Development of Tools to Assess the Effects of Lunasin on Normal Development and Tumor Progression in Drosophila Melanogaster

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DEVELOPMENT OF TOOLS TO ASSESS THE EFFECTS OF LUNASIN ON NORMAL DEVELOPMENT AND TUMOR PROGRESSION IN DROSOPHILA MELANOGASTER

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The Faculty of the Department of Biology
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Master of Science

By
Gillian E. Jones

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DEVELOPMENT OF TOOLS TO ASSESS THE EFFECTS OF LUNASIN ON NORMAL DEVELOPMENT AND TUMOR PROGRESSION IN DROSOPHILA MELANOGASTER

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Soy contains many bioactive molecules known to elicit anti-cancer effects. One such peptide, Lunasin, has been shown to selectively act on newly transformed cells while having no cytotoxic effect on non-tumorigenic or established cancer cell lines. In this study we attempt to understand the developmental effects of Lunasin overexpression in vivo and create reagents that will help us understand Lunasin’s anti-tumorigenic effects in an intact organism.

cDNA encoding lunasin and EGFP-lunasin were cloned into pUAST and microinjected into Drosophila embryos. Tissue-specific overexpression of EGFP-Lun in the resulting transgenic lines was accomplished by crossing transgenics to various GAL4 driver lines. Progeny were assessed for phenotypic alterations and no phenotypic abnormalities were observed in tissues expressing EGFP-Lunasin, supporting current studies that show Lunasin does not affect normal cells. Previous studies have localized Lunasin to the nuclear compartment. To test if this was the case for EGFP-Lun, subcellular localization of EGFP-Lun was determined via fluorescence microscopy. Salivary glands from EGFP-Lun expressing individuals were dissected, fixed, and mounted in Vectashield® with the nuclear stain, DAPI. Our results demonstrate that EGFP-Lun, like native Lunasin, is localized to the nucleus. Eight transgenic lines were
mapped to specific chromosomes and EGFP-Lun transgenic line GEJ1-L2 was balanced in preparation for use in tumor suppression studies.

In summary, we have created and characterized transgenic flies capable of overexpressing Lunasin under the control of the GAL4/UAS system. Localization of EGFP-Lunasin to the nucleus and data on the phenotypic consequence of its overexpression in flies is presented. Finally, reagents created as part of this thesis will aid experiments aimed at understanding the effects of Lunasin on benign and invasive tumors.
1.1 History of soy

Soy (*Glycine max*) has been a staple in the diets of many cultures for millennia. Originally cultivated in Asia, soy arrived in Europe in the 1700s CE and by the 1800s was being grown in the United States. There are many health benefits associated with soy rich diets. Soy consumption lowers the so-called bad cholesterol (LDL) (Carroll and Kurowska 1995; Sirtori et al. 1995) and reduces risk of coronary disease and cancers, particularly breast, colon, and prostate (Liu and Pan 2010). Soybeans contain many bioactive compounds with demonstrated anticancer activity including isoflavones, protease inhibitors (most noted is the Bowman-Birk protease inhibitor, BBI), saponins, and more recently the peptide Lunasin (Messina and Barnes 1991).

Lunasin was discovered while isolating the 2S cDNA which codes for a nutrient enhancing, methionine-rich albumin protein in soy (Odani et al. 1987). The isolated gene, Gm2S-1, codes for the methionine-rich protein, and three additional peptides: a signal peptide, a linker peptide, and Lunasin (Figure 1) (Galvez et al. 1997). Lunasin has also been found in wheat (Jeong et al. 2007a), barley (Jeong et al. 2010), amaranth (Silva-Sánchez et al. 2008), pepper (Hernández-Leñosma and De Lumen 2008), rye (Jeong et al. 2009), oats (Nakurte et al. 2013), and the herb *Solanum nigrum* (Jeong et al. 2007b). As Lunasin has been isolated from a variety of angiosperm seeds, a more thorough screening will likely reveal Lunasin in many additional sources. However, to date, soy remains the richest source of Lunasin.
Figure 1. Primary structure of Gm2S-1 albumin
Gm2S-1 is comprised of four peptides: a signal peptide (residues 1-21), Lunasin (residues 22-64), a Gm2S-1 linker peptide (residues 65-81), and alisin (residues 82-158). (Adapted from (DE LUMEN and GALVEZ 2003).)
1.2 Structure of Lunasin

Lunasin is a 43 amino acid peptide with a predicted helical region with homology to chromatin binding protein, and a carboxyl terminal cell adhesion motif (Arginine-Glycine-Aspartic acid, RGD) followed by a run of eight aspartic acid residues (Poly-D tail) (Figure 2; GALVEZ and DE LUMEN 1999). The poly-D tail, N-terminus, and predicted helix are shown to be essential for the bioactivity of Lunasin; however, there is conflicting evidence as to whether the RGD motif is essential for the anti-cancer effects (Table 1) (GALVEZ and DE LUMEN 1999; LAM et al. 2003). The Poly-D tail binds to deacetylated histones and is necessary for the antimitotic effect of Lunasin as demonstrated by Galvez and de Lumen (1999) in both murine hepatoma cells and Escherichia coli (E. coli) cells. Transfection of murine hepatoma cells with full-length lunasin resulted in a diffused chromosomal mass, indicative of mitotic disruption, whereas transfection with a Poly-D deletion mutant resulted in a loss of antimitotic activity (i.e. chromosomal DNA appeared normal and there were no morphological changes). Moreover, E. coli expressing full-length Lunasin exhibited reduced bacterial growth and abnormal filament formation, whereas E. coli expressing a Lunasin-Poly-D deletion mutant exhibited normal septa formation and normal division (GALVEZ and DE LUMEN 1999), supporting the claim that the Poly-D tail is essential for the bioactivity of Lunasin.
Figure 2. Primary structure and motifs of Lunasin

Lunasin is a 43 amino acid peptide with a predicted chromatin binding helical domain (residues 23-32), an RGD cell adhesion motif (residues 33-35), and a poly-D tail (residues 35-43).

(Adapted from Kyle et al. 2012)
Table 1. Motifs of Lunasin

Two studies have explored the motifs of lunasin and have agreed that full length lunasin is bioactive and acts as an anti-mitotic molecule. The N-terminus and poly-D tail are both essential for the anti-mitotic bioactivity of lunasin.

However, there are conflicting reports on the necessity of RGD motif the retain bioactivity as Lam et al. (2003) claim that lunasin lacking the RGD motif retains bioactivity, whereas Galvez and De Lumen (1999) argue that RGD-deletion mutants lose bioactivity.
The sequence from the N-terminus to the predicted helix is also necessary for the full bioactivity of Lunasin. Lam et al. demonstrated that an N-terminus deletion mutant was only about one third as effective as full-length Lunasin at preventing foci formation in NIH 3T3 cells (LAM et al. 2003).

The RGD cell adhesion motif is thought to be responsible for the internalization of Lunasin. Galvez et al. (2001) reported that an RGD-deletion mutant failed to prevent chemically induced transformation of mouse embryonic fibroblasts (C3H 10T1/2). The lack of bioactivity was due to an inability of Lunasin to be internalized by cells and thus it was concluded that the RGD motif is necessary for internalization of Lunasin (GALVEZ et al. 2001). In a later study by Lam et al., Lunasin lacking the RGD motif was only slightly less effective at preventing transformation of mouse embryo fibroblasts (NIH3T3) compared with non-mutated Lunasin. Additionally, they reported that RGD-deletion mutants are internalized into the nucleus as effectively as full-length Lunasin, demonstrating that the RGD motif is not required for internalization (LAM et al. 2003). Lam et al. propose that this discrepancy in the data is due to differences in internalization mechanisms between cell lines (NIH3T3 and C3H 10T1/2).

