated with varying numbers of PBs. For example, the number of PBs marked by GW182 varies at different stages of the cell cycle and in proliferating versus quiescent cells (Yang et al., 2004). The composition of PBs during these different physiological states has not yet been examined. The change in both the number of PBs and their composition when cells are stressed (Kedersha et al., 2005) suggest that changes in the number of PBs in other physiological states such as the stage of the cell cycle or the state of proliferation may also be associated with changes in PB composition.

3.3.3 dGW is required early in *Drosophila* embryonic development

There have been several screens to identify zygotically transcribed genes that affect precellular embryonic development in *Drosophila* (Merrill et al., 1988). These screens identified a total of seven genes that are thought to be expressed before the cellular blastoderm stage (Merrill et al., 1988). However, these screens did not include all genes on the fourth chromosome. In this study, *gw* was identified as an additional zygotically expressed gene on the fourth chromosome that is required for successful completion of development of the early *Drosophila* embryo. The reduction in dGW protein observed at 60–70 min AED (Fig.11), together with the onset of the mutant phenotype shortly afterwards, suggests that maternally supplied dGW is depleted at this time. Progression of development in normal embryos after this time would therefore depend on zygotic transcription of *gw*. The time of zygotic dGW expression could be verified by determining the time that the truncated protein is first detected in heterozygous gw^1 mutant embryos.

3.3.4 The gw^1 mutation is recessive lethal in embryos

The gw^1 phenotype appears to be caused by loss of functional dGW protein. Although a truncated protein lacking an RRM domain is expressed, several observations indicate that there are no obvious dominant interfering effects caused by this protein. First, embryo viability is consistent with the mutation being homozygous recessive. In a strain of gw^1/Ci^D flies, approximately 75% of the embryos hatched (Table 3). Also, all embryos examined from this strain that had one gw^1 allele did not show mitotic defects during the blastoderm stage (section 3.2.3). Further, no gross phenotypic abnormalities were observed in these adults. There is evidence suggesting that expression of a truncated GW182 family protein may have a dominant interfering effect. Expression of a fragment of human GW182 encompassing the N-terminal one third of the protein caused disruption of PBs and interfered with RNA*i* function (Jakymiw et al., 2005); however, a smaller N-terminal fragment localized to PBs and did not disrupt them (Eystathioy et al., 2002). The presence of minor dominant interfering effects caused by the gw^1 mutation that were not found in this study could be identified by additional careful observation of gw^1 heterozygous individuals during postembryonic development.

3.3.5 Lack of functional dGW causes cell cycle defects and defects in organization of blastoderm nuclei

The most stiking defects observed in embryos lacking functional dGW, either due to the *gw*^{1} mutation or depletion of dGW by injection of anti-dGW antibody, were cell cycle defects. These included defects in chromatin separation, centrosome arrangement, actin cytoskeleton dynamics as well as enlarged nuclei and uncoordinated divisions.

Interestingly, many of these defects could be accounted for by a primary defect in actin cytoskeleton dynamics, an event that occurs in late mitosis. A stabilized pseudocleavage furrow (Fig.13) could impede chromatin separation. If DNA synthesis continued, this would result in enlarged, polyploid nuclei. Eventually, this could lead to additional mitotic defects, such as mislocalized centrosomes, as a result of a mitotic catastrophe (Castedo et al., 2004).

Additional evidence could be provided to show that, in embryos expressing NLS-GFP, nuclei are actually enlarged after injection of anti-dGW. These nuclei could be larger than nuclei of control embyos at the time they were photographed, however they could be the size of normal nuclei at an earlier stage of development, which is when they were injected. This would imply that the size of nuclei is reduced as blastoderm development progresses. The apparent enlargement could be due to a cell cycle arrest shortly after anti-dGW injection. Monitoring the size of nuclei in embryos expressing NLS-GFP continuously by videomicroscopy after antibody injection could distinguish between these possibilities. If embryos are injected at NC10, when nuclei are at the cortex their size could be monitored continuously after injection. An increase in the size of nuclei with time would indicate continued DNA synthesis in the absence of mitosis, while observing a constant size would indicate mitotic arrest. Changes in size of injected nuclei could also be compared with control embryos. In these embryos a progressive decline in size of nuclei would be observed.

Defects unrelated to cell cycle progression were also observed. One of these defects was a lack of pole cell formation in embryos expressing NLS-GFP injected with anit-dGW (Fig. 12.). Pole cells are a group of cells at the embryo posterior which are germline determinants. The other defects involved abnormal movement of nuclei. First, a defect in migration of nuclei to the cortex was observed. Fewer nuclei reached the cortex

and, unlike the synchronous migration of nuclei to the cortex in normal embryos (Video 1), in *gw*^{1} mutants, movment to the cortex is asynchronous (Video2). Second, a larger displacement of nuclei during each nuclear cycle was seen in *gw*^{1} mutatant embryos (Video 2) compared to normal embryos (Video 1) which indicates a defect in anchoring of nuclei at the cortex.

Several of the defects observed in *gw*^{1} mutant embryos resemble defects in embryos overexpressing maternal cyclin B. One of these defects is in migration of nuclei to the embryo cortex (Video 1 and 2). Several studies reported slower migration of nuclei to the cortex and fewer nuclei reaching the cortex (Crest et al., 2007; Ji et al., 2004; Stiffler et al., 1999). Also, a defect observed in chromatin separation was observed (Crest et al., 2007) like in *gw*^{1} mutant embryos (Fig. 10). The similarities in defects between *gw*^{1} mutatant embryos and embryos overexpressing maternal cyclin B suggest that cyclin B expression may be misregulated in *gw*^{1} mutatant embryos.

3.3.6 The *gw*^{1} mutation may disrupt RNA*i*

This is the first report of a GW182 family protein influencing the early development of an organism through a defect in cell cycle regulation. Mutations in GW182 genes have been described in *Caenorhabditis elegans*; however they do not resemble the *gw*^{1} mutation. *Caenorhabditis elegans* encodes two GW182 protein family homologues AIN-1 and AIN-2. Mutations in either gene alone are viable and displayed few or no morphological defects. However, organisms with mutations in both *ain-1* and *ain-2* showed severe defects in development of postembryonic cells. These included a developmental timing defect requiring membrane fusion for the formation of seam cells and an additional defect in vulval development resulting in its protrusion (Ding et al., 2005; Zhang et al., 2007).

Although the specific phenotypes of mutations in *ain-1* and *ain-2* differ from the *gw*^{1} phenotype, there is evidence that mutations in *gw* genes in both species may exert some of their effects through the RNA*i* pathway. GW proteins in several species, including *Drosophila* and *Caenorhabditis elegans*, interact physically and/or functionally with Argonaute proteins, essential effectors of the RNA*i* machinery [reviewed in (Ding and Han, 2007; Eulalio et al., 2007a)]. The phenotypes caused by mutations in

Caenorhabditis elegans and *Drosophila gw* genes resemble mutations in one of their respective Argonaute genes. In *Caenorhabditis elegans*, a mutation resulting in the loss of function of the Argonaute homologue *alg-1* shows a similar phenotype to the *ain-1* mutation (Ding et al., 2005) while a mutation in *Drosophila* that severely reduces dAgo2 levels produces a phenotype similar to the gw^1 mutation. Like the gw^1 mutation, the *dago*2 mutation results in defects in pole cell formation and defects in the syncytial blastoderm including nuclear migration, nuclear division, centrosome localization and the actin cytoskeleton (Deshpande et al., 2005). The different phenotypes resulting from *gw* mutations in these two species could arise by misregulation of different target mRNAs by RNA*i*.

3.3.6a The gw^1 mutation may disrupt dAgo2 function

The similarity between phenotypes of gw^1 and *dago*2 mutations, as well as the defects caused by immunodepletion of dAgo2 (Fig.12), point to siRNA-mediated RNA*i* as a potential cause of the mitotic defects observed in gw^1 mutants. Detecting a genetic interaction in individuals carrying both mutations would be consistent with a functional linkage between these two genes. Genetic interaction analysis is a method for establishing functional linkages between genes. Functional linkages between two genes are indicated when two mutations have a combined effect not exhibited by either mutation alone. However, detecting a genetic interaction is not by itself proof of a functional linkage since it is also possible that combining two independently functioning deleterious alleles could also produce a more severe effect than either alone. Mitotic defects in homozygous *dago*2 mutants are seen in approximately 50% of syncytial embryos, and 90% of individuals are viable and fertile (Okamura et al., 2004). Although the gw^1 mutation is homozygous lethal, heterozygotes develop normally during embryogenesis. Observing more severe defects or an increase in the fraction of embryos showing mitotic defects in homozygous *dago2* mutants carrying one gw^1 allele would be consistent with these genes being functionally related.

Despite the similarities in phenotypes resulting from homozygous *dago*2 and gw^1 mutations, the gw^1mutation is more severe, resulting in lethality in all embryos. This difference could be due to a quantitative effect, moderated in the *dago2* mutant by

residual dAgo2 activity, since the mutation is not a complete null (Deshpande et al., 2005). However, the observation that null mutants in Dicer-2, which functions upstream of dAgo2 in siRNA biogenesis, are viable, fertile and show no defects in cellularization (Lee et al., 2004; Meyer et al., 2006) do not support this idea.The less severe phenotypes of both mutants in siRNA-mediated RNA*i* suggest that the gw^1 mutation may be qualitatively different due to impairment of other pathways in addition to siRNA-mediated RNA*i*.

Until recently, siRNA-mediated RNA*i* was not known to target endogenous *Drosophila* genes. This pathway was known primarily as an antiviral defense mechanism, silencing exogenous double stranded RNA derived from viral replication intermediates (Deddouche et al., 2008; Ding and Voinnet, 2007; van Rij et al., 2006; Zambon et al., 2006). However, several recent studies identified a novel pathway for silencing endogenous genomic elements that requires Dicer-2 and Ago2 but not components of the miRNA pathway. Sequencing of Ago2-associated RNAs from somatic tissues and cells combined with analysis of large-scale small RNA sequence data revealed a novel class of 21 nucleotide long endogenous short interfering RNAs (esiRNAs) (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008b). esiRNAs resemble PIWI interacting (piRNAs) that associate with the Argonaute paralogues Piwi, Aubergine and Ago3 to silence selfish genetic elements in the germline (Brennecke et al., 2007; Saito et al., 2006; Vagin et al., 2006). However, unlike piRNAs, esiRNAs are derived from a broader range of genomic elements. In addition to retroposons and other repetitive genomic elements, they also target protein-coding genes as well as a few regions predicted to generate non-coding transcripts (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008a). The majority of esiRNAs are formed via bi-directional transcription of the same genomic region (Okamura et al., 2008a); however, formation of hairpins from transcripts containing inverted repeats was also detected (Okamura et al., 2008b). Protein coding genes expressing esiRNAs are enriched for nucleic acid-related functions such as nuclease activity (including Ago2), transcription factor complexes and pericentric chromosome regions (Okamura et al., 2008a).

The discovery of esiRNAs provides a mechanism for misregulation of endogenous genes that could account for the mitotic defects observed when dAgo2 and dGW levels are reduced. It also reveals additional experimental approaches for investigating dGW functions. If the gw^1 mutation interferes with dAgo2 function, then experimentally varying levels of dGW should result in detectable variations in the levels of retroposon transcripts. An increase in retroposon activity would be expected in gw^1 mutants or in cultured cells depleted of dGW. Conversely, overexpression of dGW should repress retroposon activity.

3.3.6b The gw^1 mutation may not affect dAgo1 function

dAgo1 is another essential player in the RNA*i* pathway that physically and functionally interacts with dGW (Behm-Ansmant et al., 2006), regulating mRNA stability and translation thorough miRNAs. miRNA-mediated regulation of development has been well documented in both plants [reviewed in (Chen, 2005)] and animals (Wienholds and Plasterk, 2005). For example, the roles of *lin-4* and *let-7* in developmental timing in *Caenorhabditis elegans* (Alvarez-Garcia and Miska, 2005; Pasquinelli et al., 2000; Reinhart et al., 2000) have been extensively studied. However, in some organisms the earliest stages of development may not be influenced miRNAs. For example, in zebrafish depletion of maternal dicer mRNA impaired miRNA production however developmental defects were not apparent for the first 24 h, appearing after normal embryonic development (Giraldez et al., 2005; Wienholds et al., 2003). Similarly, in *Drosophila* the effects of depletion of maternal dAgo1 became apparent during late embryogenesis at stages 14-16 affecting denticle-forming cells and cells of the central and peripheral nervous systems (Kataoka et al., 2001). The later lethality of these mutants argues against the defects seen in the gw^1 mutation being mediated through Ago1.

3.3.7 dGW may act through mRNA regulatory pathways distinct from RNA*i*

Several proteins whose functions are not known to be related to RNA*i* regulate maternal mRNA in the early *Drosophila* embryo, influencing both embryonic patterning and the cell cycle. [reviewed in (Dahanukar et al., 1999; Tadros and Lipshitz, 2005)].Among these pathways, those that regulate the embryonic cell cycle could act in conjuction with

dGW. One of these pathways is mediated by the RNA-binding protein Smaug (Smg). Smg was originally identified as a translational suppressor of maternal *nanos* mRNA (Dahanukar et al., 1999; Smibert et al., 1996). It was subsequently found to be required for destabilizing maternal *Hsp83* mRNA (Semotok et al., 2008) (section 1.1.2b), and recent micro-array based gene expression profiling revealed its role as a major regulator of maternal mRNA, being required for destabilizing approximately two thirds of unstable maternal transcripts. These transcripts were enriched for gene ontology (GO) terms related to cell/chromosome cycle and cell proliferation and include cyclins A and C (Tadros et al., 2007).

Embryos lacking Smg display defects in cortical nuclear division cycles that resemble gw^1 mutants. In both mutants, mitotic defects become apparent in the syncytial blastoderm, and the embryos fail to cellularize. Also, the arrangement of nuclei appears irregular, divisions are asynchronous and finally, aggregated nuclei fall into the centre of the embryo (Dahanukar et al., 1999). Smg-dependent destabilization of transcripts encoding genes involved in cell cycle and cell proliferation could account for the mitotic defects observed in *smg* mutants (Tadros et al., 2007).

Smg is a likely candidate to act in concert with dGW to regulate early embryonic cell cycles for several reasons. First, mitotic defects seen in *smg* and gw^1 mutants are similar. Further, Smg's roles in translational repression and mRNA degradation are similar to other mRNA regulatory proteins that select specific mRNAs and localize to PBs. These proteins such as Ago1 in its role in RNA*i* or TTP, in its role in ARE decay (section 1.2.3b) physically interact with their target mRNAs and recruit other mRNA regulatory factors (section1.1.4). Smg, in its role as a translational suppressor, binds to the Smg response element in the 3'UTRs of target transcripts (Dahanukar et al., 1999; Smibert et al., 1996) and recruits the translational suppressor Cup, an eIF4E binding protein (Nelson et al., 2004). In its role in mRNA degradation Smg recruits the deadenylase complex Ccr4/Pop2/Not to the target mRNA (Semotok et al., 2005). Finally, there is evidence for a physical association between Smg and dGW. Smg was identified as one of several dGW-interacting proteins in our lab (Simmonds, unpublished results) and it localized to PBs (Eulalio et al., 2007b).

Pumilio (Pum) is another regulator of maternal mRNAs in the early embryo, although its potential relationship with dGW is currently less apparent than that of Smg. Pum is a maternally expressed protein that is a member of the conserved PUF family of translational suppressors [reviewed in (Wickens et al., 2002)]. It influences abdominal patterning and pole cell formation in the early embryo (Asaoka-Taguchi et al., 1999; Lehmann and Nusslein-Volhard, 1987; Macdonald, 1992). Cyclin B is one mRNA that is repressed by Pum. It was known for some time to represses translation of cyclin B in pole cells, which is required for mitotic arrest during their migration (Asaoka-Taguchi et al., 1999). Results of a recent study indicate that it represses cyclin B translation throughout the early embryo (Vardy and Orr-Weaver, 2007). Pum-mediated repression of cyclin B mRNA is modified by other factors providing insights into the effects of cyclin B misregulation in early embryos. The Pan Gu kinase complex relieves repression of cyclin B mRNA, and mutants in components of this kinase express reduced levels of cyclin B protein. The reduced levels of cyclin B protein result in the inability to block DNA replication, which permits entry into mitosis. Consequently, these mutants progress through multiple rounds of S phase without mitosis and form enlarged nuclei (Freeman and Glover, 1987; Lee et al., 2003; Shamanski and Orr-Weaver, 1991).

3.3.8 Cell cycle regulatory genes that may be affected by the *gw*^{1} mutation

The process of cell cycle regulation is complex and relies on multiple regulatory pathways including regulation of transcription, posttranscriptional mRNA regulation and regulation of protein stability, as well as signal transduction (Lodish H., 2008). dGW in its role in mRNA regulation likely influences the cell cycle by targeting transcripts encoding cell cycle regulatory proteins.

Progression through the cell cycle depends on the activites of a small number of key regulators known as cyclin-dependent kinases (Cdks) (Nurse, 2002). Although cdk levels remain constant during the cell cycle, their activities are regulated by proteins whose levels oscillate (Lee and Orr-Weaver, 2003). One extensively studied class of proteins that regulate cdk activity is the cyclins (Fig. 14). Although proteolysis is clearly a major mechanism for timely elimination of cyclins during the cell cycle (Lee and Orr-Weaver, 2003), the role of of cyclin mRNA regulation during the cell cycle is not as well

understood. Evidence supporting cyclin mRNA regulation in cell cycle progression has recently been accumulating. dGW could therefore affect cell cycle progression by regulating cyclin mRNA. In addition to cyclins, another class of Cdk regulators whose levels vary in a timely sequential manner is Cdk inhibitors (De Clercq and Inze, 2006). Models derived from considering cyclins can be extended to include these or other potential targets when evidence points to them.

A pathway for eliminating cyclin B mRNA at the end of mitosis by the endoribonuclease RNase MRP was described in *Saccharomyces cerevisiae* (Gill et al., 2004). Moreover, RNase MRP was identified as a component of a PB-like structure also containing the 5' to 3' exonuclease Xrn-1p during late mitosis. These observations support a model of cyclin B mRNA elimination at the end of mitosis by the action of these two ribonucleases in a PB-like structure (Gill et al., 2006). dGW could participate in a similar mechanism in *Drosophila,* accounting for mitotic defects seen in the gw^1 mutation. This mechanism, acting in concert with protein degradation, would eliminate residual cyclin levels that could interfere with progression to the next stage of the cell cycle. Candidate cyclin(s) that may be misregulated in gw^1 mutants can be identified by analysis of the defects that the embryos display. Delayed chromosome separation, impaired nuclear division and inappropriate centrosome position while nuclei continue to enlarge suggest that defects may be limited to mitosis. Moreover, the failure to rearrange the actin cytoskeleton points to a defect in late mitosis, when this rearrangement normally allows separation of daughter nuclei (Warn, 1986; Warn et al., 1984).

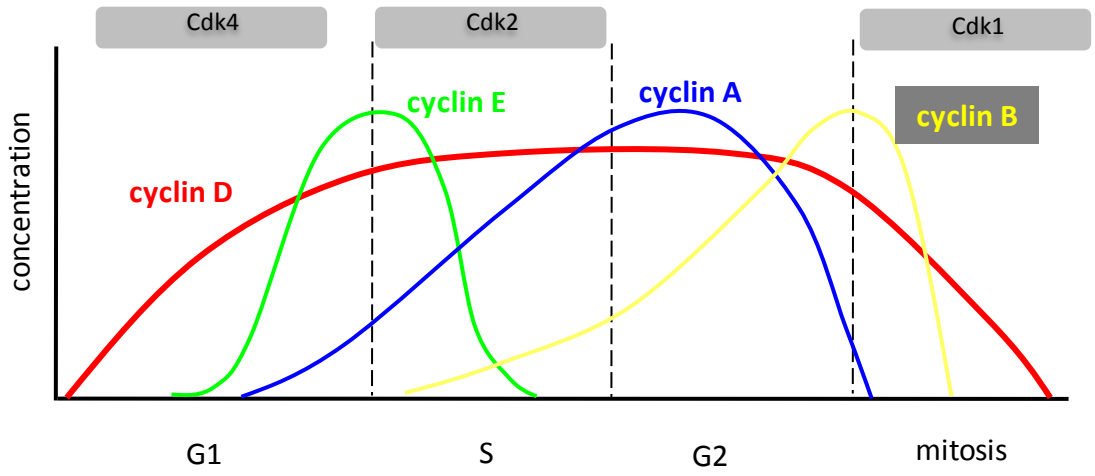


Figure 14. Regulation of the cell cycle by oscillating levels of cyclins. The graph shows relative levels of cyclins during the cell cycle. Approximate times of activity of different cdks are shown above the graph. Graph is adapted from Wikipedia 2009 (http://en.wikipedia.org/wiki/Cyclin) and based on literature reviewed in Lee and Orr-Weaver 2003; Sullivan and Morgan, 2007.

In *Drosophila,* progression through mitosis depends on the sequential destruction of cyclins A, B and B3. Cyclin A is degraded during prometaphase, cyclin B at metaphase and cyclin B3 at anaphase [reviewed in (Sullivan and Morgan, 2007)]. Further, expression of stabilized forms of these cyclins results in arrest of mitosis at the stages corresponding to the times of their normal degradation (Parry and O'Farrell, 2001). It is difficult to precisely identify the contribution of a single mitotic cyclin to progression through each stage of mitosis, because the efficiencies of mitotic arrests when stabilized cyclins were expressed varied depending on their levels and levels of the corresponding endogenous cyclin (Parry and O'Farrell, 2001). Further, there is evidence for overlapping functions of cyclins B and B3 since neither is required for mitosis but mitotic defects occur in the absence of both (Jacobs et al., 1998). Despite the difficulties in predicting precise phenotypes of defects in mitotic cyclin degradation, defects in late mitosis observed in the gw^1 mutant point to misregulation of cyclin B, cyclin B3 or both.

In the early *Drosophila* embryo, the cell cycle is modified so that multiple rounds of rapid DNA synthesis and nuclear division occur without G phases or cytokinesis (Fig. 15) [reviewed in (Vidwans and Su, 2001)]. Unlike the conventional cell cycle, where large oscillations of cyclin B occur in each cell, in the multinuclear syncytial *Drosophila* embryo variations in overall cyclin levels are less pronounced, while localized cyclin B oscillations in the region of the mitotic apparatus are thought to regulate progression through mitosis (Huang and Raff, 1999; Su et al., 1998). Cell cycle defects at this time could be caused by misregulation of global cyclin levels, as in PAN GU kinase mutants (Freeman and Glover, 1987; Lee et al., 2003; Shamanski and Orr-Weaver, 1991) or by localized cyclin misregulation. Accordingly, both global and local cyclin B and B3 levels need to be measured in gw^1 mutant embryos to determine the cause of the mutation.

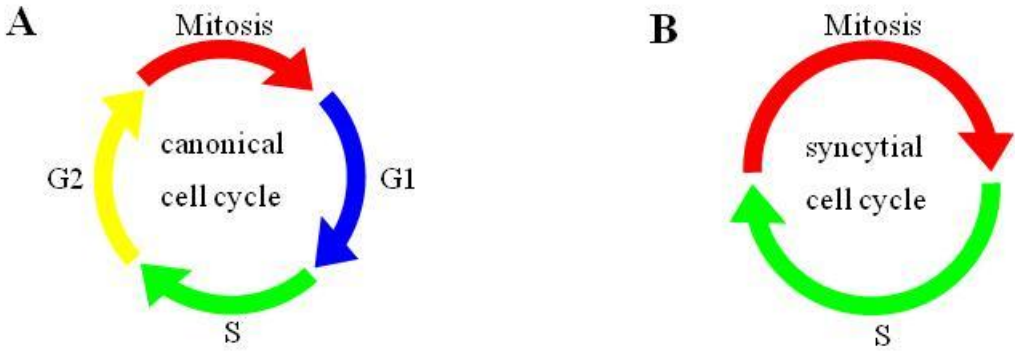


Figure 15. Modified cell cycle of the *Drosophila* syncytial embryo. (A) The canonical cell cycle. Most dividing cells pass through G1, S, G2 and M in each cycle of division. (B) Syncytial cell cycle of the early *Drosophila* embryo. Nuclei alternate between S phase and mitosis without intervening G1 or G2 growth stages.

CHAPTER 4: CHANGES IN P BODY SIZE AND NUMBER DURING THE CELL CYCLE

4.1 Overview

The mitotic defects observed in the absence functional dGW described in Chapter 3 point to a role for the *dgw* gene in cell cycle regulation. This idea is consistent with the observation of changes in PB size and number during the cell cycle in human cells. In human cells most PBs marked by GW182 disassemble before mitosis like many cellular structures such as the nucleus, nucleolus and Golgi complex. Relatively small PBs reappear in G1 and the largest PBs are seen during late S and G2 phases (Yang et al., 2004). Together these observations suggest that PB-mediated regulation of mRNA may be a conserved mechanism that contributes to regulating the cell cycle.

4.2 Objectives

The objective of this chapter was to monitor PB distribution marked by dGW throughout the cell cycle and to correlate changes in their distribution with stages of the cell cycle. Cell cycle-associated changes in dGW distribution could provide additional insights into mechanisms of cell cycle regulation by dGW. These observations would also provide a basis for comparing dGW distribution with the published results of human GW182 distribution during the cell cycle and suggest additional functional similarities or differences between these homologues.

4.3 Results

4.3.1 *Drosophila* P bodies persist during mitosis

A survey of dGW distribution in S2 cells indicated the presence of PBs during all stages of the cell cycle. Unlike the diffuse pattern shown by human GW182, (Yang et al., 2004), foci of dGW were apparent during mitosis (Fig.16). Of particular interest is the concentration of PBs seen in the region of the spindle during anaphase. This observation suggests the presence of mRNA that is being translationally suppressed or degraded in this region.

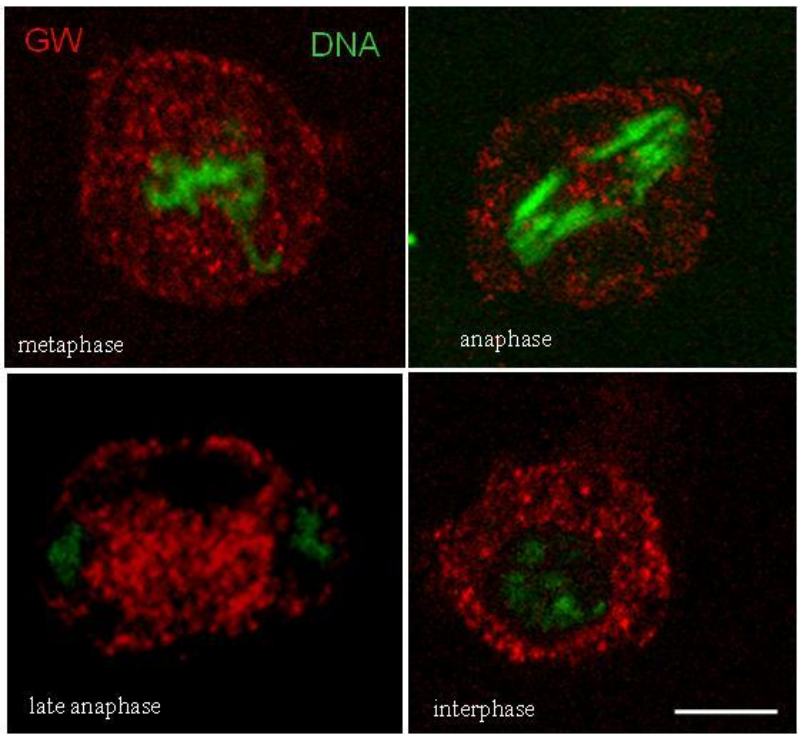


Figure 16. *Drosophila* PBs are visible throughout the cell cycle. S2 cells in mitosis and interphase stained with anti-dGW antibody (red) and PicoGreen (DNA). dGW appears to be concentrated in the region of the mitotic spindle in late anaphase. Bar is 5 μm.

4.3.2 P bodies were observed in the nuclei of live S2 cells expressing GFP-dGW

To observe the dynamics of dGW distribution during the cell cycle, GFP-dGW was monitored in live cells by video-microscopy. Surprisingly, two to three large PBs assembled in the nuclei of cells immediately after the completion of cytokinesis. These bodies persisted for approximately 25 minutes and then dispersed (Fig. 17). After dispersal of these PBs, the majority of GFP-dGW then appeared to be in the cytoplasm, which suggests that dGW may have been exported from the nucleus into the cytoplasm. The assembly of these large PBs in the nucleus indicates an abrupt accumulation of non-translating mRNAs, which may then be either degraded or dispersed as these PBs disassemble. It is not clear what the functional significance is of what appears to be a major post-mitotic mRNA regulatory event at this time. I am not aware of other reports of similar PB-like structures in the nucleus.

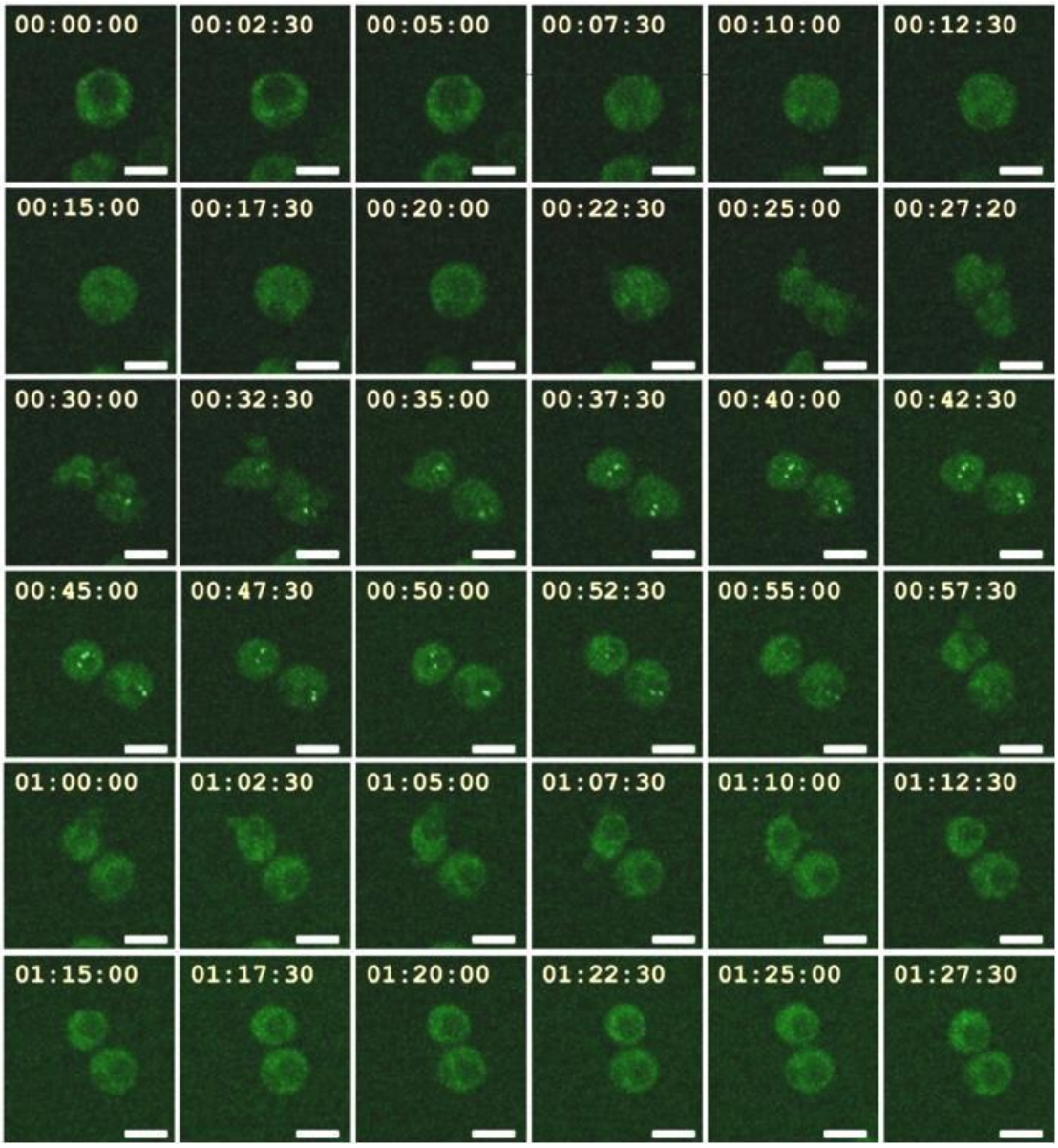


Figure 17. Post mitotic nuclear P bodies in S2 cells expressing GFP-dGW. Cell begins cytokinesis at t = 25 min. P body assembly begins at t = 35 min. Two to three large P bodies can be seen in a central area where diffuse GFP is clearing, presumably the nucleus. At t = 55 min, P bodies begin to disperse and most of the GFP-dGW re-localizes to the cytoplasm. Image is a single confocal plane. Bar is 10 μm.