1.3 Proposed mechanisms of action

Natural role in soy

Lunasin is found in abundance in developing soy cotyledons. There are three stages in the development of the cotyledon: (1) a phase of rapid cell division followed by (2) a cessation of mitosis, during which time cells grow larger and acquire and store nutrients, and (3) the dehydration of the seed. The expression of Lunasin coincides with
mitotic arrest. It is thought that Lunasin may play a role in triggering the arrest of cell division because of its demonstrated anti-mitotic activity in vitro (Jeong et al. 2003).

Effects on tumorigenic and non-tumorigenic lines

Lunasin exhibits no cytotoxic or anti-mitotic effects on non-tumorigenic or established cancer cell lines (DIA and Gonzalez de Mejia 2011; Galvez et al. 2001; Lam et al. 2003; Pabona et al. 2012). When treated or transfected with Lunasin, these cells show normal growth and cell division. In contrast, in cells newly transformed by chemical carcinogens or viral oncogenes, Lunasin has been shown to suppress transformation, arrest cell division, and induce chromosomal fragmentation and apoptosis (DIA and Gonzalez de Mejia 2011; Galvez et al. 2001; Lam et al. 2003; Pabona et al. 2012).

Inhibition of Core Histone Acetylation

Under steady-state conditions, core histones are typically in a repressed, deacetylated state. During a transformation event the histones are acetylated, which relaxes the chromatin, and results in the initiation of transcription (Galvez et al. 2001). Interruption of acetylation is perceived by the cell as abnormal, and in response the cell commits itself to apoptosis. Lunasin competes with histone acetyl-transferases (HATs) to bind the histones that are exposed during transformation events (Galvez et al. 2001). If HAT binds, transcription is initiated, S phase proteins are produced, and mitosis progresses. However, binding of Lunasin to exposed, deacetylated histones disrupts the acetylation process. This results in commitment of the newly transformed cells to apoptosis, thereby preventing the replication of neoplastic cells (De Lumen 2005). In this
Inhibit mitosis by increasing expression of CDK-inhibitors, p21 and p27

Cyclin-dependent kinases (CDKs) are important in controlling cell cycle progression (MALUMBRES and BARBACID 2009). Deregulation of the cell cycle is attributed to low levels of CDK inhibitors, such as p21 and p27, and is a hallmark of transformed cells (XIONG et al. 1993). Additionally, low levels of p27 are correlated with a poor prognosis in breast cancer patients (STEEG and ABRAMS 1997). Lunasin has been shown to increase the expression of p21 and p27, resulting in an arrest at the G2/M stage in transformed colon cancer cells (KM12L4) and mouse embryonic fibroblasts (NIH3T3) (DIA and GONZALEZ DE MEJIA 2011; LAM et al. 2003).

Additionally, earlier work has shown that a reduction in p21 and p27 gene expression correlated with an increase in drug resistance (ABUKHDEIR et al. 2008; CHU et al. 2007). It is possible that the increased levels of these CDK-inhibitors, as induced by Lunasin, will reduce the prevalence and slow the rise of drug resistance in patients.

Decrease metastasis via modification of expression of ECM genes

Angiogenesis and metastasis are often preceded by deregulation and degradation of the extracellular matrix (ECM). Lunasin has been found to modify the expression of numerous genes involved in cell-adhesion and ECM regulation (Table 2; DIA and GONZALEZ DE MEJIA 2011). Although several matrix metalloproteases (MMPs), which
Table 2. Effect of Lunasin on gene expression of extracellular matrix and cell adhesion in human KM12L4 colon cancer

(+ Values represent upregulated genes and (-) values represent those downregulated by lunasin. (Table from DIA and GONZALEZ DE MEJIA 2011).
contribute to the breakdown of the ECM, are upregulated (average of 2.05 fold; n=7) in the presence of Lunasin, MMP10 is considerably downregulated (7.71 fold).

Additionally, Lunasin upregulated the expression of COL14A1 (collagen, type XIV, α1), a protein that, when knocked down, results in increased cell proliferation in renal cell carcinoma cell lines (DIA and GONZALEZ DE MEJIA 2011).

α5β1 integrin plays a key role in cell adhesion, growth, and proliferation (LU et al. 2006). Elevated levels of α5β1 are associated with increased invasiveness of colorectal cancer (CRC) cell lines (GONG et al. 1997) and loss of α5β1 function results in decreased metastasis in CRC cells (STOELTZING et al. 2003). Lunasin is able to downregulate the expression of α5 integrin and has the most potent anti-mitotic effect on cell lines which highly express the α5β1 integrin (DIA and GONZALEZ DE MEJIA 2011), suggesting a role as an anti-metastatic molecule.

Induce apoptosis by activating the mitochondrial apoptosis pathway

Mitochondrial-mediated apoptosis can be triggered by a change in membrane permeability, which is regulated by proteins in the Bcl-2 super family and cytochrome c (TSUJIMOTO et al. 2006). Lunasin has been found to activate the mitochondrial apoptosis pathway in colon cancer cells (KM12L4) by increasing levels of pro-apoptotic molecules such as Bax, nuclear clusterin, and caspases 2, 3, and 9, while reducing the expression of Bcl-2, a pro-survival molecule (DIA and GONZALEZ DE MEJIA 2011).

Reduce inflammation by inhibiting proinflammatory cytokines

Chronic inflammation has long been implicated as an initiating factor in many cancers (colon, liver, bladder, and stomach) (COUSSENS and WERB 2002). During times
of inflammation, leukocytes induce DNA damage through the production of genotoxins including reactive oxygen species (ROS) and nitric oxide (NO). Recently, it has been suggested that Lunasin may help reduce inflammation by inhibiting the release of NO and the pro-inflammatory cytokines, TNF-α and IL-1β (LIU and PAN 2010). The discovery of the anti-inflammatory effects attributed to Lunasin may be significant in the search for therapeutics for other ailments directly caused or amplified by inflammation including arthritis, heart disease, diabetes, and Alzheimer’s disease.

**Upregulate the tumor suppressor, PTEN**

PTEN, a phosphatase and tensin homolog, is a tumor suppressor that plays a regulatory role in cell cycle progression and apoptosis, and is often found mutated or downregulated in cancers (SIMPSON and PARSONS 2001; TAMURA et al. 1998). Genistein, a soy isoflavone, has been found to impart antitumor effects via activation of PTEN pathways (DAVE et al. 2005; RAHAL and SIMMEN 2009), leading researchers to question if Lunasin shares this anti-tumor mechanism. Pabona et al. found that human breast cancer cells (MCF-7) treated with Lunasin exhibited elevated levels of PTEN expression and nuclear localization, and induced apoptosis via the PTEN pathway (PABONA et al. 2012).

1.4 Lunasin as a Natural Chemotherapeutic

Aside from the demonstrated benefits of Lunasin, public opinion may very well play a role in the development of Lunasin as a therapeutic. Society is beginning to place a high value on products derived from natural sources in order to reduce our dependence on manmade chemicals which often have unforeseen side-effects. Because of the
multitude of anti-cancer pathways influenced by Lunasin, this short peptide holds promise in combating numerous cancers, including those which have developed drug resistance. The next stage in Lunasin research is to test the efficacy of Lunasin \textit{in vivo}. I argue that \textit{Drosophila} is the natural choice for a model organism.
“The idea was to learn about the general by studying the particular; to use a specific organism as a model for all others.” – Kellogg and Shaffer, 1993

1.5 Drosophila as a model organism

Since the time of Thomas Hunt Morgan, *Drosophila* has been a powerful model organism. Morgan originally chose the fruit fly because of the ease of collection, fast generation time, and small size. Since his time, much information has been collected that leads researchers to continue to choose *Drosophila* as a model organism.

The power of *Drosophila* as a model lies in the simplicity of conducting genetic screens made possible by the ease and affordability of rearing multiple generations in a short period of time. *Drosophila* has an eight to ten day life cycle (Figure 3) at 25 °C with females laying between 500 and 1000 eggs per animal (Hanson and Ferris 1929). Vast quantities of eggs allow researchers to analyze hundreds or even thousands of mutant lines over multiple generations, a feat matched only by bacteria and yeast (Beckingham *et al.* 2005).

*Drosophila* has a streamlined genome consisting of four chromosomes. Although once thought to have only 5,000 genes (Garcia-Bellido and Ripoll 1978), due to the complete sequencing of the *Drosophila* genome in 2000 (Adams *et al.* 2000), it is now known that *Drosophila* has approximately 13,600 genes (Adams *et al.* 2000); a relatively low number as compared to the estimated 20-30,000 genes in humans (Venter *et al.* 2001). Due to the small amount of genetic material, *Drosophila* has relatively little genetic redundancy as compared to vertebrate models (Beckingham *et al.* 2005). This allows for easier genetic mapping and balancing (discussed below).
Mated females lay embryos and first instar larvae hatch approximately 24 hrs later. First instar larvae consume as many nutrients as possible before molting and becoming second instar larvae. One day later, the second instar larvae molt and become third instar larvae. The third instar larvae continue to acquire as many nutrients as possible before pupating. Three to five days after pupation, adult flies eclose. Within six hours after eclosing, flies are sexually mature. (Figure adapted from Weigmann et al. 2003)
Perhaps the most important aspect of a model organism is the ability to draw comparisons to other organisms. As many processes and biochemical pathways are conserved from flies to humans (BECKINGHAM et al. 2005), *Drosophila* provides an informative model with which to study human disease states. About 80% of human genes have homologues in *Drosophila* and about the same percentage of identified human disease genes have *Drosophila* cognates (75%) (REITER et al. 2001; ST. JOHN and XU 1997).

1.6 Availability of a sophisticated tool box in *Drosophila*

The expansive ‘tool box’ that exists for *Drosophila* research is rivaled only by bacteria and yeast, making fruit flies a very attractive model. The iconic tool used by *Drosophila* geneticists is non-lethal phenotypic mutations (e.g. red eye, \(w^+\)). These mutations allow researchers to follow specific genes as well as whole chromosomes through multiple generations. The use of these mutations has led to the development of another powerful tool, balancer chromosomes.

Balancer chromosomes are chromosomes that contain multiple inversions capable of suppressing crossover events between homologous chromosomes during meiosis. These chromosomes are marked by dominant, homozygous lethal mutations (e.g. CyO, Tb, Sb, ScO, etc.) and allow investigators to (1) maintain recessive lethal mutations in a population and (2) to know the genotype of individuals without having to genotype each animal (BELLEN et al. 2010). Balancer chromosomes are available for the X chromosome, as well as all three *Drosophila* autosomes.
The GAL4/UAS system is a tool derived from yeast which allows for tissue specific overexpression of a gene of interest (Brand and Perrimon 1993). GAL4 is a transcription activator protein that binds to the UAS (Upstream Activation Sequence) enhancer element and drives expression of downstream genes (Figure 4). GAL4 expression can be spatially restricted by placing GAL4 under the control of tissue specific proteins (e.g. GMR-GAL4 expresses GAL4 in the eye). By forcing the ectopic expression of a gene, it is possible to study the biology of an otherwise lethal gene in vivo (Potter et al. 2000).

There exist a number of GAL4 driver lines that allow for the expression of GAL4 in specific tissues (Table 3) and can be obtained from Bloomington Stock Center (http://flystocks.bio.indiana.edu/). In practice, one need only create a transgenic line with a UAS-cDNA construct for a gene of interest and cross that to a GAL4 driver line that expresses GAL4 in the desired tissue. The resulting progeny will have both the activator protein (GAL4) and the target gene and will therefore express the gene of interest only where GAL4 is expressed (Figure 4).

Mosaic Analysis with a Repressible Marker (MARCM) is a technique that combines GAL4/UAS and a recombination system (Flipase/Flipase Recombination Target (FLP/FRT)). MARCM allows researchers to create fluorescently labeled (Green Fluorescent Protein, GFP) clones that are homozygous for a target gene in an otherwise heterozygous animal (Lee and Luo 1999). This can be used in the study of tumor micro-environments by creating clones which express an activated oncoprotein (e.g. Ras\textsuperscript{V12}), effectively creating fluorescently labeled tumors (Miles et al. 2011; Srivastava 2013).
Figure 4. Schematic of GAL4/UAS gene expression technique

Virgins from a GAL4 driver line are mated with transgenic males containing a UAS construct (a GFP fusion is shown here). Progeny (F₁) will contain both the GAL4 driver and the UAS construct and are therefore capable of expressing genes directly downstream of the UAS (Brand and Perrimon 1993).
<table>
<thead>
<tr>
<th>GAL4 Driver</th>
<th>Genotype</th>
<th>GAL-4 Expression Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>Cg-GAL4, UAS-GFP/CyO</td>
<td>hemocytes</td>
</tr>
<tr>
<td>Eyeless</td>
<td>w; Ey-GAL4/CyO</td>
<td>eye disc, ventral nerve cord, brain</td>
</tr>
<tr>
<td>Glass Multiple Reporter</td>
<td>GMR-GAL4</td>
<td>eye</td>
</tr>
<tr>
<td>Larval serum protein 2</td>
<td>w; LSP2-GAL4</td>
<td>third instar fat body</td>
</tr>
<tr>
<td>Pannier</td>
<td>Pnr-GAL4/TM₆Tb</td>
<td>dorsal cells along the length of fly</td>
</tr>
<tr>
<td>Patched</td>
<td>Ptc-GAL4, UAS-GFP</td>
<td>antero-posterior compartment boundary</td>
</tr>
<tr>
<td>Scalloped</td>
<td>Sd-GAL4 (x)</td>
<td>wing</td>
</tr>
<tr>
<td>Tubulin</td>
<td>yw;; Tub-GAL4/TM₃Sb</td>
<td>ubiquitous (tubulin)</td>
</tr>
<tr>
<td>Ultrabithorax</td>
<td>w;; Ubx-GAL4/Tb</td>
<td>abdomen, haltere, wing</td>
</tr>
<tr>
<td>Vestigial</td>
<td>Vg-GAL4 (M+Q)</td>
<td>wing imaginal disc</td>
</tr>
</tbody>
</table>

**Table 3. GAL4 driver lines chosen for this study.**
1.7 Biomedical advances attributed to fruit fly research

Fruit flies have been used to model a variety of diseases and genetic disorders. From diseases associated with aging (Michno et al. 2005) to neurological diseases such as Alzheimer’s and Parkinson’s diseases (Beckingham et al. 2005; Muqit and Feany 2002), Drosophila provides a valuable model to understand disease states in humans and has led to many advances in modern medicine.

One such advancement in medicine is the approval of a therapeutic for the treatment of tuberous sclerosis (TS). In patients with TS, tumors develop in multiple organs. Research on Drosophila elucidated the signaling pathway responsible for the misregulation of tissue growth, and furthermore led to the discovery that reducing the expression of a gene (S6 kinase) prevents this misregualtion (Tanenbaum 2003). Rapamycin is a drug known to inhibit a portion of the S6 kinase pathway, and was approved by the FDA for clinical trials in TS patients. In 2008, Bissler et al. published findings that a 12 month regimen of Rapamycin resulted in a decrease in angiomyolipomas in 50% of patients (Bissler et al. 2008).