4. 4 Discussion

4.4.1 The distribution of *Drosophila* PBs does not resemble human P body distribution during the cell cycle

This survey of *Drosophila* PBs, marked by dGW at various stages of the cell cycle, showed that PBs were visible throughout the cell cycle (Fig. 16). In contrast, human PBs marked by GW182 dispersed during mitosis and reassembled in G1(Yang et al., 2004). This difference between human and *Drosophila* PBs can be accounted for by the difference in the number of GW paralogues in the two species. The *Drosophila* genome encodes only one *gw* gene, while the human genome encodes three (Fig. 2). The role of dGW in mitotic PBs could be carried out by one of the other human GW paralogues while GW182 may regulate other stages of the cell cycle. Alternatively, in human cells macroscopic PBs may not be required for regulation of mitotic events. Some mRNA regulatory pathways such as RNA*i* (Chu and Rana, 2006) and ARE-mediated decay (Stoecklin et al., 2006) can occur in the absence of visible PBs. Observation of the distribution of other human GW paralogues during the cell cycle may distinguish between these possibilities. Another human GW paralogue showing a similar distribution to dGW during the cell cycle would indicate that it performs a mitotic function similar to dGW.

4.4.2 A model for regulating progression through mitosis that includes dGW

In gw^{1} mutant embryos, mitosis is impaired, while continued enlargement of nuclei suggests that DNA synthesis continues. This suggests that dGW may not affect all stages of the cell cycle. Localization of PBs in the region of the mitotic spindle is consistent with this idea (Fig.16). Detailed observations of cyclin B protein localization during mitosis in syncytial and cellularized *Drosophila* embryos showed that it accumulates on the mitotic spindle during metaphase, starts to disappear during late metaphase and is no longer detectable in anaphase (Huang and Raff, 1999). Cyclin B mRNA was also detected on the mitotic spindle in syncytial *Drosophila* embryos (Lecuyer et al., 2007). Localization of cyclin B protein, mRNA and dGW to the mitotic spindle suggests a mechanism regulating progression through mitosis by highly refined spatial and temporal

regulation of cyclin B protein levels. Huang and Raff (1999) proposed that cyclin B protein accumulates on the spindle to maintain high levels of cdk1 activity and preserve the integrity of the spindle to prevent premature initiation of anaphase. Localization of cyclin B mRNA to the spindle could maintain high levels of cyclin B protein by localized translation. dGW could regulate progression through mitosis by suppressing or degrading cyclin B mRNA at the same time that cyclin B protein is being degraded in the vicinity of spindle. This would insure that there is no further accumulation of newly synthesized cyclin B protein in this region to inhibit the initiation or progression through anaphase (Fig.18).

4.4.3 A model for post-mitotic nuclear P body function

Transient assembly of PBs in the nuclei of live cells expressing GFP-dGW having just completed cytokinesis indicates a rapid accumulation of non-translating mRNA in PBs at this time (Fig 17). Nuclear clearing of the GFP signal after dispersal of the PBs suggests that dGW is exported to the cytoplasm after disassembly of the PBs. PB-associated mRNAs could be either degraded or dispersed upon disassembly of these PBs. I am not aware of previous reports of such a process.

dGW contains a UBA domain whose function has not been analyzed; however, there is evidence supporting a role for proteins with this domain in nuclear export of mRNA. The UBA domain is found in mRNA export factors of *Saccharomyces cerevisiae* (Mex67p) and several metazoans (NXF), including human, *Caenorhabditis elegans* and *Drosophila* (Herold et al., 2000). This domain was originally named for its role in interacting with ubiquitinated proteins. It has been associated with two nuclear export functions. One of these functions, carried out by Mex67p, is the interaction with ubiquitinated Hpr1, which is another nuclear export factor. This interaction results in transient protection of ubiquitinated Hpr1 from degradation by the 26S proteasome acting as a regulatory step in the nuclear export pathway (Gwizdek et al., 2006). The other nuclear mRNA export function is through interaction of the UBA domain with nucleoporins facilitating

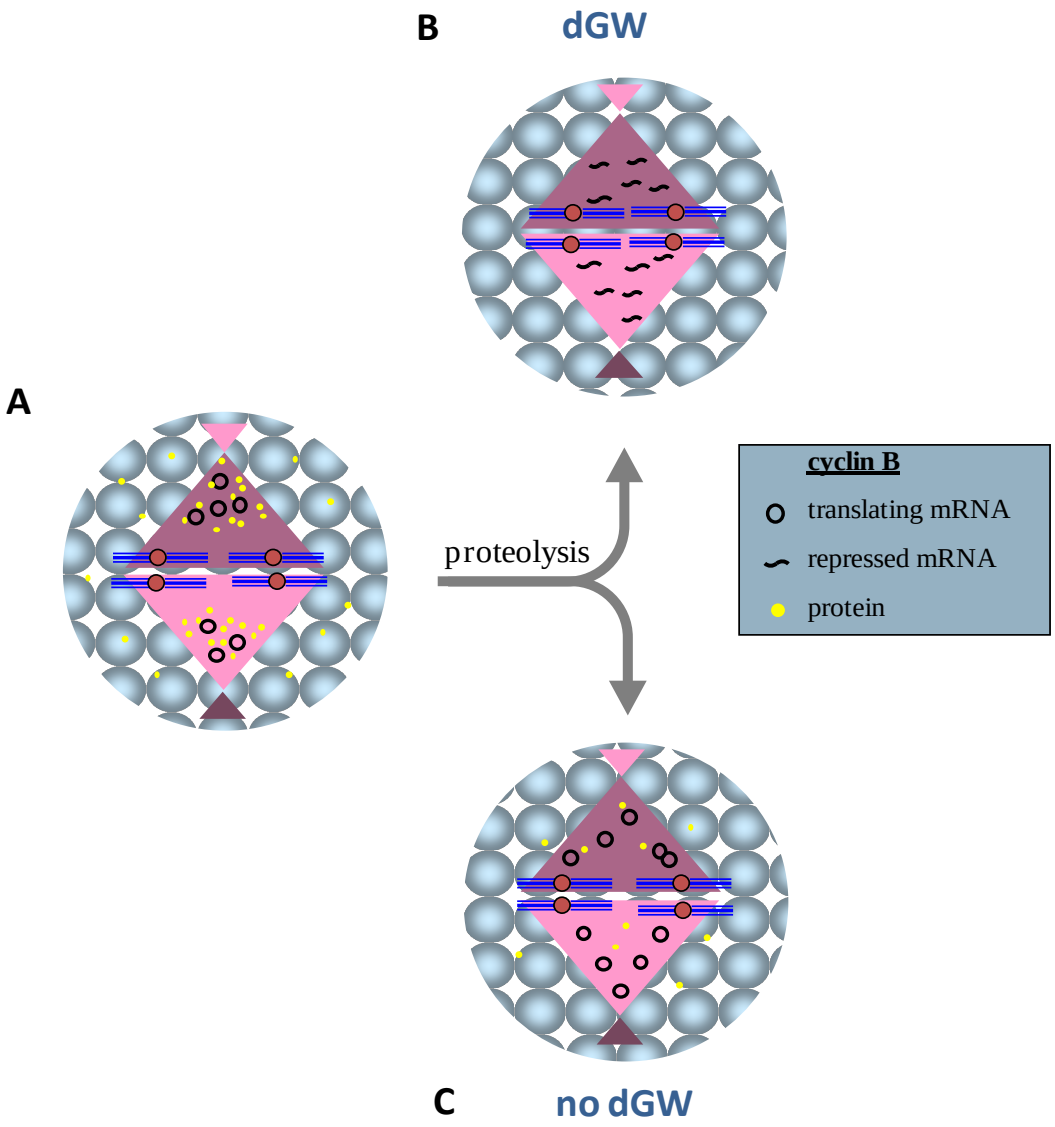


Figure 18. Loss of dGW leads to inappropriate expression of cell cycle regulatory proteins. (A) A metaphase cell with spindle-localized cyclin B mRNA and protein. Spindle-localized cyclin B mRNA maintains high levels of cyclin B protein on the spindle to inhibit the onset of anaphase. (B*)* An anaphase cell with functional dGW. Cyclin B mRNA is repressed or degraded. After proteolysis, cyclin B protein is eliminated to allow progression through anaphase. (C) An anaphase cell without dGW. Cyclin B mRNA is active and cyclin B protein continues to be translated. Proteolysis cannot adequately reduce cyclin B levels to allow progression through anaphase.

passage through the nuclear pore (Strasser et al., 2000; Suyama et al., 2000). The UBA domain in dGW could function in export of dGW and associated mRNAs from the nucleus into the cytoplasm upon dispersal of nuclear PBs. This idea could be tested by observing the effect of destroying the UBA domain on trafficking of dGW after dispersal of nuclear PBs. Impairment in export of dGW to the cytoplasm could also impair subsequent stages in the cell cycle.

The functional significance of a major posttranscriptional regulatory event that may be represented by the transient assembly of post mitotic nuclear PBs is not known. However, a reasonable hypothesis could be made based on the timing of this event. mRNAs associated with dGW in nuclear PBs may be exported in association with dGW from the nucleus to the cytoplasm or degraded while in the nucleus. Either possibility would result in elimination of mRNA from the nucleus after formation of the nuclear membrane at the end of mitosis (Fig. 19). This suggests a mechanism for restoring compartmentalization of mRNA in the cytoplasm during interphase.

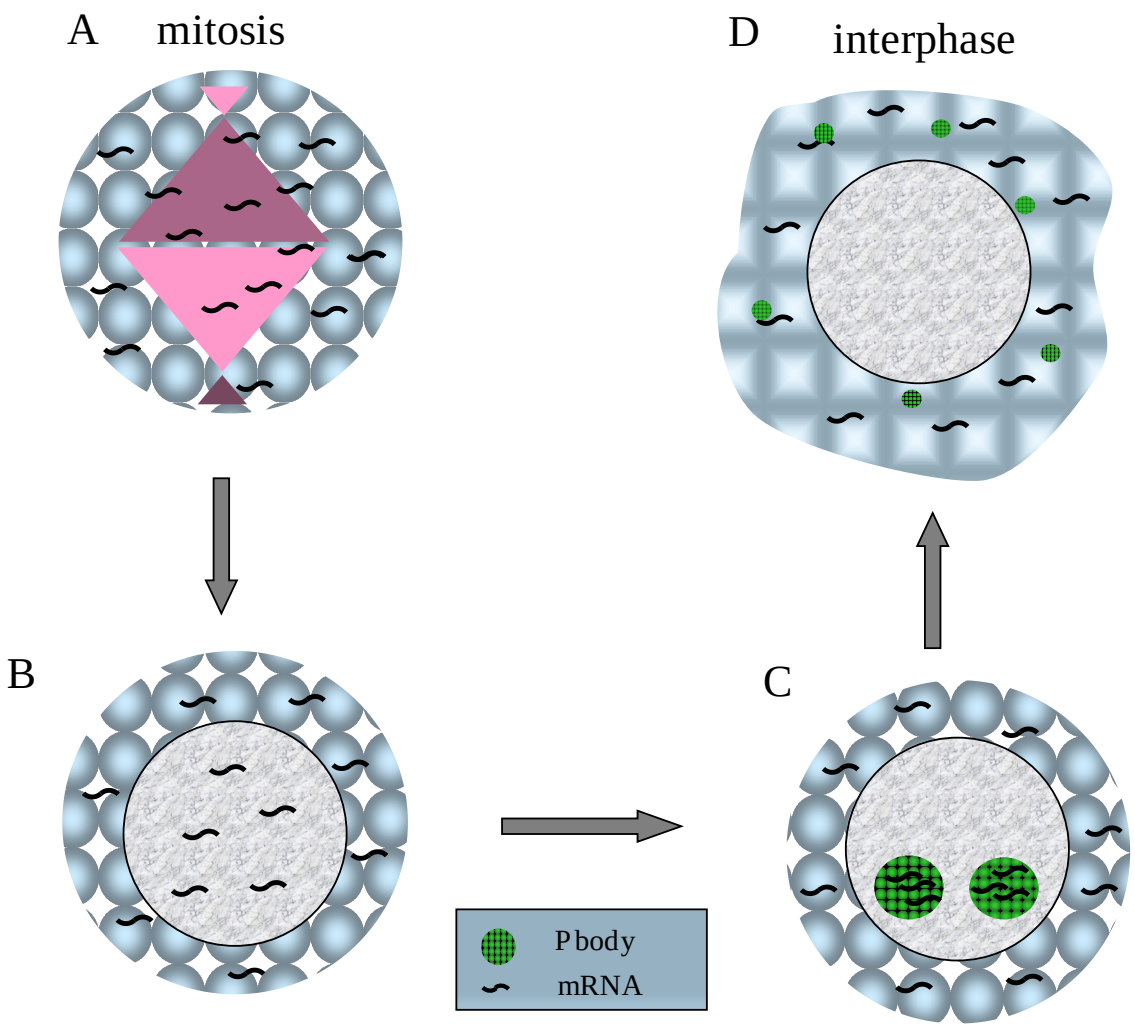


Figure 19. PBs may reorganize nuclear mRNA after mitosis. (A) During mitosis mRNA is distributed throughout the cell. (B) When the nuclear membrane forms after mitosis, some mRNA will be trapped in the nucleus. (C) This mRNA may be targeted to nuclear PBs at the end of mitosis. (D) mRNAs can then be degraded in the nucleus or be exported to cytoplasm with dGW.

CHAPTER 5: *DROSOPHILA* RNASE MRP

5.1 Overview

5.1.1 Rationale for studying RNase MRP

To determine the cause of the mitotic defects that were observed in gw^1 mutants, I was interested in identifying mRNAs and mRNA regulatory pathways that are affected by this mutation. Given the role of GW proteins in silencing mRNA, gene products that are targets of the gw^1 mutation should be overexpressed. Consistent with the phenotype of the gw^1 mutation, these gene products should affect the later part of mitosis. Cyclin B is a cell cycle regulator that matches both of these criteria. The gw^1 mutation (Schneider et al., 2006) and failure to degrade cyclin B both result in a failure to complete mitosis in *Drosophila* (Parry and O'Farrell, 2001) (Fig. 13). Localization of dGW to the region of the mitotic spindle (Fig.16) where cyclin B mRNA localizes (Lecuyer et al., 2007) is also consistent with cyclin B mRNA being a target of dGW during mitosis.

Although little is currently known about how mRNA regulation influences cell cycle progression, one mRNA regulatory pathway with a clear role in this process has been described. In this pathway, RNase MRP, a site-specific endoribonuclease, has been shown to regulate the cell cycle by initiating the degradation of cyclin B mRNA at the end of mitosis in *Saccharomyces cerevisiae* (Gill et al., 2004) and in human cells (Thiel et al., 2005). Further, a component of this pathway has been localized to a specialized PB-like structure in *Saccharomyces cerevisiae* (Gill et al., 2006). An association of RNase MRP with PBs in other species has not yet been reported. Demonstrating such an association in *Drosophila* would indicate the presence of a conserved posttranscriptional mechanism regulating cell cycle progression involving RNase MRP acting in PBs, cellular sites with a well established role in mRNA regulation.

5.1.2 Function and Composition of RNase MRP

RNase MRP is an essential, highly conserved eukaryotic ribonucleoprotein complex (Piccinelli et al., 2005) consisting of a non-coding RNA subunit and at least 10 proteins (Salinas et al., 2005; Schmitt and Clayton, 1992; Welting et al., 2004; Yuan et al., 1991).

Studies in *Saccharomyces cerevisiae* and mammals have identified three RNase MRP substrates that are cleaved in a site-specific manner. These substrates are associated with diverse cellular processes. In mammalian cells, RNase MRP processes mitochondrial transcripts complementary to the origin of replication of the mitochondrial chromosome to produce primers for mitochondrial DNA replication (Chang and Clayton, 1987). In *Saccharomyces cerevisiae* it cleaves the rRNA (ribosomal RNA) precursor to produce mature ribosomal RNA (Lygerou et al., 1996; Schmitt and Clayton, 1993) and B-type cyclin (*CLB2*) mRNA to regulate cell cycle progression (Gill et al., 2004). Defects associated with processing of the rRNA precursor and regulation of cyclin B mRNA have also been reported in human cells however direct cleavage of these substrates by RNase MRP has not yet been demonstrated (Thiel et al., 2005; Thiel et al., 2007). An orthologue of MRP RNA was predicted in the *Drosophila* genome by Piccinelli and colleagues (Piccinelli et al., 2005); however, expression of this gene had not yet been verified.

The RNA subunit of RNase MRP (MRP RNA) is evolutionarily related to the RNA subunit of RNase P (P RNA), which is found in all kingdoms as well as in the genomes of mitochondria and chloroplasts (Xiao et al., 2002). Like RNase MRP, RNase P is a ribonucleoprotein complex involved in RNA processing and shares many common protein subunits with RNase MRP (Rosenblad et al., 2006). Further, both RNAs can adopt similar structures (Piccinelli et al., 2005). RNase P, however, acts on different substrates. Its most extensively studied substrate is the precursor tRNA, which it cleaves to produce mature tRNA. Precursors to the 4.5S RNA component of the signal recognition particle and transfer-messenger RNA are among other documented RNase P substrates (Kazantsev and Pace, 2006; Kirsebom, 2007). Transfer-messenger RNA is a bacterial molecule with both tRNA and mRNA-like properties. It is recruited to aberrant mRNAs that are stalled during translation and recycles the stalled ribosome. For example, if an mRNA is stalled due to lack of a stop codon, transfer-messenger RNA mediates a process that rescues the stalled ribosome , marks the resulting incomplete polypeptide with a signal that directs its subsequent degradation and facilitates degradation of the aberrant mRNA (Shpanchenko et al., 2005).

MRP RNA is the first non-coding RNA to be associated with a human disease. Ridnapaa et al, (2001) described a set of mutations in the human MRP RNA gene (*RMRP*) that co-segregate with the phenotype of the autosomal recessive disease, cartilage-hair hypoplasia (CHH). CHH is a pleiotropic disease characterized by short stature (dwarfism) combined with a variety of other symptoms such as low abundance of hair, immunodeficiency, hypoplastic anemia and predisposition to cancers. Various *RMRP* mutations are currently known to be associated with other allelic diseases such as Omenn syndrome and anauxetic dysplasia (AD) (Martin and Li, 2007; Thiel et al., 2005). Short stature is common to all of these diseases; however, each disease presents a characteristic spectrum of other disorders affecting a broad range of organs. These include mental retardation, impaired spermatogenesis, respiratory disorders and Hirschsprung disease, a developmental disorder resulting in a blockage of the large intestine due to the absence of ganglion cells (Makitie et al., 2001a; Makitie et al., 2001b; Martin and Li, 2007; Toiviainen-Salo et al., 2008). Recent studies have identified correlations between molecular defects and disease symptoms in patients with various *RMRP* mutations. Mutations that affect ribosomal RNA processing are correlated with characteristic AD symptoms of severe skeletal defects with normal abundance of hair. Mutations that result in increased cyclin B mRNA levels are correlated with symptoms characteristic of CHH. These patients show less severe skeletal defects but have higher incidences of immunodeficiency, hematological abnormalities and cancer (Thiel et al., 2005; Thiel et al., 2007).

5.2 Objectives

My first objective was to verify expression of the predicted *Drosophila* MRP RNA (dMRP RNA) gene. My second objective was to determine if dMRP RNA shares functional homology with MRP RNA in other species. Towards this objective, I characterized the phenotype of a *Drosophila* strain with a mutation in the dMRP RNA gene. My final objective was to identify a potential relationship between RNase MRP and PBs.

5.3 Results

5.3.1 The Predicted *Drosophila* MRP RNA gene

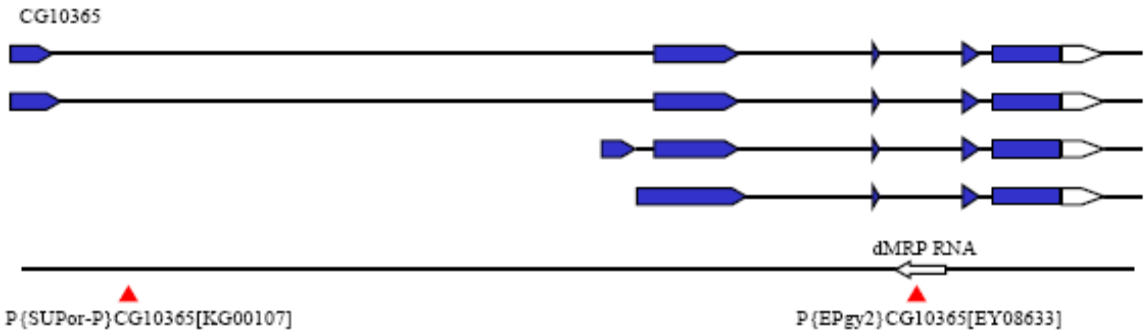
A single *Drosophila* gene encoding MRP RNA was predicted by Piccinelli et al., (2005) using a bioinformatics approach to identify genes for P RNA and MRP RNA from a broad range of eukaryotic genomes. Expression of this gene was previously detected as part of a screen for small non-coding RNAs. In this screen it was identified as smnRNA:342, a small non-coding RNA of unknown function (Yuan et al., 2003). dMRP RNA is encoded on the third chromosome within an intron of CG10365, a predicted protein-coding gene of unknown function (FlyBase; Fig. 20 A).

MRP RNA is transcribed by RNA polymerase III (Pol III) in all genomes except yeast (Dieci et al., 2007). The presence of genomic sequences resembling Pol III promoter elements upstream of transcribed *dmrp* sequences suggests that dMRP RNA is also transcribed by Pol III. These include a TATA-like sequence and a proximal sequence element (PSE), both resembling Pol III promoter elements characteristic of *Drosophila* U6 spliceosomal RNA genes (Hernandez et al., 2007). A typical Pol III termination element consisting of a short run of T residues (Dieci et al., 2007) is present downstream of transcribed sequences (Fig. 20 B).

5.3.2 The predicted dMRP RNA is expressed throughout development and localizes to the nucleolus

Expression of dMRP RNA was detected throughout the *Drosophila* life cycle. This is consistent with roles of RNase MRP in the essential cellular processes of ribosome biogenesis, mitochondrial DNA replication and cyclin B mRNA degradation (Fig. 21 A, B). In *Saccharomyces cerevisiae* and in human cells, the major fraction of MRP RNA is in the nucleolus, specifically in the dense fibrillar component marked by fibrillarin where it processes rRNA (Jacobson et al., 1995; Schmitt and Clayton, 1992). dMRP RNA colocalizes with *Drosophila* fibrillarin in the nucleolus (Fig. 21 C), indicating the presence of a conserved RNase MRP complex that processes rRNA in *Drosophila* as in other organisms.

A



B

ctgcaatcaa aaatgggaaa tgtatattta ataaaataca caaaaacatg tggttcacat

agatctaaaa atatttaatg agttcgcccc gcgcgggttt tgaacccgta tctttcggat

tggggtgtgc tattcatatc gcgttcggct ggttatgatt cccaactcgt ttttccgctg

atggcgcgta taaatagcgc tgatagcagc aaatgtgatg ccggtttgag tcttccatgc

ttgtctctcg gggccacaaa acgagttcct ggtaactcaa ctgataatgc cctgggcgaa

agtccccggg cctaggatag aaagtatcaa ggtgtaaaaa gtgtgcacaa aacacccacc

acccctgtgg tgggtggtgc attcgcctat attctgcgga atttcgcctg gcgtatggat

gaagaggatt ttatccgaat ccttacgcgc caggttgtct gcggaaatct gccagagtaa

tcttagatat ggacgagttg gtaggactcg gcgggtggtg ttcacacact ttctcgtctg

agaaaccgcc tacacagaat ggggcttaca ttgggaaact cggacggcgc actcccgttt

ttatacaacg atatcataac atatatatat ataaccccca ccccccttgg aatcatgatc

tttatacttt actcac

-44 -23 +1

Figure 20. The *dmrp* RNA gene. (A) dMRP RNA is encoded in the second to last intron of CG10365, transcribed from the opposite strand. Translated sequences are blue; red triangles indicate P element insertions. P[EPgy2] CG10365[EY08633] disrupts *dmrp*. (B) *dmrp* sequence with potential Pol III regulatory elements. The sequence of the entire intron encoding *dmrp* is shown. *dmrp*-transcribed sequences including the terminator element are underlined. Positions of the PSE (-44 nt) and TATA-like element (-23 nt) upstream of the transcription start site (+1) are indicated above the sequence. Green nucleotides in promoter elements are identical in all reference sequences (Hernandez, Valafar et al., 2007); blue nucleotides are the same in at least one of the reference sequences. Red nucleotides indicate the Pol III terminator.

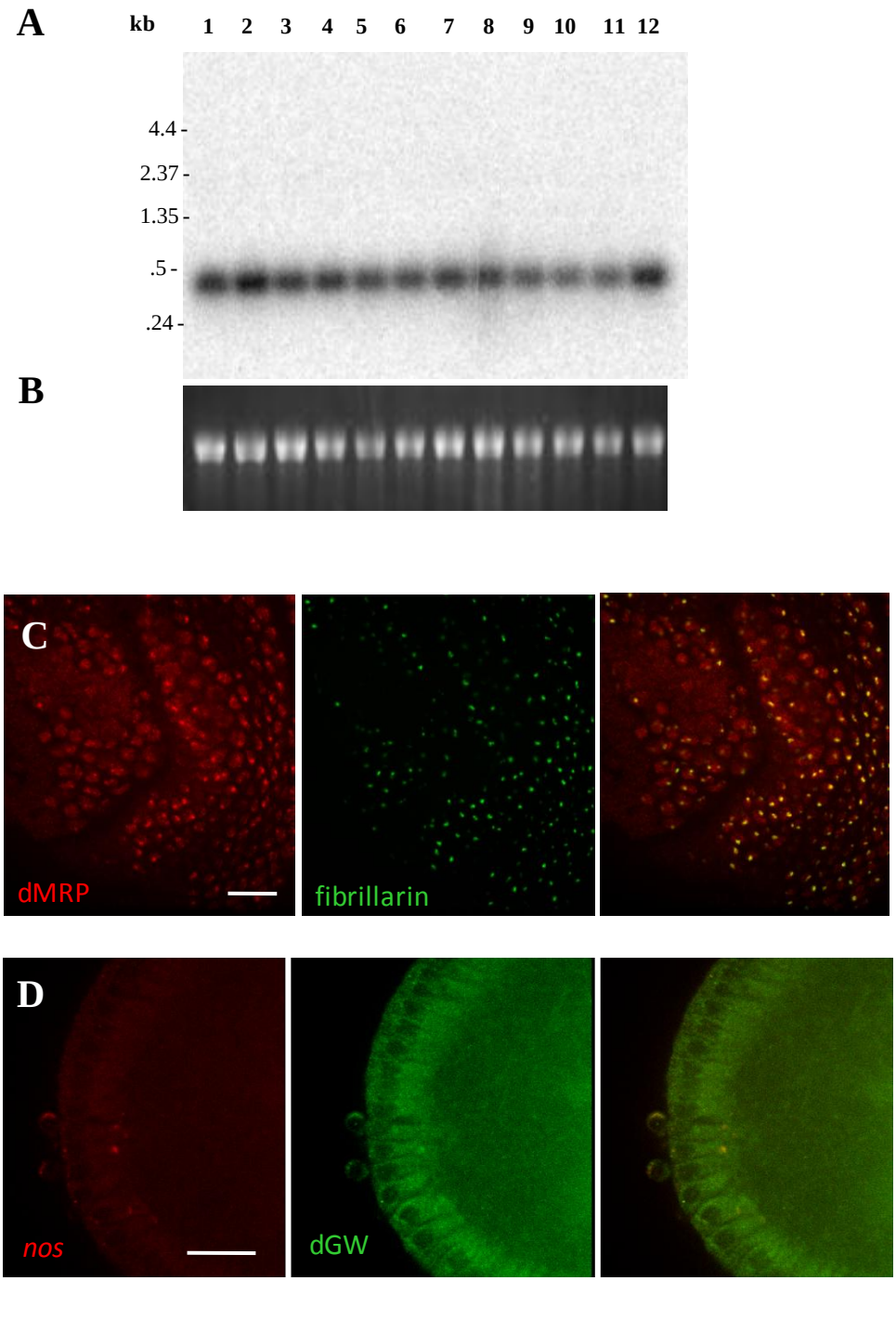

A
kb
1 2 3 4 5 6 7 8 9 10 11 12
4.4 -
2.37 -
1.35 -
.5 -
.24 -
B
C
dMRP
fibrillarin
D
nos
dGW

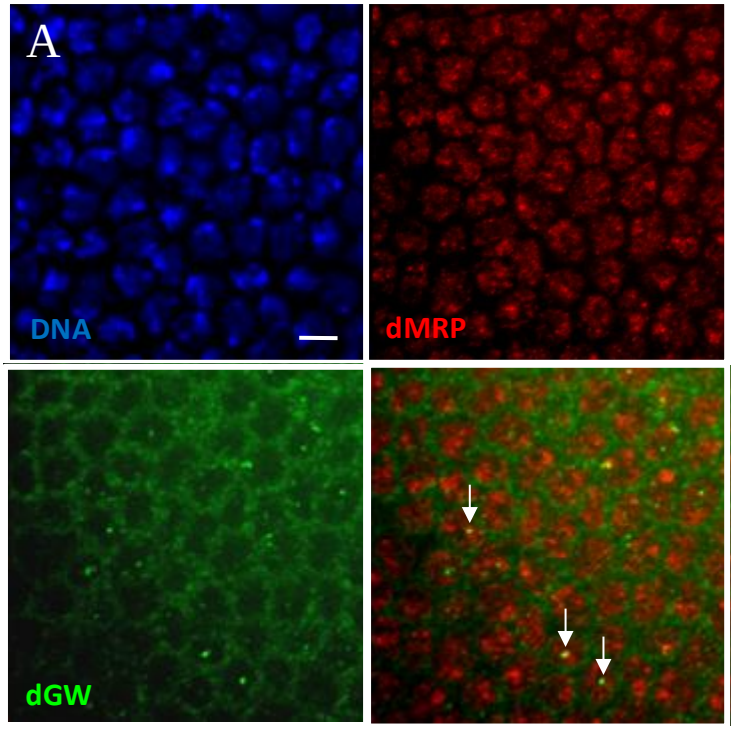
Figure 21. Expression and nucleolar localization of dMRP RNA . (A) dMRP RNA is expressed throughout *Drosophila* development. A Northern blot of total *Drosophila* RNA was probed with dMRP antisenese RNA. dMRP RNA corresponding to the predicted size of 383 nucleotides was detected throughout the *Drosophila* life cycle. 1) stage 1-2; 2) stage 3-4; 3) stage 5-6; 4) stage 7-9; 5) stage 10-14; 6) stage 14-16; 7) late stage [16-23 hr.]; 8) 1^{st} instar; 9) 2^{nd} instar; 10) 3^{rd} instar; 11) pupae; 12) adult. (B) rRNA as a loading control was visualized by ethidium bromide staining. (C) dMRP RNA colocalizes with fibrillarin in cellularized *Drosophila* embryos. dMRP RNA was detected by FISH and fibrillarin with rabbit anti-fibrillarin antibody. Bar is 10 μm. (D) *Nos* mRNA was detected by FISH. The most intense *nos* signal was in the cytoplasm of pole cells and in granules in the embryo's posterior plasm. Distinct localization patterns shown by *nos* and dMRP probes indicate that both hybridizations are specific. Bar is 50 μm.

A hybridization reaction was also performed with an antisense probe directed against nanos (*nos*) mRNA as a control for the specificity of hybridizations (Fig. 21 D). Both probes showed distinct patterns of hybridization, each localizing to the expected cellular and subcellular regions. In contrast to dMRP RNA which was detected in the nucleoli of most cells, *nos* mRNA was detected in the cytoplasm of embryonic pole cells and granules in the posterior plasm. These granules are likely to be polar granules that direct formation of the abdomen and pole cells (Ephrussi et al., 1991).

5.3.3 dMRP RNA also localizes to non-nucleolar structures

One of the functions of the non-nucleolar fraction of RNase MRP is regulation of the cell cycle by degrading cyclin B mRNA (Gill et al., 2004; Thiel et al., 2005; Thiel et al., 2007). There is evidence suggesting that this occurs by a mechanism involving PBs. In *Saccharomyces cerevisiae*, a protein subunit of RNase MRP, Pop1 was localized to a specialized PB named the temporal asymmetric MRP (TAM) body (Gill et al., 2006). The TAM body appears transiently during mitosis at the time of *Clb2* mRNA and protein degradation (Spellman et al., 1998; Yeong et al., 2000). It has been proposed that the TAM body is the site of Clb2 mRNA degradation that regulates exit from mitosis (Gill et al., 2006).