1.8 Modeling of tumors in Drosophila

Many Drosophila genes have homologs to human oncogenes and tumor suppressors making Drosophila a powerful/useful model to study tumorigenesis and metastasis. Potter et al (2000) identified more than fifty Drosophila genes with cognates to mammalian tumor genes (Table 4). Research on Drosophila has led to the elucidation of a number of pathways that directly regulate tumor development in humans including the Notch, Hippo, JAK-STAT, LATS, and Hedgehog signaling pathways.
### Table 4. Fly homologues to mammalian genes

Potter et al. amassed a list of over 50 tumor related fly genes that have homologues to human genes demonstrating the relevance of *Drosophila* as a model for tumor studies. (From Potter et al., 2000)
The Notch signaling cascade, which has been implicated in several human cancers including breast, ovarian, and skin cancers, was first identified in flies (ALLENSPACH et al. 2002; MILES et al. 2011). Additionally, genetic screens in Drosophila led to the identification and characterization of three key genes involved in the Hedgehog pathway which is a major regulator of tissue growth and is linked to the transformation of healthy tissue to tumors in several cancers (BECKINGHAM et al. 2005; WATKINS et al. 2003).

While studies of cell cultures provide invaluable information, Drosophila affords us the opportunity to study the microenvironments of tumors in situ. Using our vast toolbox, we can readily manipulate cancer related genes in vivo and visualize the effects on tumor growth and metastasis.

1.9 Modeling of metastasis in Drosophila

The majority of cancer fatalities in humans are attributed to secondary metastatic growth (CHAMBERS et al. 2002). Drosophila provides a useful model for studying metastasis as many biochemical mechanisms and pathways associated with metastasis are conserved between humans and flies (e.g. ECM turnover) (POTTER et al. 2000).

There are several established systems in Drosophila which allow us to study metastasis. One such model is the Ras\textsuperscript{V12}/lgl model (PAGLIARINI and Xu 2003; SRIVASTAVA 2013). Ras is an oncogenic protein and Ras\textsuperscript{V12} codes for an activated isoform of the protein. Flies overexpressing Ras\textsuperscript{V12} exhibit benign, non-metastatic tumors. However, when combined with lethal giant larvae, a tumor suppressor encoded by the gene lgl, aggressive metastatic tumors result (PAGLIARINI and Xu 2003;
Thus we can induce a metastatic tumor by creating *Drosophila* with a genetic background of Ras$^{V12}$/gl.

### 1.10 Limitations of *Drosophila* as a tumor model

Although there are structures in flies that perform similar functions to mammalian organs (e.g. *Drosophila* Malpighian tubules and mammalian kidneys; Singh and Hou 2008), several human organs have no direct homologous organ in flies. This can make modeling of organ specific cancers difficult. Regardless, there is much to be learned about the highly conserved biochemical characteristics of tumors that can be studied by mimicking tumor environments in the fly.

Instead of blood vessels, *Drosophila*, like all members of the phylum Arthropoda, utilize an open circulatory system. Hemolymph serves as their nutrient transport system and the hemocoel, an open cavity, houses and transports hemolymph. Although true angiogenesis does not occur in *Drosophila*, in tumors produced by mutations in the *lats* gene, “lumen like” structures, resembling blood vessels, form and serve to distribute nutrients (Potter *et al.* 2000), mimicking angiogenesis. In this way, we are able to study angiogenesis in flies as it relates to tumors; however, care must be taken to ensure conclusions are not overstated.

Lunasin is currently a focus of investigation for its role as a potential anti-cancer therapeutic. This study aimed to create tools that will help determine whether Lunasin, when expressed *in vivo*, exerts any effect on normal development and tumor progression. We attempted to (1) create transgenic *Drosophila* capable of overexpressing Lunasin, (2) assess the effects of Lunasin on normal development, and (3) balance transgenic *Drosophila* in preparation for tumor suppression studies.
2.1 Construction of transgenic flies capable of expressing Lun and EGFP-Lun

Design and synthesis of DNA encoding \textit{lunasin} and \textit{EGFP-lunasin}

The experimental design required two gene constructs: \textit{lunasin} and a fluorescently tagged \textit{lunasin}. The Lunasin DNA sequence was obtained from published reports. To untagged lunasin, a start and stop codon were added to the 5’ and 3’ ends, respectively. For fluorescently tagged \textit{lunasin}, the \textit{EGFP} (Enhanced Green Fluorescent Protein) sequence was fused \textit{in silico} to the \textit{lunasin} DNA sequence on the 5’ end according to published reports. In both constructs, restriction sites for EcoRI and XhoI were added to the 5’ and 3’ end, respectively. Once designed, these sequences were synthesized \textit{in vitro} and the sequence verified by Integrated DNA Technologies (IDT; http://www.idtdna.com/site). The synthesized DNA was received from IDT in the proprietary vector, pIDTSMART.

Cloning of \textit{lunasin} and \textit{EGFP-lunasin} into pUAST

Plasmids were digested with EcoRI and XhoI. Restriction digests of pUAST and the pIDTSMART plasmids containing the synthesized genes were set up in 20 μL aliquots as follows: 1 μL EcoRI, 1 μL XhoI, 2 μL 10X buffer H, <1 μg plasmid, and sterile water to bring the volume up to 20 μL (Invitrogen; cat. nos. 10502 and 15231). The reaction mixture was incubated at 37 °C for 1 hr in a thermal cycler (Protocol 1) and subsequently electrophoresed on a 1% agarose gel. The agarose gels were stained with either 0.02 mg ethidium bromide (10 mg/mL) or 3 μL, 10,000 X GelRed® (Biotium; cat. no. 41003) added to molten agarose. Loading dye, 10 X, was mixed with DNA was mixed to a final concentration of 1X (Invitrogen; cat. no. 10816) and loaded alongside a
1Kb Plus DNA ladder (Invitrogen; cat. no. 10787; Supplementary figure 3) and was electrophoresed for 60 minutes at 100 V.

Electrophoresed DNA was gel extracted utilizing the QIAEX II gel extraction kit according to manufacturer’s instructions (QIAGEN; cat. no. 20021; Protocol 2).

Ligations of pUAST and **EGFP-lunasin** were set up in 10 µL reactions with a vector: insert ratio of 1:22. Reactions were set up as follows: 50 ng pUAST digested with EcoRI and Xhol, 105 ng **EGFP-lun** digested with EcoRI and Xhol, 0.5 µL ligase, and 1 µL ligase buffer (New England BioLabs; cat. no. M0202S.) Ligations of pUAST and **lunasin** were set up in 20 µL reactions with a vector: insert ratio of 1:9 as follows: 175 ng pUAST, 25 ng **lunasin**, 1 µL ligase, and 2 µL ligase buffer. All ligation reactions were incubated at 10 °C for 16 hr in a thermal cycler (Protocol 3).

Ligated plasmids (pUAST::**EGFP-lun** and pUAST::**lun**) were transformed into DH5α Escherichia coli (E. coli) cells as follows: cells were thawed and kept on ice. Between 50 and 150 µL cells were combined with 10 µLs of ligation reaction, and incubated on ice for 20 min. The mixture was then heat shocked at 42 °C for 90 s, and immediately placed on wet ice for 2 min. 300 µL of Luria broth (LB) (SIGMA; cat. no. L7275) was added to the transformation mixture and the transformation mixture was incubated at 37 °C for 45 min in a shaking incubator. A portion of the transformation mixture (between 100 and 200 µL) was plated on LB-agar plates containing Ampicillin (SIGMA; cat. no. A0166-5G) at a final concentration of 50 µg/mL. Plates were inverted and incubated at 37 °C for 16-20 hr (Protocol 4).