To investigate the potential localization of *Drosophila* RNase MRP to PBs, the localization of dMRP RNA with the PB component dGW was compared. In interphase embryos, most of the dMRP RNA was detected in the nucleolus where it occasionally colocalized with dGW (Fig.22 A). The significance of the nucleolar localization of dGW is not clear; however, it suggests that dGW may perform a function in addition to regulating cytoplasmic mRNA. During mitosis, both dMRP RNA and dGW were dispersed into numerous foci of various sizes that occasionally colocalized (Fig.22 B). Given that PBs contain mRNA targeted for repression and degradation (Liu et al., 2005b; Sheth and Parker, 2003), colocalization of dMRP RNA with the PB component dGW during mitosis is consistent with a role for *Drosophila* RNase MRP in degrading an mRNA encoding a cell cycle regulator, such as cyclin B.


A
DNA
dMRP
dGW

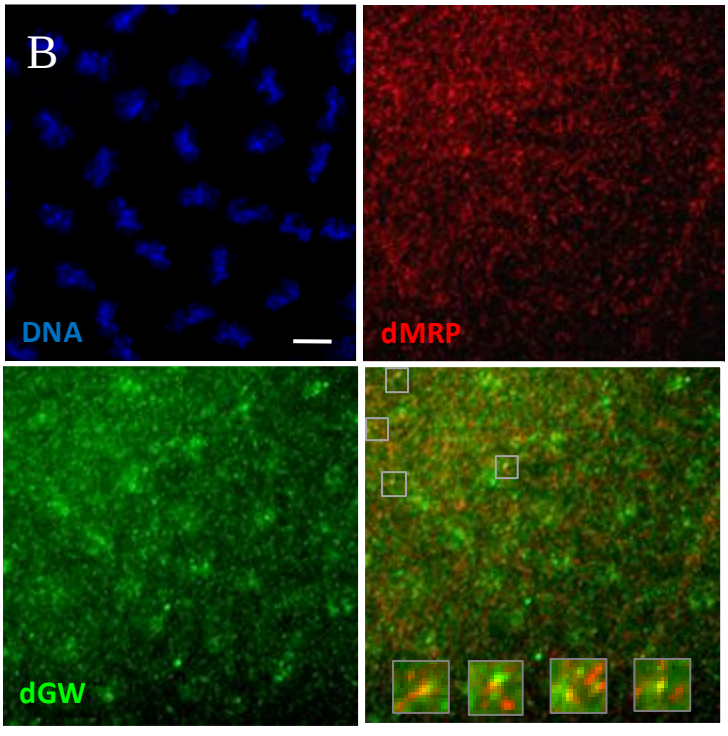

B
DNA
dMRP
dGW

Figure 22. dMRP RNA and dGW occasionally colocalize in *Drosophila* embryos. DNA stained with Pico-green (recoloured blue) shows nuclei in interphase (A) and mitosis (B). In each adjacent panel dMRP RNA is red and dGW is green. In the merged panel, only dMRP and dGW are shown, for clarity. (A) In interphase, dMRP RNA is in the nucleus. dGW occasionally colocalizes with dMRP in the nucleus. (B) In mitosis, dGW and dMRP RNA occasionally colocalize or are seen in closely apposing foci as shown in boxed areas. Each boxed area is shown magnified 3X in an inset. Bar is 10 μm.

To investigate a potential role for RNase MRP in replication of mitochondrial genomes (Chang and Clayton, 1989), localization of dMRP RNA was examined in third instar larval muscle tissues stained with cytochrome c. Some cytoplasmic dMRP foci were observed in structures marked by cytochrome c, suggesting that *Drosophila* RNase MRP may also function in replication of mitochondrial genomes (Fig. 23).

5.3.4 Characterization of a mutant dMRP RNA strain

5.3.4a A P element insertion disrupts dMRP RNA

dMRP is encoded in an intron of the uncharacterized gene CG10365, transcribed from the opposite DNA strand. The P element P[EPgy2] CG10365[EY08633] is inserted in transcribed dMRP sequences (Fig.20 A). Since the P element insertion in an intron does not disrupt the transcribed sequences of CG10365, the lethal phenotype may be caused by disruption of the dMRP transcribed sequences. To test this idea a complementation test was performed to determine if another lethal P element insertion in CG10365 would complement the P[EPgy2] CG10365[EY08633] insertion and result in a viable heterozygote. The results of this test show that the heterozygous progeny of a cross between the lethal P-element insertion strains P[EPgy2]CG10365[EY08633] and P[SUPor-P]CG10365[KG00107] were indeed viable. Also, the proportion of progeny of this genotype was consistent with random segregation of chromosomes carrying each insertion, demonstrating that the viability of the heterozygote was not impaired (Table 5). These results show that each insertion impairs the function of a distinct genetic unit and are consistent with the idea that P[EPgy2] CG10365[EY08633] does not disrupt the function of CG10365.

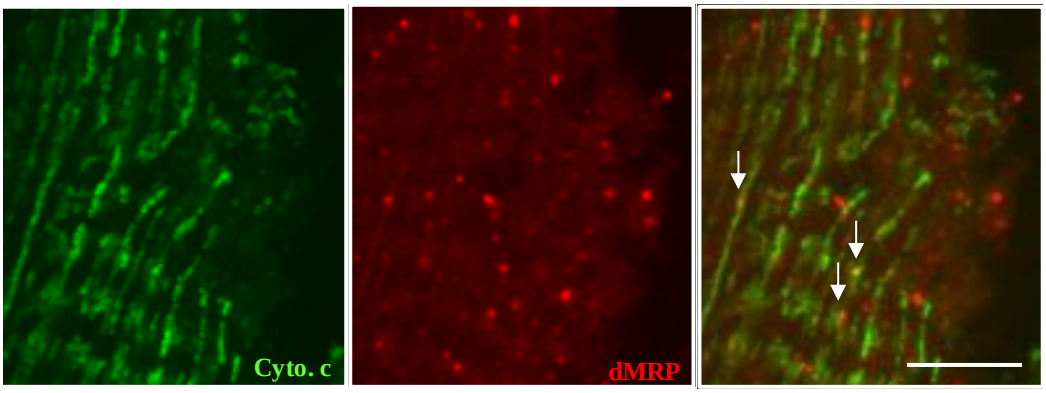


Figure 23. dMRP RNA localizes to mitochondria in *Drosophila* larval muscle.

Some dMRP foci localize to mitochondria stained with cytochrome c (cyto. c).

Bar is 5 μm.

Table 5. Complementation Test: Two P element Insertions Disrupt Distinct Functions in CG10365

P{SUPor-P}CG10365[KG00107] /TM3, Sb^1 Ser X P{EPgy2}CG10365[EY08633] / TM3, Sb^1 Ser^1

Progeny	# observed	# expected
P{SUPor-P}CG10365[KG00107] / TM3, Sb^1 Ser^1	66	70.7
P{EPgy2}CG10365[EY08633] / TM3, Sb^1 Ser^1	80	70.7
P{EPgy2}CG10365[EY08633] /P{SUPor-P}CG10365[KG00107]	66	70.7
TM3, Sb^1 Ser^1 / TM3, Sb^1 Ser^1	0	0
total	212	

5.3.4b dMRP mutants are impaired in growth and development

A significant impairment in growth of homozygous dMRP mutants compared to the normal w^{1118} strain was observed beginning at 3 days AED. At 5 days AED, the mean cross sectional area of homozygous dMRP mutants was approximately one third of the w^{1118} strain (Fig. 24). Homozygous dMRP mutants were also delayed in development, undergoing the first molt approximately one day later than normal (Fig. 25 A). Mouth hooks examined after death had 2- 4 teeth (Fig. 26 B), characteristic of second instar larvae (Apatov, 1929). Growth and development of heterozygous dMRP larvae were indistinguishable from the w^{1118} strain (Fig. 24 and 25).

Homozygous dMRP mutants showed a bimodal pattern of mortality. Approximately 70% of individuals died between 5 and 6 AED while the remaining individuals lingered for up to 17 days, which is several days after normal individuals have reached adulthood (Fig. 26A). Analysis of dMRP RNA levels by Northern blot showed a progressive decline in homozygous mutants from 1 to 5 days AED, while levels remained constant in dMRP heterozygotes (Fig. 27). This progressive decline in dMRP levels is likely due to depletion of maternal dMRP RNA and correlates with the timing of impairments in growth and development prior to mortality (Fig. 25 and 26).

5.4 Discussion

5.4.1 *Drosophila* MRP RNA is homologous to MRP RNA genes in other species

The evidence presented in this chapter supports the idea that dMRP RNA, a non-coding RNA component of the RNase MRP complex, is a structural and functional *Drosophila* homologue of conserved MRP RNA genes previously characterized in other eukaryotes. Analysis of structure and function of this gene was undertaken on the basis of evidence provided by Piccinelli and colleagues (Piccinelli et al., 2005) who identified dMRP RNA along with numerous other potential MRP RNA genes in other species using a bioinformatics approach to screen for MRP RNA genes in a broad range of eukaryotes.

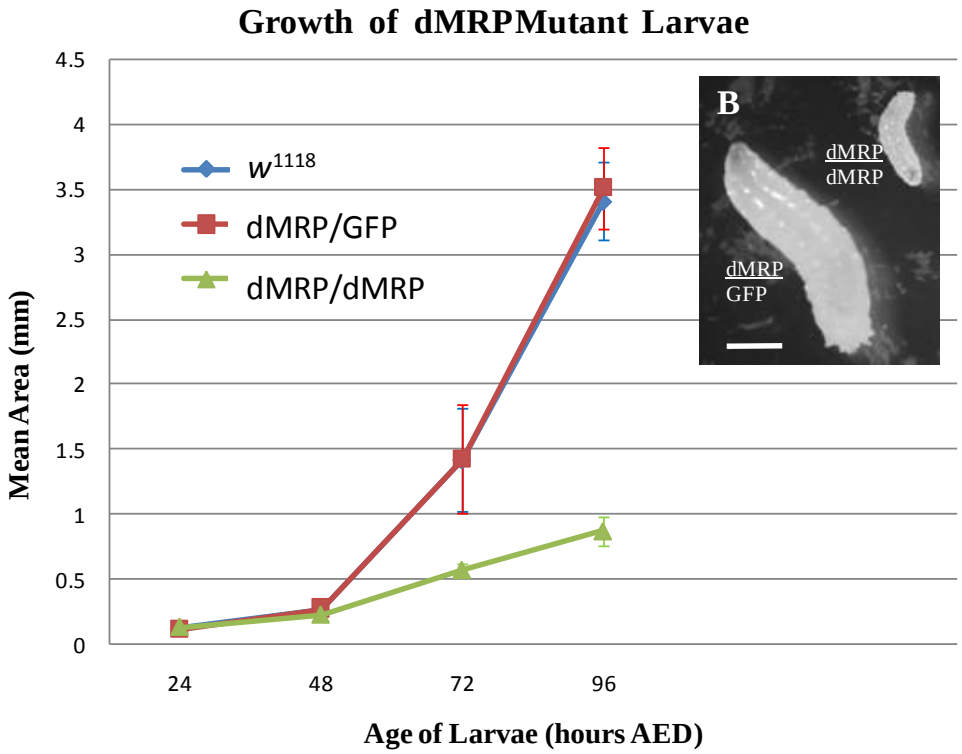


Figure 24. Homozygous dMRP mutant larvae are delayed in growth. (A) dMRP/MRP mutants showed a significant growth delay at 72 h AED. For w^{1118}, dMRP/GFP, and dMRP/dMRP genotypes, the mean cross-sectional area for 20 individuals for each day from 1 to 4 days of age was determined using Image J. (B) Comparison of 5-day-old dMRP/dMRP and dMRP/GFP mutants. Bar is 1mm.

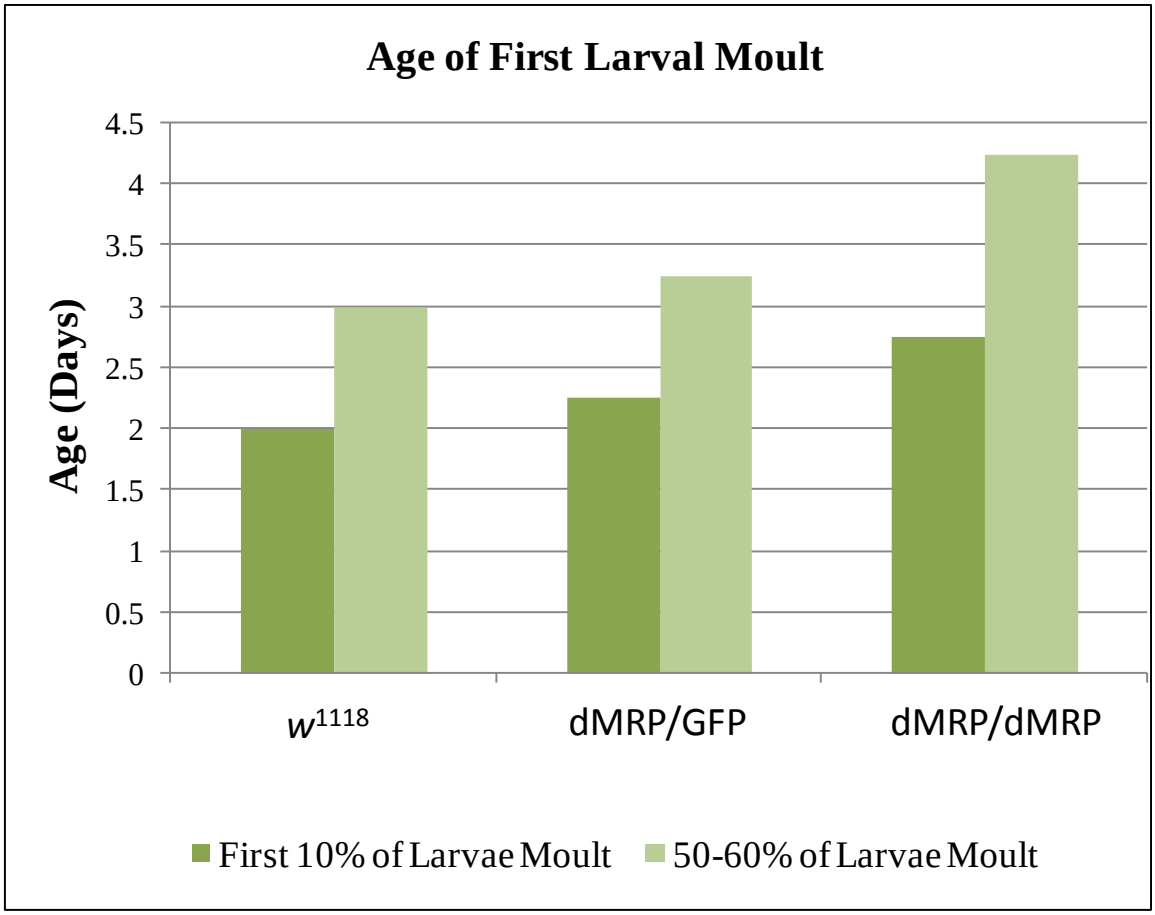


Figure 25. Homozygous dMRP mutant larvae are delayed in development.

The first molt in dMRP/dMRP mutants occurred approximately one day later than normal.

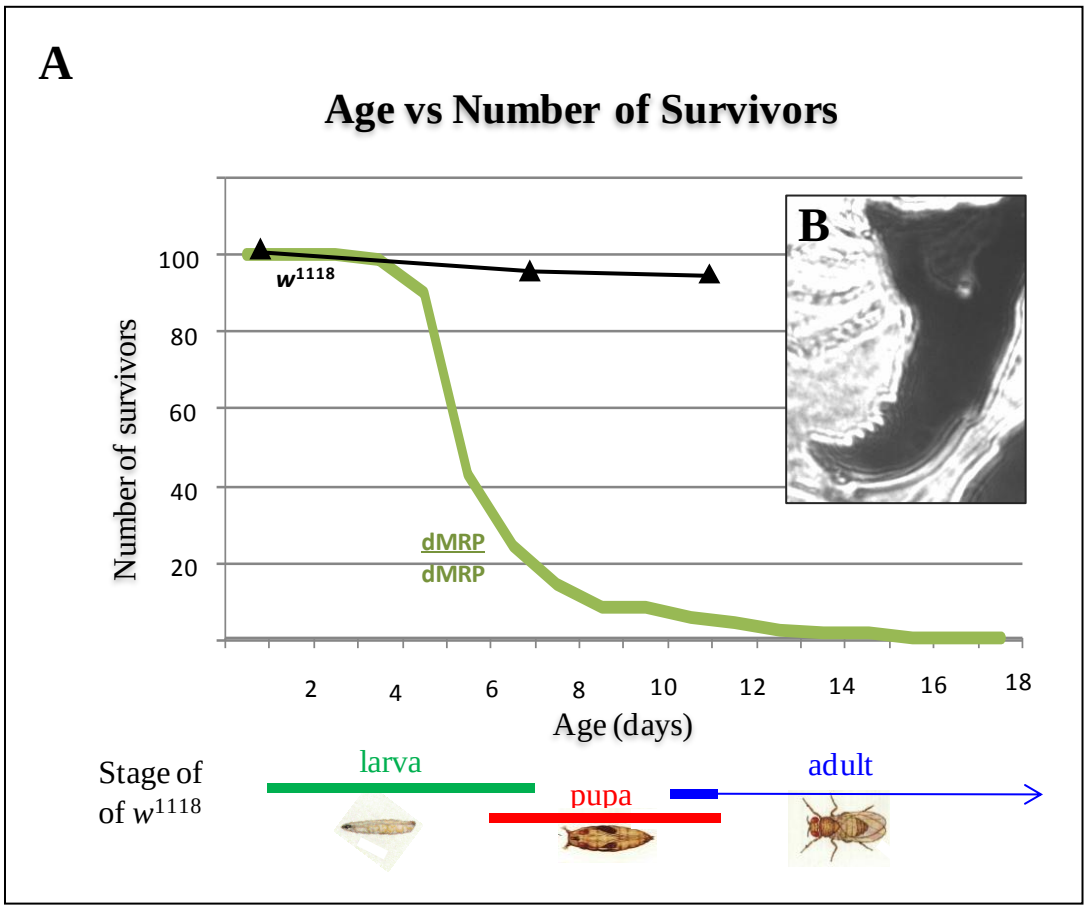


Figure 26. Homozygous dMRP mutants die during the second instar stage.

(A) A bimodal mortality pattern of homozygous dMRP mutant individuals was observed. Approximately 70% of individuals died between 5 and 6 days AED, while the remaining individuals lingered as second instar larvae for up to 17 days. Coloured bars below the graph show the time of development of normal (w^{1118}) individuals. Blue arrow indicates that individual adults were not observed after eclosion but normally survive past the indicated time. (B) Representative mouth hook from 1 of 50 homozygous dMRP larvae examined after death. All mouth hooks had 2- 4 teeth, indicative of the 2nd instar stage.

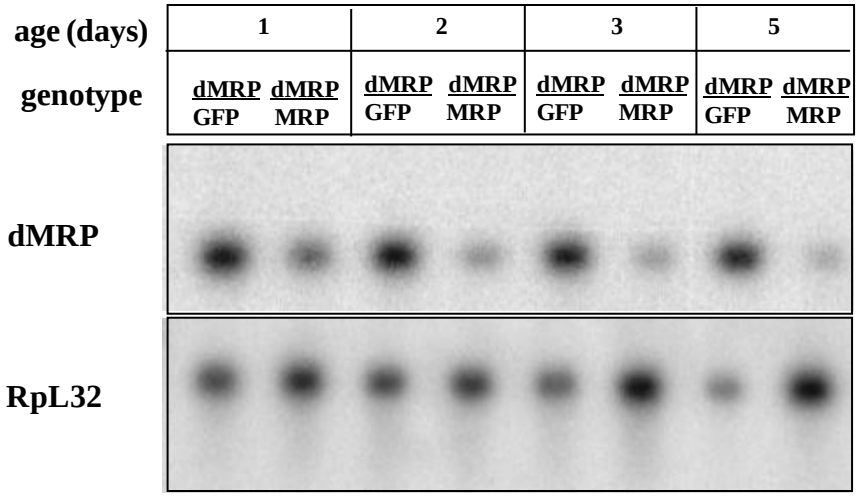


Figure 27. dMRP RNA levels decline at the time of growth arrest and death in dMRP homozygous mutants. Northern blot of total RNA from homozygous dMRP/dMRP mutants and their heterozygous dMRP/GPF siblings. The same blot was re-probed with RpL32 as a loading control.

In addition to the structural and sequence similarities between transcribed dMRP RNA and MRP RNAs in other species identified by Piccinelli et al. (2005), genomic sequences flanking dMRP RNA resembling RNA Pol III regulatory elements (Hernandez et al., 2007) were identified in this study (Fig. 20). The presence of these elements suggests that in *Drosophila,* as in most species (Dieci et al., 2007), MRP RNA is transcribed by RNA Pol III.

A number of additional observations support the idea that this gene is functionally homologous to MRP RNA in other eukaryotes. Expression of dMRP RNA throughout development is consistent with its role in essential cellular processes such as ribosome biogenesis, mitochondrial DNA replication and cell cycle regulation (Chang and Clayton, 1987; Gill et al., 2004; Lygerou et al., 1996; Schmitt and Clayton, 1993) (Fig. 21A). Further, subcellular localization of dMRP RNA is similar to localization of RNase MRP in other species. Like in human cells (Jacobson et al., 1995) and in *S Saccharomyces cerevisiae* (Gill et al., 2006), dMRP RNA localizes to the nucleolus (Fig. 21B), where it was reported to function in rRNA processing (Lygerou et al., 1996; Schmitt and Clayton, 1993).

The role of RNase MRP in mitochondrial DNA replication has been controversial. The enzyme was initially isolated from mouse cells and shown to cleave an RNA representing the primer for mitochondrial DNA replication (Chang and Clayton, 1987). This activity was dependent on complementarity of a segment of MRP RNA to sequences of the RNA substrate (Bennett and Clayton, 1990). However, the cleavage site on the substrate is 6-10 nucleotides from the *in vivo* cleavage site suggesting that the *in vitro* activity may be an artifact (Kiss and Filipowicz, 1992). In favour of a role for RNase MRP in mitochondria, there is reasonable evidence for localization of MRP RNA to mitochondria of mouse cardiomyocytes by *in situ* hybridization (Li et al., 1994). Localization of dMRP RNA to cytochrome c containing structures in this study (Fig. 23B) provides additional evidence supporting a role for RNase MRP in mitochondria. Future identification of potential defects in mitochondrial function in the dMRP mutant strain could provide additional evidence for a role for RNase MRP in mitochondrial DNA replication and contribute to the advancement in understanding of this basic cellular process.

Figure 23 shows that not all mitochondria appear to contain MRP RNA. RNase MRP may only be present in mitochondria whose genomes are replicating. The third instar larval stage is a time when muscle cells in wandering larvae would be actively respiring, but rapid growth would have ceased. Consequently, the number of replicating mitochondrial genomes may be reduced at this stage. A higher proportion of replicating mitochondria containing dMRP RNA could be detected in younger, rapidly growing tissues or rapidly dividing cells such as S2 cells

In *Drosophila* embryos, dMRP RNA localized to some foci containing dGW during mitosis (Fig. 22). This partial colocalization is consistent with a role in degrading cyclin B mRNA in a PB-like structure, as in *Saccharomyces cerevisiae* (Gill et al., 2006). Localization of MRP RNA to a subset of PBs in these static images may be due to the dynamic nature of PBs. Some PB components transit rapidly in and out of PBs so that they can only be detected under certain conditions. This is particularly evident for proteins involved in NMD (see Table 1). One condition that allowed detection of NMD factors in PBs involved the use of mutants with reduced levels of XRN1. In these mutants, the reduced rate of mRNA degradation results in accumulation of mRNA in enlarged PBs [reviewed in (Eulalio et al., 2007a; Parker and Sheth, 2007)]. A *Drosophila* strain with a mutation in the *Drosophila* homologue of XRN1, Pacman, was recently isolated (Zabolotskaya et al., 2008). If dMRP RNA transits in and out of PBs, a higher frequency of localization of dMRP RNA to PBs could be observed in this mutant.

It is also possible that RNase MRP does not function with dGW and that the level of colocalization that was observed in Fig. 22 is not physiologically significant. It may, however, function in mRNA degradation independently of dGW and localize to a class of PB that does not contain dGW. XRN-1 is required in *Saccharomyces cerevisiae* for RNase MRP-mediated *CLB2* mRNA degradation (Gill et al., 2004) and colocalizes with RNase MRP in TAM bodies (Gill et al., 2006). Consequently, observing colocalization of dMRP RNA with Pacman suggests that these two enzymes may function together in mRNA degradation. Functional relationships between RNase MRP and dGW or RNase MRP and Pacman could be established in further experiments by observing genetic interactions between each pair of mutations.

5.4.2 dMRP RNA mutants resemble human *RMRP* mutants

The similarity in phenotype of the dMRP mutant strain with human patients with *RMRP* mutations further supports the idea that these genes are homologous. The impairment in growth of dMRP mutant larvae resembles the short stature thought to be caused by an intrinsic defect in proliferation of human patients' cells with *RMRP* mutations (Pierce and Polmar, 1982). It is not clear what process in which RNase MRP is involved is causing the impairments in growth and development seen in the dMRP RNA mutants. It is unlikely that it is an impairment of cell cycle regulation by a defect in cyclin B mRNA degradation because cyclins A, B and B3 are not expressed in endoreplicating cells (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990), which constitute most of the larval tissues. The dMRP RNA mutant may be most similar to a class of human *RMRP* mutations that is associated with impaired rRNA processing that does not alter cyclin B mRNA degradation. This class of mutations, is associated with a severe growth defect seen in the disease Anauxetic dysplasia (Thiel et al., 2005; Thiel et al., 2007).

The potential impact of a defect in mitochondrial function on dMRP mutants cannot be inferred from studies of human *RMRP* mutations because defects in mitochondrial function have not been yet been detected in these patients (Hermanns et al., 2005). However, mutations in a variety of mitochondrial functions have been described in *Drosophila*. These mutations can affect a number of diverse processes such as apoptosis (Abdelwahid et al., 2007), spermatogenesis (Hales and Fuller, 1997) and growth in both mitotic and endoreplicating tissues (Morris et al., 2008). The last effect is consistent with the impairment in growth displayed by dMRP RNA mutants. It is therefore possible that the defects in growth of dMRP RNA mutants are caused by impaired rRNA processing and/or impaired mitochondrial functions. Defects in development may be an indirect consequence of growth defects. In *Drosophila* larvae there may be a requirement to reach a certain size before proceeding to the next stage of development.

5.4.3 Unexpected Observations

5.4.3a Localization of dGW in the nucleus

Detection of dGW in nuclei of *Drosophila* embryos has not been previously reported in the published literature and was therefore surprising for a protein with a well established role in cytoplasmic mRNA regulation (Fig 22A). However, a GFP fusion of dGW was also observed in the nuclei of post mitotic S2 cells in this study (Fig. 17). Together these two observations of dGW in the nucleus detected by two different methods strongly suggests that nuclear localization of dGW is not an artifact. dGW was seen for the first time in the nuclei of embryos after they were treated with proteinase K as part of the *in situ* hybridization protocol to expose RNA that may be masked by associated proteins (Fig.17). It is possible that nuclear dGW was also exposed from masking by associated proteins by this treatment. Applying this modification to immunolocalization of dGW could allow its detection where it was previously not observed, such as in fixed *Drosophila* S2 cells. This procedure could also reveal previously undetected nuclear localization of GW proteins in other species.

The role of nuclear dGW is not known; however, it is possible that dGW may be functioning in RNA*i*-mediated gene silencing in the nucleus as a component of the RISC. Although most published studies report post-transcriptional gene silencing by RISC activity in the cytoplasm, there is emerging evidence for RISC activity in the nucleus. The expression of two small nuclear RNAs, 7SK and U6, was reduced by targeting each of them with siRNA (Robb et al., 2005). Also, these siRNAs localized to their targets in the nucleus (Berezhna et al., 2006). Further, Robb et al. (2005) detected the RISC components Ago1 and Ago2 in the nucleus by subcellular fractionation. However, it has been noted that the Ago2 cannot be detected in the nucleus by immunofluorescence [reviewed in (Jakymiw et al., 2007)]. It is possible that, like detection of dGW, detection of Argonaute proteins in the nucleus may be hindered by associated proteins. This would explain why Ago1 and Ago2 can be detected by immunoblotting of fractionated cellular extracts but not by immunofluorescence.

Transcriptional gene silencing mechanisms that depend on RISC activity also occur in the nucleus. Transcriptional gene silencing through heterochromatin formation

has been studied in several organisms [reviewed in (Matzke and Birchler, 2005)]. Recently, a transcriptional gene silencing mechanism that is independent of heterochromatin formation was reported in mammalian cells. Gene silencing was induced by small antigene RNAs that are complementary to promoters. Further, there is evidence for involvement of Argonaute proteins in this process. Not only does it require Ago1 and Ago2 expression, but both Argonaute proteins associate with promoter DNA in cells treated with antigene RNAs (Janowski et al., 2006).

5.4.3b Colocalization of *nos* mRNA with dGW

In a control experiment to test for *in situ* hybridization probe specicifity, *nos* mRNA was unexpectedly observed to colocalize with dGW protein. Colocalization of these two molecules suggests a possible functional interaction between them. This observation is also consistent with evidence of an association between dGW and Smg because Smg as a suppressor of *nos* mRNA translation would also be be expected to associate with *nos* mRNA (Dahanukar et al., 1999; Smibert et al., 1996). A physical association between Smg and dGW was detected in our laboratory (Simmonds unpublished), and Smg was localized to PBs (Eulalio et al., 2007b). This observation supports the idea proposed in sectin 3.3.5 that dGW may function with Smg to regulate translation and/or stability of mRNA in the early embryo.

CHAPTER 6: DISCUSSION

6.1 Overview of Results

The results of this study largely support my central hypothesis: **dGW may be structurally and functionally homologous to the human GW182 protein; however, as the single GW protein in the *Drosophila* genome, it may perform additional functions that are carried out by the other two human GW paralogues, which have been less extensively characterized.** Additional results implicating dGW in regulation of the cell cycle as well as evidence for novel distribution patterns of dGW and potential novel pathways that dGW interacts with are summarized below.

1. dGW appears to be functionally homologous to human GW182

Several lines of evidence suggest functional homology between dGW and GW182. First, most of dGW is usually seen in cytoplasmic foci that resemble PBs. Second, these foci have a similar composition to human PBs, containing several proteins involved in regulating mRNA translation and stability. These include the *Drosophila* homologues of Pacman, a 5' to 3' exonuclease, LSm4, a component of the heptameric LSm2-8 decapping activator complex, and Ago2, a component of the RNA*i* pathway. Third, all three human GW paralogues, when expressed in *Drosophila* cells, localize to these foci, most likely by interacting with the endogenous *Drosophila* proteins. Fourth, like human PBs, these foci require intact RNA to maintain their integrity. Treatment with RNase A resulted in their dispersal. Finally, like in the human homologues, a physical intraction was detected between dGW and dAgo2.

2. dGW is involved in regulating the cell cycle

A role for dGW in cell cycle regulation was identified by observing cell cycle defects of in a *Drosophila* strain carrying a mutation in *dgw*.

3. dGW may participate in additional functions that are not carried out by GW182

Identifying a role for dGW in regulating the cell cycle prompted further observations of dGW distribution throughout the cell cycle. These observations revealed several novel

dGW distribution patterns. Observation of endogenous dGW throughout the cell cycle revealed the the presence of PB-like aggregates during mitosis. This distribution appears different form the distribution of GW182, which is diffuse during mitosis. The difference in distributions of these two GW proteins may represent a functional difference between them. Since the human genome encodes three GW proteins and only one is encoded in the *Drosophila* genome, dGW may be performing additional roles carried out by the other two human GW paralogues. Another unexpected distribution pattern of endogenous dGW was observed during metaphase. At that time, dGW aggregates appeared to be concentrated in the region of the mitotic spindle. Localization of dGW to the spindle suggests a role for dGW in regulating the expression of spindle-localized mRNAs.

4. A GFP fusion of dGW forms post-mitotic nuclear PBs of unknown function

Live imaging of a GFP fusion of dGW was employed as another approach to observing the distribution of dGW during mitosis. This led to the observation of several large PB-like structures in the nuclei of cells shortly after they completed cytokinesis. Although the significance of these post-mitotic nuclear PBs cannot be concluded from this observation alone, the appearance of these PB-like structures during this particular time during the cell cycle suggests a role for dGW in removing fully processed mRNA from the nucleus after mitosis.