**Confirmation of successful cloning**
Transformants carrying the putative pUAST recombinant constructs were inoculated in 5 mL LB broth with Ampicillin (50 μg/mL) and incubated for 12-16 hrs at 37 °C. Plasmids were subsequently isolated utilizing a QIAGEN Plasmid Plus Mini kit (QIAGEN; cat. no. 27104; Protocol 5).

To confirm successful cloning of pUAST::EGFP-lun, plasmids were digested with EcoRI and XhoI (Protocol 1) and the sample electrophoresed in a 1% gel stained with 0.02 mg Ethidium Bromide (10 mg/mL) added to molten agarose.

To confirm successful cloning of pUAST::lun, we first performed whole colony PCR of putative pUAST::lun transformants utilizing two oligonucleotide primers that flank Lunasin: pUAST.FOR and GEJ.pUAST.REV (Table 5). Reactions were set up as follows: 22.5 µL SuperMix (Invitrogen; cat. no.10572-014), 200 nM pUAST.FOR, 200 nM GEJ.pUAST.Rev, and water added to 25 µL. PCR amplification parameters were as follows: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 40 s, primer annealing at 57 °C for 30 s, and extension at 72 °C for 30 s; final extension at 72 °C for 4 min (Protocol 6).

Bacterial stocks confirmed to contain the pUAST recombinant plasmids were inoculated in 400 mL LB broth with Ampicillin (50 μg/mL) and plasmids subsequently isolated utilizing QIAGEN Plasmid Plus Maxi kit (QIAGEN; cat. no. 12963; Protocol 7).

The pUAST::lun and pUAST::EGFP-lun constructs were sequenced as follows: 2 µL 5X BigDye® Terminator v1.1/3.1 sequencing buffer, 2 µL Ready Reaction premix (ABI; cat. no. 4336917), 0.5 µL UAAS-2 primer (Table 5), 20 ng template DNA, and water to 10 µL. Reactions were run for 25 cycles as follows: 30 sec denaturation at 95 °C, 15 sec annealing at 50 °C, and 4 min extension at 60 °C. PCR products were cleaned
using a QIAGEN DyeEx® Spin kit (QIAGEN; cat. no. 63206) according to manufacturer’s directions. Reactions were dried in a speedvac for 15 min with no heat, and re-suspended in 20 μL Hi-Di Formamide (Applied Biosystems; cat. no. 4311320). Samples were then loaded into ABI Prism sequencer and analyzed via ABI Prism software.

Microinjection of recombinant constructs (*performed by GSI)

Microinjection of the plasmid constructs was performed by Genetic Services Incorporated. Briefly, plasmid constructs and helper plasmids coding for transposases were injected into the posterior end of Drosophila embryos where germ cells are located. Successfully transformed individuals were screened for w⁺, red eyes.

2.2 Assessing the effects of EGFP-Lunasin on normal development

Confirmation of EGFP-Lun expression

To confirm EGFP-Lun expression, UAS-EGFP-lun transgenics were crossed to a Tubulin-GAL4 line which drives ubiquitous transgene expression. Progeny were screened for fluorescence as an expression indicator.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Use</th>
<th>Sequence (5' - 3')</th>
<th>MW (g/mol)</th>
<th>TM (°C)</th>
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<td>Sequencing</td>
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<td>58.1°C</td>
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<td>Whole Colony PCR</td>
<td>CCG AGC GGA GTA CTG TCC TCC GAG CGG AGA CTC TAG CGA GCG</td>
<td>12966.4</td>
<td>80.3°C</td>
</tr>
<tr>
<td>GEJ.pUAST.REV</td>
<td>Whole Colony PCR</td>
<td>GTC ACA CCA CAG AAG TAA GG</td>
<td>6144</td>
<td>60.4°C</td>
</tr>
</tbody>
</table>

Table 5. Primers used in this study
Evaluation of EGFP-Lun effects on normal development

EGFP-Lun transgenics were crossed to several GAL4 driver lines (Table 3). Crosses were set up in vials with nutrient agar (LabExpress; cat. no. 7002) using five virgins (♀) from GAL4 driver lines and five male (♂) transgenic flies. Individuals determined to express EGFP-Lun were carefully assessed to evaluate developmental effects. Legs, thorax, abdomen, and head were visually examined to identify any phenotypic abnormalities.

Confirmation of nuclear localization of EGFP-Lun

Nuclear localization of EGFP-Lun was determined by imaging salivary glands stained with Vectashield® mounting medium containing DAPI (Vector Labs; cat. no. H-1200). Salivary glands were first dissected in chilled 1X PBS (137 mM NaCl + 2.68 mM KCl + 10.14mM Na₂HPO₄ + 1.76 mM KH₂PO₄) and the glands fixed for 10-20 minutes in a fixative (0.1 M PIPES pH 7.2 + 4% paraformaldehyde), followed by two washes with a wash buffer (1XPBS + 0.1 % TritonX-100 + 0.01% sodium azide). The discs were then cleaned of excess tissue and mounted on a glass slide in Vectashield® mounting medium. Tissues were imaged at a final magnification of 100X utilizing a blue filter to detect DAPI fluorescence and a green filter to detect EGFP fluorescence.
2.3 Preparation of fly lines for use in tumor suppression studies

Mapping and balancing select fly lines

The transgene (*lunasin* or *EGFP-lunasin*) was mapped to either the X chromosome or chromosome ii or iii. We mapped lines GEJ1-L5, and GEJ2-L3 and confirmed the mapping done by GSI for lines GEJ1-L1- L3, and L8. Selected lines were balanced using standard genetic cross schemes.
3.1 Creation of transgenic *Drosophila* capable of expressing Lunasin and EGFP-Lun

**Gene synthesis**

We created transgenic flies that carried an EGFP tagged and an un-tagged version of Lunasin. The tagged construct will allow us easily to visualize the expression and localization of the protein. The untagged construct will allow us to determine whether any effects demonstrated by EGFP-Lun are a result of the EGFP tag or Lunasin itself. The genes coding for Lunasin and EGFP-Lunasin were synthesized by IDT. We then cloned these genes into the *Drosophila* overexpression vector, pUAST (Figure 5.)

We obtained the sequence for Gm2S-1 from NCBI and used the primary structure obtained from Lam *et al.* (2003) to identify the region coding for Lunasin. To generate the EGFP-Lun fusion construct, we attached the sequence for *EGFP* to the 5’ end of Lunasin; a study by Galvez *et al.* (1999) demonstrated that Lunasin tagged with GFP on the N-terminus resulted in fluorescently labeled Lunasin with no observable differences in bioactivity. To the untagged *lunasin* sequence, we added a start codon and stop codon to ensure translation potential. Native Lunasin lacks translation start and stop sites as it is part of a larger protein (Gm2S-1) that is post-translationally cleaved into smaller active peptides (Figure1). Finally, to allow for directed cloning into pUAST, we placed the
First, pIDTSMART and pUAST vectors were digested with the restriction enzymes, EcoRI and XhoI. Gene fragments and pUAST vector were gel extracted. Finally, *lun* and pUAST and *EGFP-lun* and pUAST were ligated.
restriction sites for EcoRI (G↓AATTC) and XhoI (C↓TCGAG) on the 5’ and 3’ end, respectively, of our constructs (Figure 6.)

Gene constructs, \textit{lunasin} and \textit{EGFP-lunasin}, were synthesized by Integrated DNA Technologies (IDT) and cloned into a plasmid vector, pIDTSMART (Figure 7). pIDTSMART is a proprietary vector with a high copy number due to the pUC origin of replication. Additionally, the plasmid contains an Ampicillin resistance marker, which we used as a selection tool. The gene sequences were verified by IDT using M13 (-20) Forward and M13 (-27) Reverse sequencing primers.

\textbf{Cloning of lunasin and EGFP-lunasin into pUAST}

pUAST is a \textit{Drosophila} specific expression vector that, when injected into \textit{Drosophila} embryos along with a helper plasmid, integrates into the genome. pUAST contains five GAL4 binding sequences, a multiple cloning site, and an Ampicillin resistance gene (Figure 8).

We digested the plasmids (pUAST, pIDTSMART::EGFP-lun, and pIDTSMART::lun) with the restriction enzymes, EcoRI and XhoI. Electrophoresis of the resulting products revealed bands of approximately: 9 Kb (Figure 9a), 2 Kb and 850 bp (Figure 9b), and 2 Kb and 150 bp (Figure 9c), which corresponded to the expected size fragments from pUAST, pIDTSMART::EGFP-lun, and pIDTSMART::lun, respectively. The bands corresponding to cut pUAST (~9 Kb), \textit{EGFP-lun} (~850 bp), and \textit{lunasin} (~150 bp) were gel purified in preparation for the ligation of \textit{lunasin} and \textit{EGFP-lun} genes into pUAST. DNA concentration and yield was determined via absorbance spectrums of the purified DNA.
Figure 6. Designed *lunasin* and *EGFP-lun* constructs utilized in this study
Lunasin and EGFP-Lunasin cDNA sequences with start (ATG) and stop (TAA) codons flanked by EcoRI restriction site (G↓AATTC) on the N-terminus and XhoI restriction site (C↓TCGAG) on the C-terminus.

*lunasin* and *EGFP-lunasin* constructs designed *in silico* to contain both start and stop codons. Restriction sites for EcoRI and XhoI were added to allow for directed cloning into pUAST. *EGFP* was fused to the 5’ end of *lunasin* as per Galvez and de Lumen (1999).
Figure 7. Plasmid maps of synthesized gene constructs

*lunasin* and *EGFP-lunasin* (blue arrows) were synthesized and cloned into pIDTSMART plasmid vectors containing an Ampicillin resistance gene, and pUC origin of replication. Gene sequences were verified by IDT.
Figure 8. Plasmid map of pUAST

pUAST contains an (a) Ampicillin resistance gene (red arrow), (b) five tandemly arranged GAL4 binding domains, and a (c) multiple cloning site

Figure from ADDGENE Vector Database.
Figure 9. Electrophoresis of plasmids digested with EcoRI and XhoI

Plasmids were digested with restriction enzymes, EcoRI and XhoI, and electrophoresed on 1 % agarose gels stained with ethidium bromide in preparation for gel extractions of (A) pUAST (8904 bp), (B) EGFP-Lun (864 bp), and (C) Lun (147 bp).
The *lunasin* and *EGFP-lun* genes were ligated into pUAST and transformed into competent DH5α *E. coli*. Only bacteria that carry plasmids containing the gene inserts should have grown on Ampicillin selection plates. As digested pUAST has non-compatible sticky ends, the plasmid should not be capable of self ligation. Thus for a bacterium to have a closed plasmid capable of conferring Ampicillin resistance, it must be ligated to a fragment with compatible ends (i.e. digested *lunasin* or *EGFP-lun*). Although unlikely, it is possible that two digested pUAST plasmids could fuse together, resulting in one super-plasmid. As such, it was necessary to confirm the insertion of the genes into pUAST.

**Confirmation of successful cloning (pUAST::EGFP-lun and pUAST::lun)**

The most direct method of confirming successful ligations of pUAST and *lunasin/EGFP-lun* was to isolate plasmids from putative clones, digest the plasmid with EcoRI and XhoI, and electrophorese the products. If we observed bands corresponding to expected size fragments (for pUAST::lun clones, bands of ~9Kb and ~150 bp, for pUAST::EGFP-lun clones, bands of ~9 Kb and ~850 bp) we could conclude that the clone was likely to be correct.

Figure 11 illustrates one such trial resulting in two distinct DNA bands at approximately 8.9 Kb and 850 bp, corresponding to pUAST and *EGFP-lun*, respectively. Thus, this sample, E9, was chosen for sequencing to ensure that no mutations had occurred.
Figure 10. Gel analysis of pUAST::EGFP-lun clone

Digestion of putative pUAST::EGFP-lun clone (lane ‘E9’) with EcoRI and XhoI produced fragments of 2 sizes: one of approximately 9 kb corresponding with pUAST, and another of approximately 850 bp corresponding with EGFP-lun.
This approach was not useful for confirming pUAST::*lun clones. Attempts to visualize digested *lunasin* bands from putative clones repeatedly failed, and it was ultimately deduced that *lunasin*, being only 147 bp, was not present in high enough concentrations to be visualized via gel electrophoresis.

To circumvent this obstacle, we performed whole colony PCR on putative pUAST::*lun clones, utilizing two oligonucleotide primers which flank the multiple cloning site: pUAST.FOR and GEJ.pUAST.REV (Table 5). When electrophoresed, empty pUAST generates a fragment of 377 bp while a successful clone generates a band of 490 bp. Samples were run alongside a 1 Kb+ ladder and a positive control for empty pUAST. Lanes 2-5, 7 and 8 contain bands of approximately 500 bp indicating recombinant pUAST plasmids (Figure 11).

Recombinant plasmids, pUAST::*EGFP-lun* and pUAST::*lun*, were isolated from liquid cultures to prepare samples for sequencing and microinjection into *Drosophila* embryos. Plasmid isolation (Maxiprep) of pUAST::*EGFP-lun* (sample E9) yielded 97 μgs of plasmid DNA with an OD\textsubscript{260}/OD\textsubscript{280} of 1.90 (Supplementary figure 2a). Plasmid isolation of pUAST::*lun* (sample 1b) yielded 941 μgs of plasmid DNA with an OD\textsubscript{260}/OD\textsubscript{280} of 1.93 (Supplementary figure 2b).

Before sending our recombinant pUAST constructs for microinjection, we sequenced each clone to ensure the integrity of *lunasin* or *EGFP-lun*. A change in nucleotide compositions in these genes could change the primary structure of the translated peptide,
Figure 11. Gel analysis of potential pUAST::lun clones

Whole colony PCR conducted on transformed colonies results in two possible band sizes. Empty vector generates a 377 bp fragment; the presence of *lunasin* generates a 490 bp fragment. Lane 1 contains empty pUAST as a positive control for empty vector. Lanes 2-9 contain potential clones. Samples in lanes 2-5, 7, and 8 show bands at 490 bp. Samples in lanes 6 and 9 show a band at approx 377 bp.
which may affect the 2° and 3° protein structures and consequently alter the behaviour of the protein \textit{in vivo}.

The resulting sequences for pUAST::\textit{EGFP-lun} and pUAST::\textit{lun} (Supplementary figure 3 and Supplementary figure 4) matched the gene sequences synthesized by IDT (Figure 12 and Figure 13). Once we confirmed that the pUAST constructs contained the correct gene sequence with no alterations, we sent between 50 and 100 μgs of recombinant plasmid (Figure 14) to GSI to be microinjected into \textit{Drosophila} embryos.

**Microinjection of recombinant pUAST constructs**

Microinjection has a low success rate as \textit{Drosophila} embryos are delicate and often die when the waxy, vitelline membrane is punctured. Even microinjecting several hundred embryos may yield only a handful of viable individuals. We received eight EGFP-Lun transgenic lines (GEJ1-L1 to L8) and five untagged Lunasin transgenic lines (GEJ2-L1 to L5) from GSI.

### 3.2 Assessing the effects of Lunasin on Normal development

**Confirmation of EGFP-Lun overexpression**

To confirm expression in \textit{EGFP-lun} transgenics, we crossed transgenics to Tubulin-GAL4 and visualized the larvae via fluorescence microscopy (Figure 15). Progeny of GEJ1-L1 through GEJ1-L8 crossed with Tubulin-GAL4 all fluoresced when placed under blue light indicating that each line was capable of expressing EGFP-Lun when under control of a GAL4 driver.
Figure 12. Sequence alignment of EGFP-lun

Sequence alignment was performed using bioinformatics software (ClustalW2).