5. The gw^1 mutation may not involve RNAi

Several lines of evidence suggest that the defects observed in the gw^1 mutant strain may not be the result of dGW acting with the RNA*i* pathway. Published analyses of phenotypes of *Drosophila* strains carrying mutations in several genes in the RNA*i* pathway either differ from the gw^1 mutant phenotype, or are less severe. Further, the results of a screen in our laboratory identifying proteins that form a complex with dGW, identified the mRNA regulatory protein Smg. Smg functions by a mechanism that is analogous to the RNA*i* pathway. Smg binds to specific mRNAs and this binding leads to degradation or translational repression of the mRNA; however, it is not known to employ small guide RNAs. Although there are no published reports indicating the involvement of GW proteins in mRNA regulatory pathways other than RNA*i* , these observations open

the possibility that dGW may function in conjunction with other mRNA regulatory pathways that select specific mRNAs for degradation or translational suppression.

6. dGW may function with RNase MRP

RNase MRP was chosen as a candidate enzyme that may function with dGW to regulate the cell cycle. This endoribonuclease complex is involved in degradation of cyclin B mRNA in a cyclic manner at the end of mitosis and localizes to a PB-like structure at this time. A *Drosophila* orthologue of MRP RNA, a non-coding RNA component of RNAse was predicted in the *Drosophila* genome, but its expression was not previously studied. In this study, expression of this gene was verified. Further, to examine a potential functional relationship between dMRP RNA and dGW, the localization of these molecules was compared in *Drosophila* embryos during mitosis. No distinct PB-like structures containing both of these molecules were observed; however, among numerous dispersed granules that both of these molecules formed during mitosis, a small mumber contained both dMRP RNA and dGW. Further tests are required to determine if this small degree of colocalization is physiologically significant.

6.2 Non-protein-coding functions of mRNAs and cellular processes influenced by mRNA regulation

In the last few years, significant strides have been made in the advancement of understanding of non-protein-coding functions of mRNAs. During this time, unexpected structural roles were discovered for some mRNAs. One of these structural roles involves localization of mRNA to specific sites to nucleate subcellular complexes [sections 1.1.3c and 1.14; (Condeelis and Singer, 2005) (Lecuyer et al., 2007)]. Another structural role of an mRNA involves the transport of a protein [section 1.1.3; (Jenny et al., 2006)]. Also, there is growing evidence for a variety of several cellular processes to be under the influence of regulated translation or stability of localized mRNA. These include cell polarity, synaptic plasticity and cell cycle regulation (section 1.1.4). In this study, implication of dGW in cell cycle regulation, contributed additional evidence for this process being influenced by regulation of mRNA stability and/or translation. These

recent advances illustrate a growing awareness of the the impact of mRNA structure and regulation on various cellular processes.

6.3 A variety of RNA granules regulate mRNA

There has been a growing interest over the last few years in characterizatizing various RNA granules in which mRNAs and their regulatory factors accumulate. RNA granules were initially identified in specialized cells such as germ cells and embryos of some species such as *Caenorhabditis elegans* [reviewed in (Strome, 2005)] and *Drosophila* [reviewed in (Lipshitz and Smibert, 2000)] and in neurons [reviewed in (Martin and Zukin, 2006)]. It is now becoming apparent that RNA granules are present in most cells. The most extensively studied granules present in most cells are SGs, sites where stalled translation pre-initiation complexes accumulate and PBs, sites where mRNAs that have been removed from translation are stored or degraded (Eulalio et al., 2007a; Parker and Sheth, 2007).

Several lines of evidence suggest that different types of granules may be functionally and structurally related. Analysis of protein compositions of various RNA granules shows that they partially overlap. Some proteins appear to be unique to a particular type of granule. For example, TIA-R and TIA-1 appear to be characteristic stress granule components (Table 2). Other proteins, such as DCP1, are common to many types of granules. This component of the decapping complex has been localized to most types of RNA granules except SGs (Table 2). Live imaging of RNA granules has revealed interactions between them, particularly between SGs and PBs. These include fusion of SGs and PBs, engulfment of PBs by SGs and formation of these granules in close proximity to each other. The interactions and overlapping compositions of RNA granules suggest the presence of a spectrum of granules in cells. This spectrum of granules may represent granules with distinct functions. For example, granules formed as a result of mRNAs recruited by NMD may not contain components of the RNA*i* pathway such as Ago2. The presence of distinct types of granules was suggested as an explanation for the observation of a reporter mRNA, translationally repressed by miRNA-mediated silencing, not always localizing to foci containing the decapping component, Dcp1(Pillai et al., 2005). Other granules may represent different stages of RNP assembly or

remodeling of RNPs that contain mRNAs at different stages of the mRNA cycle (Fig. 1). For example, mRNAs that are translationally repressed by miRNA mediated-silencing may acquire PB components sequentially. These mRNAs could first form a complex with Ago-1, then acquire a GW protein and finally associate with mRNA decay machinery.

6.4 *Drosophila* as a model organism for studying P body function

6.4.1 Genes encoding conserved P body components are encoded in the *Drosophila* genome

During the course of this study, several genes that function in mRNA regulation were characterized in the genome of *Drosophila melanogaster*. These genes are *Drosophila* homologues of human GW182 (dGW), the 5' to 3' exonuclease XRN-1 (Pacman), the component of the RNA*i* pathway Ago2 and the previously uncharacterized orthologue of Lsm4. Expression of these genes in *Drosophila* cells showed that as their homologues in other species, their products form cytoplasmic foci similar to PBs.

Two lines of evidence suggest that these cytoplasmic foci have a similar composition to human PBs and also may be functionally homologous to them. First, the high level of colocalization that was observed between dGW and the three human GW genes when co-expressed in S2 cells (Fig. 7), suggests that they are functionaly homologous. The human GW proteins would have to recognize *Drosophila* PB components and interact with them to be able to localize to *Drosophila* PBs. Second, like human PBs (Sen and Blau, 2005) the integrity of *Drosophila* PBs depends on the presence of intact RNA. In S2 cells, PBs marked by GFP-dGW dispersed when the cells were treated with RNase A (Schneider et al., 2006). The dependence of the integrity of these PBs on intact RNA suggests that they consist of ribinucleoprotein complexes. These ribinucleoprotein complexes may regulate mRNA translation and stability, rather than being aggregates of mRNA regulatory proteins sequesterd away from mRNAs.

This study initially focused on the *Drosophila* orthologue of the metazoan GW protein family (Table 1; Fig. 2). Several features of dGW indicate that *Drosophila* is an ideal experimentally tractable organism for studies that are relevant to human GW proteins. dGW shows a high degree of sequence similarity with the three human GW

paralogues. In contrast, the *Caenorhabditis elegans* GW proteins lack an RRM domain, which suggests that their function may not be homologous to human GW proteins. Finally, the presence of a single GW gene in the *Drosophila* genome compared with multiple paralogues in other species simplifies functional analysis of these proteins.

6.4.2 Understanding the role of larger microscopically visible PBs

A paradox surrounding PB function is the observation that these structures form in response to an increase in non-translating mRNAs (section 1.2.3a), yet in some cases translational repression (Decker et al., 2007) and mRNA decay can occur in the absence of visible PBs. For example, PB dispersal by depletion of GW182 does not affect decay of a reporter mRNA targeted by ARE-mediated decay (Stoecklin et al., 2006) or expression of proteins whose mRNAs are targeted by siRNA-mediated decay (Serman et al., 2007). Similarly, dispersal of PBs by LSm1 or LSm3 depletion does not affect miRNA-mediated repression (Chu and Rana, 2006; Eulalio et al., 2007b) or NMD (Eulalio et al., 2007b). These results suggest that the smallest functional PB unit is below the level of detection by most immunofluorescent microscopic methods. However, the presence of larger, easily observable PBs suggests that there is a purpose for their formation. Several functions have been proposed for larger PBs. They may sequester mRNAs more efficiently from other processes such as translation or degradation by the exosome, resulting in more efficient repression and storage (Decker et al., 2007; Eulalio et al., 2007b). Alternatively, they could play a role in sequestering RNA binding proteins to limit their concentration in the cytoplasm (Decker et al., 2007). Further, it is possible that different sizes of PBs are required for optimal regulation of mRNAs involved in different processes. For example, large PBs may function to efficiently transport suppressed mRNAs to specific regions of a cell (Decker et al., 2007), particularly over long distances such as in neurons [reviewed in (Martin and Zukin, 2006)]. Finally, smaller PBs could more efficiently regulate mRNAs dispersed over a larger area of the cell. Consequently, aggregation of RNPs into PBs of various sizes may be regulated to suite the requirements of individual cellular processes. Regulated aggregation of RNPs could account for the formation of single large RNA granules found in some cells such as the TAM body in *Saccharomyces cerevisiae* (Gill et al., 2006) or the chromatoid body in

mammalian male germ cells (Kotaja et al., 2006). A future challenge will be to identify mechanisms of PB size regulation. Correlating PB size with a variety of cellular processes in a variety of cell types could be accomplished in an experimentally tractable model organism such as *Drosophila*.

6.4.3 Using genetic or genetic mosaic mutants to analyze P body assembly in *Drosophila*

A major unresolved question in understanding how PBs function is understanding the mechanism of their assembly. A number of proteins have been implicated in PB assembly on the basis of experiments showing that PB are dispersed in response to depletion of thes proteins (Table 1). In human cells, PBs were dispersed following siRNA-mediated depletion of several PB components. These include GW182, LSm1, LSm4, eIF4E-T, Rck/p54, RAP55 and Ge-1 (Andrei et al., 2005; Chu and Rana, 2006; Ferraiuolo et al., 2005; Jakymiw et al., 2007; Kedersha et al., 2005; Lian et al., 2007). siRNA-mediated depletion of many *Drosophila* homologues of these components in S2 cells, gave rise to similar results. These include dGW, LSm1, Me31B (Rck/p54) and Ge-1 (Eulalio et al., 2007b). However, there are inconsistencies in published data on which proteins are required for PB assembly. For example, in *Saccharomyces cerevisiae*, genetic depletion of LSm1 enhances PB formation (Sheth and Parker, 2003), while siRNA-mediated depletion of LSm1 in human and *Drosophila* cells results in PB dispersal (Chu and Rana, 2006; Eulalio et al., 2007b). Also, siRNA-mediated depletion of Tral, the *Drosophila* orthologue of human RAP55, does not lead to PB dispersal (Eulalio et al., 2007b) unlike depletion of RAP55 (Yang et al., 2006). Some of these inconsistencies could be due to differences in the functions of these proteins in different species (Eulalio et al., 2007b).

Some of the inconsistencies in the role of specific proteins on PB assembly described above could be unrelated to the effects of its depletion but rather due to unpredictable effects caused by introduction of siRNA. The results of one study showed that PBs can be dispersed by transfection with some siRNAs that are directed against mRNAs that are not PB components. These included the transmembrane proteins CD9 and CD81 as well as rabbit β-globin and mouse lymphotoxin, proteins that were not expressed in the experimental human cell line (Serman et al., 2007). According to the

authors of this study, it is unlikely that PB dispersal was caused by random off-target effects of these siRNAs because it is unlikely several different siRNAs, with no sequence similarity between them, would all target PB components. These results led to the conclusion that introduction of some siRNAs can interfere with PB assembly independently of the targeted gene. Detection of an siRNA with no endogenous target localized to PBs (Jakymiw et al., 2005) is consistent with this conclusion. Consequently, the results of experiments that conclude that a protein is required for PB assembly by siRNA-mediated silencing of that protein must be interpreted with caution. How introduction of siRNA can lead to PB dispersal is unknown; however, since PB assembly requires non-translating mRNA (section 1.2.3*a*), dispersal of PBs by introducing siRNAs may interfere with targeting non-translating mRNA to PBs (Serman et al., 2007).

Potential effects of introduction of siRNA on the integrity of PBs could be verified by comparison with genetic depletion of the same component in the same organism. *Drosophila* would be a suitable organism for this purpose, because genetic mutants in some PB components have already been identified, and phenotypes of some of these mutants have been characterized. For example, the effect of Ago2 on PB assembly could be examined in the viable Ago2 mutant strain (Deshpande et al., 2005), and the effect of Ago1 on PB assembly could be examined in individuals lacking maternal Ago1 (Kataoka et al., 2001) which are viable throughout early embryonic development. There have been no published reports of PB morphology in these organisms.

Drosophila strains with mutations in other PB components such as dGW could also be examined; however, analysis of the phenotype with respect to PB formation may be difficult if the component is essential. For example, the rapid degeneration of all embryonic structures in the gw^{1} mutant strain (Video 2) would require examining PBs in embryos within a very narrow time range when levels of the protein are being depleted. If this approach does not yield interpretable results, then genetic mosaic mutants could be made (Xu and Rubin, 1993). With this approach, homozygous mutant clones of cells could be produced at a later time during development when the effects of dGW depletion may not be as drastic. The viability of human HEp-2 cells without detectable levels of GW182 (Bloch et al., 2006) suggests that GW proteins may not be essential for viability of all cell types.

Drosophila strains with mutations in other PB components that have not yet been characterized could also be examined. For example, there is a *Drosophila* strain with a P-element inserted one nucleotide upstream of transcribed *dlsm4* sequences (pBac[WH]f04861) which could disrupt *dlsm4* expression. If this or other *Drosophila* strains with mutations in PB components are difficult to characterize because of a rapid onset of lethal effects, then genetic mosaic mutants could be created.

6.5 Functions of *Drosophila* GW

6.5.1 dGW functions in cell cycle regulation by a mechanism that may not involve miRNA

Characterizations of the gw^1 mutant strain led to the surprising finding of mitotic defects as the cause of death in early gw^1 mutant embryos. This finding suggests a role for regulating the cell cycle at the level of mRNA. Prior to this study, there was evidence for regulation of cyclin B mRNA by RNase MRP (Chapter 5) and Pumilio (section 3.3.5); however, little else is currently known about the cell cycle regulation by regulating mRNA stability and translation. One previously published report of changes in size and number of PBs marked by GW182 during the cell cycle did however point to a potential involvement of GW proteins in cell cycle regulation (Yang et al., 2004).

Additional evidence from this study suggests that it is unlikely that dGW directs miRNA-mediated silencing during early embryonic development. Embryos that lack maternal dAgo1 show defects in development at a much later stage than gw^1 mutant embryos (Kataoka et al., 2001). This conclusion differs from the current view that the most important function of GW proteins is in miRNA-mediated gene silencing. The results of several studies have indicated that GW proteins are involved in both miRNA- and siRNA-mediated silencing; however, they appear to be involved miRNA-mediated gene silencing to a greater degree (Chu and Rana, 2006; Liu et al., 2005a; Rehwinkel et al., 2005). Evidence from another study supports the idea that formation of most PBs marked by GW182 is a consequence of miRNA-mediated silencing (Pauley et al., 2006). In this study, depleting Drosha, which functions in miRNA biogenesis upstream of GW proteins, resulted in dispersal of most PBs marked by GW182. The presence of PBs in

normal embryos at the time of the onset of the *gw*^{1} mutant phenotype, together with evidence for the lack of a requirement for miRNA-mediated silencing at this time during development, indicates that the formation of PBs at this time may not be a consequence of miRNA-mediated silencing. It is therefore possible that in cells with a reduced level of miRNA-mediated silencing activity, PBs containing GW proteins may form as a consequence of other mechanisms of translational suppression. These findings emphasize the importance of extending studies that examine protein function in cultured cells to additional specialized cell types, at various stages of development of a whole living organism. This is especially important for proteins like the GW proteins that are restricted to metazoan genomes. Such proteins may participate in a diverse range of processes in various differentiated cells or in their development. Some of these processes involve intercellular communtication between different cell types to form organs and organ systems, processes cannot be studied in cultured cells.

6.5.2 Does dGW act through pathways other than RNAi?

Involvement of GW proteins in the two main branches of the RNA*i* pathway, siRNA and miRNA, has been well documented (Chu and Rana, 2006; Liu et al., 2005a; Rehwinkel et al., 2005), but they have not been associated with other mRNA regulatory pathways. NMD and ARE-mediated decay pathways were examined for dependence on GW proteins, and none was found (Rehwinkel et al., 2005; Stoecklin et al., 2006). However, a comprehensive analysis of the composition of RNP complexes containing GW proteins is still lacking and little is known about the functions of most regions of GW proteins. Functions have been assigned to two broad regions of dGW. An N-terminal region is associated with Ago1 binding and a larger N-terminal region is associated with PB targeting (Fig.2) (Behm-Ansmant et al., 2006). The requirement of a larger fragment of dGW for PB targeting suggests that this requires molecular interactions in addition to the interaction with Ago1.