Sequence 1 (EGFP__Lun) is the sequence for EGFP-lun as synthesized by IDT.

Sequence 2 (sampleUAAS-2) is the experimental data from the sequencing of potential clone, E9. Stars (*) below the sequences identify nucleotide homology.
Table 13. Sequence alignment of pUAST::lun

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</table>

Figure 13. Sequence alignment of pUAST::lun

Sequence alignment was performed using ClustalW2 bioinformatics software.

“pUAST::Lun” is the constructed sequence of pUAST::lun (assembled from the sequence of pUAST and the sequence of our synthesized lunasin) and “pUASTLun SEQ5” is the experimental sequence obtained from pUAST::lun clone, 1b. Aqua shading denotes lunasin, whereas gray represents pUAST sequence flanking the lunasin insert.
Figure 14. Plasmid maps of pUAST clones

Recombinant pUAST constructs contain 5X GAL4 binding sites, an Ampicillin resistance gene, and a multiple cloning site where EGFP-lunasin (A) and lunasin (B) was inserted.
Figure 15. GAL4 mediated overexpression cross scheme

Virgins of the GAL4 line are crossed with transgenic/UAS males. In this instance, Tubulin-GAL4/Tm3Sb virgins are crossed with EGFP-Lun transgenics. Offspring will be either UAS EGFP-Lun/Tubulin-GAL4 or UAS EGFP-Lun/Tm3Sb. UAS EGFP-Lun/Tubulin-GAL4 individuals will fluoresce under blue light (490 nm).

(Adapted from Duffy 2002).
Forcing ectopic expression of EGFP-Lun

Once we established that EGFP-Lunasin was being expressed in our transgenic lines, we sought to assess whether this expression affected normal development of *Drosophila* by examining animals for gross phenotypic abnormalities. To drive expression of EGFP-Lun in a tissue specific manner, we employed the GAL4/UAS system. Progeny resulting from crosses between EGFP-Lun transgenic lines (GEJ1-L1 to GEJ1-L8) and GAL4 driver lines showed no phenotypic abnormalities (Table 6). Not every transgenic line was crossed with all ten GAL4 drivers. However, it is most important to note that for each transgenic line, when EGFP-Lun was expressed ubiquitously (i.e. under control of Tubulin-GAL4 driver), there were no observed phenotypic abnormalities. This suggests that if EGFP-Lun expression were to be restricted to a portion of the body, there would likewise be no resulting abnormalities.

Subcellular localization of EGFP-Lun

Native Lunasin is known to localize to the nucleus, and thus it was necessary to ensure that EGFP-Lun acted in the same way. If EGFP-Lun does *not* localize to the nucleus, any conclusions concerning the effects of Lunasin on normal development would be irrelevant. To determine the subcellular localization of EGFP-Lun, we imaged animals expressing EGFP-Lun in the salivary glands. Cells in the salivary glands undergo multiple rounds of DNA replication without completing cytokinesis, resulting in polytene chromosomes and enlarged nuclei to house the chromosomes. These enlarged nuclei allow for simple imaging of the cells.
Table 6. Summary of UAS-EGFP-lun crosses to various GAL4 lines

<table>
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<th>GEJ1</th>
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<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>w;Ey-GAL4/CyO</td>
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<td>X</td>
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<tr>
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<td>X</td>
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<td>-</td>
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<td>X</td>
<td>-</td>
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<td>Pnr-GAL4/Tm6Tb</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>w;;Ubx-GAL4/Tb</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vg-GAL4 (M+Q)</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

X = progeny of cross were viable and exhibited no phenotypic abnormalities  
- = cross not conducted

Table 6. Summary of UAS-EGFP-lun crosses to various GAL4 lines

When crossed to Tub-GAL4, which drives ubiquitous expression of UAS constructs (EGFP-Lun, in this instance), progeny exhibited no phenotypic abnormalities.

GEJ1-L2 was crossed to each GAL4 driver and similarly exhibited no phenotypic abnormalities.
Ey-GAL4 and Sd-GAL4 are known to drive expression of EGFP-Lun in the salivary glands. Progeny resulting from crosses between our transgenic GEJ1-L2 and these GAL4 lines express EGFP-Lun in the salivary glands. Glands were mounted in Vectashield with DAPI® as this medium greatly slows degradation of fluorescent signals while staining the nucleus with DAPI. In this way, we were able to compare the location of EGFP-localization to those of nuclear boundaries. Our results revealed direct alignment between nuclear boundaries and EGFP-Lunasin localization regions (Figure 16) indicating that, when expressed in Drosophila, EGFP-Lun localizes to the nucleus.

3.3 Development of tools to be utilized in tumor suppression studies.

Mapping transgenes to specific chromosomes

Mapping transgenes to specific chromosomes allows investigators to track the presence of these genes when conducting crosses, thus it was essential that we map the location of our transgenes. To do this, we first determined whether the transgene is located on an autosome (ii or iii) or X chromosome by crossing virgin double balanced flies (w; Sco/CyO; Sb/TM6 Tb) with male transgenic flies. If the transgene is on an autosome, both male and female progeny will be a mix of red eyed (w+) and white eyed (Figure 17). If on the X chromosome, all male progeny will have white eyes (w), and females will have a mix of red and white eyes, as male progeny receive the Y chromosome from the transgenic male, and therefore would not receive a copy of the transgene (marked by presence of w+).
Figure 16. Localization of EGFP-Lun to Nucleus

EGFP-Lun expression was driven in salivary glands. Glands were dissected, preserved, and mounted in Vectashield ® mounting medium with DAPI, a nuclear stain. Fluorescence microscope images of salivary glands expressing EGFP-Lun with (A) a blue filter detect DAPI fluorescence and (B) a green filter show EGFP-Lun localization. A composite image (C) demonstrates overlap of expression area.
Figure 17. Schematic for mapping transgene to either X chromosome or autosomes

To determine if transgene is inserted on X chromosome or an autosome, male transgenics are crossed with double balanced virgins (F₀). If the transgene (pw⁺) is inserted on the X chromosome, male progeny (F₁) will all be white eyed (w). If on an autosome, male progeny will be a mix of white eyed (w) and red eyed (w⁺).
If the transgene is on an autosome, we then set out to determine onto which chromosome, ii or iii, the trangene inserted (Figure 18). Our transgenics were created utilizing Flipase mediated recombination, and as crossing over and recombination do not occur on chromosome iv (or the Y chromosome) of *Drosophila* (*Sandler* and *Szauter* 1978), transgenes do not insert on chromosome iv. We determined whether the transgene was inserted on chromosome ii or iii by crossing selected progeny (w+, CyO, Tb) from the cross described above (♀ double balanced x ♂ transgenics) with double balanced virgins (Figure 18). If the resulting w+ progeny are Sco/CyO, the transgene must be on chromosome iii. Conversely, if the w+ progeny are Sb/Tb, the transgene is necessarily on chromosome ii.

GEJ1 transgenic lines L1-L3, and L8 were mapped by GSI (Table 7) and later confirmed by our lab. The transgene for L1 inserted on the X chromosome, L2 mapped to chromosome iii, and L3 and L8 mapped to the ii chromosome. We mapped GEJ1-L5 and GEJ2-L3 to the X chromosome.