One important domain whose function in GW proteins has not yet been investigated is the RNA binding (RRM) domain. This domain is common to all human GW proteins and dGW (Fig. 2). The RRM domain is one of the most abundant protein domains found in eukaryotes, present in 0.05% to 1% of all human proteins. It is found in

many proteins that are involved in posttranscriptional gene expression including pre-mRNA processing, export, translation and degradation of mRNA and rRNA processing. The biochemical functions of this domain are diverse. It can bind to RNA, DNA and protein [reviewed in (Clery et al., 2008; Maris et al., 2005)]. Recently the RRM of mammalian poly-A specific ribonuclease (PARN) was found to participate in a novel interaction. This interaction involves simultaneous binding to both the 7-methylguanosine cap and the poly A tail of an mRNA, which results in stimulating the rate of deadenylation (Monecke et al., 2008; Nagata et al., 2008). The broad range of biochemical functions associated with RRM domains suggests a potentially diverse range of additional functions of GW proteins, including, interactions with other proteins. The importance of this domain in the function of dGW is illustrated by the phenotype of the gw^1 mutation which expresses a truncated dGW lacking a portion of the protein that contains the RRM domain (Schneider et al., 2006).

The idea that dGW may regulate mRNA through pathways other than RNA*i* is supported by the identification of two proteins in our laboratory, Smg and Glorund (Glo), that are part of a complex with dGW. Smg was also independently identified as a PB component by another group [Table 1 (Eulalio et al., 2007b)]. Both of these proteins regulate mRNA translation and/or stability in a very similar way. They both recognize the same element, the Smg response element, in the 3'UTR of mRNAs (Dahanukar et al., 1999; Kalifa et al., 2006; Smibert et al., 1996) and recruit additional mRNA regulatory proteins. Smg recruits known PB components (section 3.3.5). Glo-associated proteins are different from those recruited by Smg and have not been identified as PB components. Glo appears to function primarily in *Drosophila* ovaries (Kalifa et al., 2009).

Identification of Smg and Glo as components of a complex with dGW suggests a model for how GW proteins may function. In this model, GW proteins would interact with proteins that target specific mRNAs for translational suppression and/or degradation such as the Argonaute proteins, Smg, Glo and RNase MRP functioning as an adapter or scaffold linking the targeted mRNAs with the general mRNA decay machinery (Fig. 28). In this model GW proteins would play a central role in coordinating multiple mRNA regulatory pathways.

Although there is evidence suggesting that GW proteins may not be required for PB assembly (section 6.3.3), this evidence does not preclude a role for GW proteins in facilitating this process. There is also evidence supporting the idea that GW proteins may function as a scaffold. First, FRAP analysis showed a slow rate of exchange of GW182 between PBs and the cytoplasm, indicating that GW182 is a relatively stable PB component (Kedersha et al., 2005), a characteristic that is consistent with a scaffold protein. Second, a glutamine-rich domain, which is common to all human GW proteins as well as dGW, could have a function in protein aggregation. The glutamine-rich region could function as a prion domain. Prion domains are also enriched in glutamine and promote self assembly of proteins (Michelitsch and Weissman, 2000; Prusiner, 1989). Glutamine-rich regions have been shown to promote assembly of other RNA granules. A glutamine-rich prion-related domain is found in the stress granule component TIA-1 and has been functionally linked to the assembly of SGs (Gilks et al., 2004). A glutamine-rich region of *Saccharomyces cerevisiae* LSm4, which is not conserved in other species, has been functionally linked to PB assembly (Decker et al., 2007). Recently, expression of a mutant cytosolic form of prion protein resulted in assembly of a large RNA granule resembling a chromatoid body in somatic cells (Beaudoin et al., 2009). Functional analysis of glutamine-rich regions in GW proteins may identify a potential role for these proteins in aggregating mRNPs into larger RNA granules and provide additional evidence for involvementof GW proteins in PB assembly. For example, if glutamine-rich regions enhance PB assembly, then deleting these regions or substituting individual glutamines with a non-polar amino acid such as alanine would be expected to reduce the aggregation potential of GW proteins and interfere with PB assembly. Conversely, increasing the length of glutamine-rich regions or enriching them in glutamine residues would be expected to increase aggregation potential and enhance PB assembly.

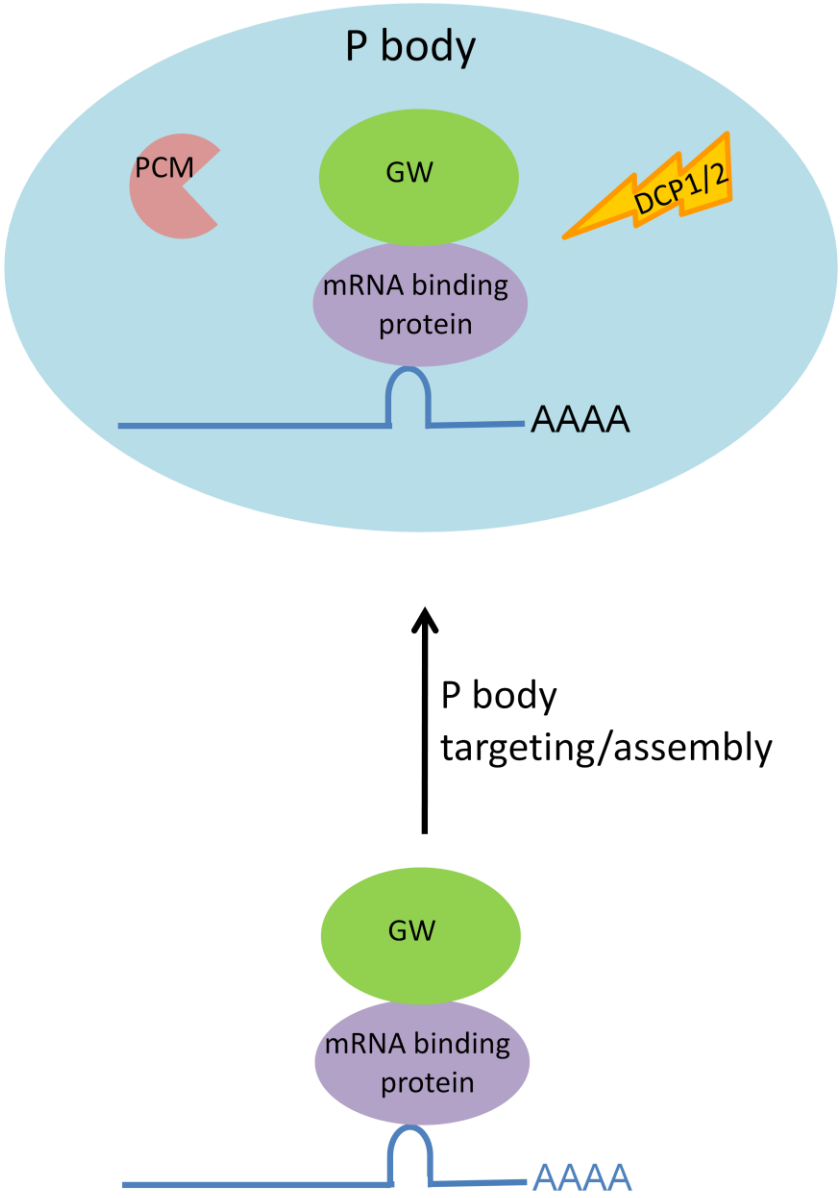


Figure 28. A model for the mechanism of GW protein function. GW proteins may function as an adaptor between mRNP complexes containing translationally repressed mRNAs and the general mRNA degradation machinery. Specific mRNAs can be translationally suppressed by different mRNA binding proteins such as the Argonautes, Glo, Smg or RNase MRP. The PB targeting region of GW at the N-terminus either directs the mRNP complex to PB or functions as a platform for PB assembly.

6.6 The importance of gene regulation by non-coding RNA in basic and human health research

In this study, components representing two mechanisms of gene regulation employing non-coding RNA were characterized. The first mechanism, RNA*i*, employs small 21-25 nucleotide-long RNAs that function as guides for selection of targets for the purpose of regulating their actions [reviewed in (Meister and Tuschl, 2004)]. The second, was an enzymatic activity associated with dMRP RNA that regulates mRNA stability [reviewed in (Martin and Li, 2007)]. Current evidence indicates that a variety of non-coding RNAs regulate a significant proportion of the human genome. Understanding the mechanisms of action of these RNAs may lead to advancement in understanding basic cellular processes and improved or novel therapies for human disease.

6.6.1 Gene regulation by RNA*i*

A comprehensive knowledge of RNA*i* pathways is valuable not only in understanding how a significant proportion of the human genome is regulated but also in developing RNA*i*-based therapies for human disease. GW proteins, as components of RISC complexes that mediate both miRNA and siRNA gene silencing pathways, play a central role in the overall regulation of gene expression. Current estimates predict that approximately 350 miRNA genes in mammalian cells regulate the expression of over 25% of the transcriptome (Lewis et al., 2005; Lewis et al., 2003; Lim et al., 2005) [reviewed in (Leung and Sharp, 2007)]. Because RISC components also function in siRNA-mediated gene silencing (section 3.1.2), their impact on gene expression is likely to exceed 25% of the transcriptome if estimates of gene regulation by endogenous siRNAs are included.

The ability to silence virtually any gene with exogenous siRNAs has been a widely-used tool in experimental research for determining gene functions. This tool is being developed into therapeutic agents to treat human diseases caused by overxpression or misexpression of proteins. Given the potential of being able to target any gene of known sequence, developing RNA*i*-based therapies is particularly important for diseases with limited treatment options [reviewed in (Kim and Rossi, 2007)]. Clinical trials are

currently underway for the treatment of a variety of diseases, including age-related macular degeneration, viral infections and cancer (Kim and Rossi, 2007). Understanding the functions of components of the RISC complex may result in improving the effectiveness of siRNA therapeutic treatments.

6.6.2 Gene regulation by other non-coding RNAs

Several lines of evidence strongly suggest that the number of genes regulated by non-coding RNAs is much larger than estimates based on gene regulation by miRNAs and siRNAs. There are several other classes of non-coding RNAs that are known to regulate gene expression. Some of these are associated with other branches of the RNA*i* pathway such as rasRNA and piRNA (Siomi et al., 2008) while others are unrelated to the RNA*i* pathway. These include MRP RNA (Gill et al., 2004) and long non-coding RNAs such as Xist and AIR [reviewed in (Kapranov et al., 2007b)]. Recent evidence suggests that a large number of yet undiscovered regulatory RNAs may be transcribed by the human genome. According to estimates by a recent collaborative study to characterize functional elements of the human genome (Encyclopedia of DNA Elements ENCODE) (Birney et al., 2007), up to 93% of the human genome is transcribed. With only 1-2% of the human genome corresponding to exons of known protein-coding genes (Kapranov et al., 2007b) these results indicate that most of the human genome is transcribed into non-coding RNA. Although some of these transcribed sequences could encode short proteins or peptides, these results suggest the presence of additional unknown transcribed non-coding sequences in the human genome that may play a role in gene regulation (Kapranov et al., 2007b). The results of further analysis of some of these unannotated sequences indicate the presence of a network of overlapping sense and antisense transcripts at the promoters and termini of expressed protein coding genes that may play a role in gene regulation (Kapranov et al., 2007a). Deciphering the functions of the non-coding transcriptome of the human genome and genomes of other organisms may lead to novel insights into understanding cellular and developmental processes.

6.7 The Importance of Localized mRNA in Human Health Research

Evidence for the involvement of localized mRNAs in several cellular processes suggests that disruption of mRNA localization may lead to human disease. The potential importance of localized mRNAs in mechanisms leading to the development of cancers was previously described in section 1.14a. These include the roles of localized actin mRNAs in determining intrinsic cell polarity and the requirement of the APC tumour suppressor for mRNA localization. Continued interest in this relatively unexplored area of research will undoubtedly identify functions of localized mRNAs in additional areas of human health research. Two noteworthy additional areas are described below.

6.7.1 Association of the chromatoid body with totipotency

Investigation into the potential involvement of the CB, an RNA granule which functions in posttranscriptional gene regulation (section 1.3.2b) (Kotaja et al., 2006), in determining totipotency of cells may have implications in future stem cell research. An association between the presence of the CB and totipotency was proposed on the basis of studies of this structure in planarians. Planarians have a strong regenerative capability that is thought to reside in the neoblast, a totipotent somatic stem cell. In planarians, CBs were observed both in germline cells, as in mammals(Kotaja et al., 2006), and in regenerating neoblasts [reviewed in (Parvinen, 2005)]. Their size and presence in germline and non-germline cells are correlated with the degree of cellular differentiation. As regenerating neoblasts differentiate, CBs become progressively smaller and less prominent and eventually disappear (Shibata et al., 1999). Similarly, CBs in fertilized eggs and in early blastomeres gradually diminish as development progresses, until they disappear during gastrulation (Sato et al., 2001). The inverse correlation between CB prominence and the degree of cellular differentiation suggests that this structure may be functionally associated with pluripotency or totipotency [reviewed in (Parvinen, 2005)]. Posttranscriptional regulatory mechanisms may therefore function in modifying gene expression in differentiating cells.

It is not clear from these observations if CB formation is a cause or consequence of potency; however, other evidence suggests that the CB may play a role in determining potency. Similarities in morphology and composition between the CB and germ plasm,

which is found in oocytes and developing embryos in some organisms, suggest that the CB may be the functional equivalent of germ plasm. Both the CB and germ plasm are composed of dense fibrous material, contain homologues of the RNA helicase VASA, a marker of germ cells, and are sites of mRNA regulation (Parvinen, 2005). Because germ plasm is essential for specification of the fate of these cells (Ikenishi, 1998), it is likely that the CB plays a role in specifying the fate and therefore the potency of the cells in which it is found. If the CB can influence potency, then posttranscriptional mechanisms could be investigated for reprogramming adult somatic cells into pluripotent stem cells.

Human somatic cells can be reprogrammed to generate pluripotent stem cells by inducing expression of four transcription factors; Oct3/4, Sox2, c-Myc and Klf4 (Maherali et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Current methods of inducing expression of these reprogramming factors involve introducing viral expression vectors into adult cells. However, tumours can develop from reprogrammed cells. These tumours are thought to arise as a result of genetic alterations caused by the viral vectors, such as insertional mutagenesis, and effects of continued expression of the reprogramming factors [reviewed in (Rossant, 2007)]. A major challenge in developing stem cells for patient use is to reduce the frequency of tumours arising from stem cells.

The results of a recent study suggest that continued expression of reprogramming factors may not be required for maintenance of stem cells (Woltjen et al., 2009). Tumour induction could be avoided if stem cells could be reprogrammed without genetic alterations resulting from expression vectors. The association of the CB with totipotency suggests that adult cells could be reprogrammed into stem cells by transiently altering posttranscriptional gene regulation. Analysis of the mRNAs contained in the CB and how they are regulated could provide insights into reprogramming adult stem cells. Reprogramming adult stem cells by this approach could reduce the frequency of tumours arising from them.

6.7.2 Localized neuronal mRNAs in the development of epilepsy

Epileptogenesis is the change in brain tissue that occurs following an insult such as trauma or stroke that leads to development of chronic epilepsy. There is a growing body

of evidence pointing to a role for localized mRNAs in epileptogenesis. Two proteins, BDNF and TrkB, have been implicated in epileptogenesis because of changes in their expression levels during this process. Increased expression levels of these proteins, following an epileptogenic stimulus have been reported by a number of studies. Conversely, reduced levels of BDNF or TrkB activity lead to reduced epileptogenesis [reviewed in (Tongiorgi et al., 2006)]. Further, accumulation of BNDF and TrkB mRNAs in dendrites observed during epileptogenic but not during chronic seizures, indicates that dendritic localization of these mRNAs is associated with development of epilepsy (Merlio et al., 1993; Simonato et al., 2002; Tongiorgi et al., 2004). Taken together, these results suggest that increased levels of BDNF and TrkB expressed from localized transcripts could produce extremely high local levels of these proteins that could lead to lasting or permanent changes in tissues resulting in chronic epilepsy. It has been proposed that inhibiting dendritic targeting of mRNAs could be used as a novel treatment for some forms of epilepsy (Simonato et al., 2002).

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