**Balancing transgenic lines**

A balanced line provides a stable, self perpetuating stock that ensures a consistent genotype. Because we set out to create tools for use in tumor suppression studies, we chose to balance a line that would be the most useful in this study. We chose to balance GEJ1-L2 because the *EGFP-lun* transgene was mapped to chromosome iii and the mutant genes for the selected tumor modeling system, *Ras*\(^{V12/lgl}\), are located on the chromosome ii. To ensure that both introduced chromosomes (transgene and invasive tumor gene) are maintained together in the stock, each need to be balanced over a balancer chromosome.
Thus, a transgenic line in which \textit{EGFP-lun} is on chromosome iii is ideal for tumor studies utilizing \textit{Ras}^{v12}/lgl flies.

To balance GEJ1-L2, we first crossed our transgenic line to a double balanced line (Figure 19). Male w; +/CyO; pw^+ /Tb progeny were then selected and recrossed to virgin double balanced flies. Male and virgin females of the target genotype (w; Sco/CyO; EGFP-Lun/TM6,Tb) were then crossed to produce a stable, balanced line. GEJ1-L2 was successfully balanced and has the genotype: w; Sco/CyO; w^+,EGFP-Lun/TM6,Tb. This line is now available for use in tumor suppression studies.
If transgene \( pw^+ \) inserted on autosome:

\[
\begin{align*}
F_0 & \quad pw^+ \\
F_1 & \quad w; \frac{\text{Sco}}{\text{CyO}}, \frac{\text{Sb}}{\text{Tm}_6\text{Tb}} \\
& \quad w; \frac{\text{Sb}}{\text{CyO}}, \frac{\text{Tm}_6\text{Tb}}{w} \\
& \quad w; \frac{\text{CyO}}{\text{Tm}_6\text{Tb}} \\
F_2 & \quad w; \frac{\text{Sb}}{\text{CyO}}, \frac{\text{Tm}_6\text{Tb}}{w} \\
& \quad w; \frac{\text{Sco}}{\text{CyO}} \quad \frac{pw^+}{w} \\
& \quad w; \frac{\text{Tm}_6\text{Tb}}{w}
\end{align*}
\]

**Figure 18. Schematic for mapping to chromosome ii or iii**

(F0) Cross \( w^+ \) males with double balanced virgins (w; Sco/Cyo; Sb/TM6Tb).  (F1) Select \( w^+ \); CyO; Tb male progeny and cross once more with double balanced virgins.

Determine whether \( pw^+ \) is on chromosome ii or iii.  If \( w^+ \) animals (F2) are Sb/Tb, then the transgene inserted on chromosome ii; if \( w^+ \) animals are Sco/Cyo, the transgene inserted on chromosome iii.
<table>
<thead>
<tr>
<th>Line Name</th>
<th>Chromosome</th>
<th>Homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEJ1-L1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>GEJ1-L2</td>
<td>iii</td>
<td>N</td>
</tr>
<tr>
<td>GEJ1-L3</td>
<td>ii</td>
<td>N</td>
</tr>
<tr>
<td>GEJ1-L4*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GEJ1-L5</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>GEJ1-L6*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GEJ1-L7*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GEJ1-L8</td>
<td>ii</td>
<td>Y</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line Name</th>
<th>X chromo.</th>
<th>Autosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEJ2-L1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GEJ2-L2</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>GEJ2-L3</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>GEJ2-L4*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GEJ2-L5</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>

**Table 7. Transgenes mapped to specific chromosomes**

Lines in which no mapping data were collected are denoted with an asterisk (*).
Figure 19. Schematic for balancing transgenic line, GEJ1-L2

For balancing a line that has the transgene on the third chromosome such as GEJ1-L2, select red eyed (w+) transgenics and cross to the double balanced line (w; Sco/CyO; Sb/Tm6Tb). Select w; +/CyO; pw+/Tb progeny and mate once more with the double balanced line. From the progeny, select w+, Sco/Cyo, and Tb virgins and males and cross to each other to maintain a balanced line.
Lunasin has been shown to exhibit anti-metastatic activities in newly transformed cell lines, while having no effect on the health or mitotic abilities of non-tumorigenic cell lines (De Lumen 2005; Dia and Gonzalez de Mejia 2011; Lam et al. 2003; Pabona et al. 2012). All of these experiments were conducted in vitro and therefore it was not known how Lunasin acts when expressed in vivo. To advance Lunasin as a chemotherapeutic agent, model organisms capable of overexpressing Lunasin must be developed, and in vivo studies must be conducted to predict how Lunasin might act in humans. We predicted that Lunasin would not impart any phenotypic effects when expressed in non-tumorigenic Drosophila. The lack of phenotypic abnormalities in EGFP-Lunasin expressing individuals would contribute to the body of evidence supporting the therapeutic potential of Lunasin. Additionally, the tools created in this study can be used in future studies (e.g. tumor suppression, and Alzheimer’s and Parkinson’s diseases).

Transgenic Drosophila are capable of expressing Lunasin and EGFP-Lun.

Animal models are essential in studying the in vivo effects of drugs, and Drosophila provides a convenient and powerful tool to model many disease states, including tumorigenesis and metastasis (Pagliarini and Xu 2003; Rudrapatna et al. 2012). We set out to create transgenic Drosophila that are capable of overexpressing Lunasin and a tagged Lunasin construct for use in developmental and tumor suppression studies.

The successful construction of pUAST::EGFP-lun and pUAST::lun plasmids for microinjection into Drosophila embryos was confirmed through sequencing efforts.
(Figure 12 and Figure 13). Our results show that our *EGFP-lun* transgenic lines were capable of producing EGFP-Lunasin.

**EGFP-Lun localizes to the nucleus.**

We chose to create a fluorescently tagged Lunasin construct to allow for visualization of the Lunasin peptide in subsequent studies. This fusion protein (EGFP-Lun), when excited with blue light, allows us to easily confirm Lunasin expression as well as monitor the expression location and, to a limited degree, gauge expression levels. However, in order to make relevant conclusions regarding the effects of EGFP tagged Lunasin *in vivo*, it was first necessary to establish that this fluorescently tagged construct acts in the same way as untagged Lunasin. Native Lunasin localizes to the nucleus, and although it remains unclear how this occurs, it is suggested that this behaviour is required for the observed anti-mitotic bioactivity attributed to Lunasin. (DE LUMEN 2005; GALVEZ *et al.* 2001; GALVEZ and DE LUMEN 1999). Our findings demonstrate that EGFP-Lun indeed localizes to the nucleus, suggesting that our EGFP-Lun construct acts in a way similar to untagged Lunasin and thus may potentially be used in place of untagged Lunasin in future studies. However, as Lunasin is less than one fourth the size of EGFP, it is possible that the added mass may cause steric hindrance and alter the bioactivity of the fusion protein. Thus, it is essential that additional assays (i.e. microarrays) be conducted to ensure that the effects of tagged Lunasin mirror that of untagged Lunasin at a molecular level.

In non-tumorigenic flies, expression of EGFP-Lunasin results in no phenotypic abnormalities.
Many chemotherapeutics have the undesirable side effect of killing healthy cells and cancerous cells. Previous *in vitro* studies have demonstrated that Lunasin selectively inhibits mitosis and induces apoptosis in newly transformed cells while having no deleterious effects on the health or mitotic ability of non-tumorigenic cell lines (DE LUMEN 2005; DIA and GONZALEZ DE MEJIA 2011; GALVEZ *et al.* 2001; LAM *et al.* 2003; PABONA *et al.* 2012). Although this makes Lunasin a desirable candidate for development as a therapeutic, these studies have solely been conducted in cell cultures. We sought to determine if the overexpression of Lunasin *in vivo* affects normal development. Our findings demonstrate that, when expressed ubiquitously, EGFP-Lun results in viable individuals with no phenotypic abnormalities. Although no effects were noticeable at the organism level, it is important to also identify changes at the molecular level.

**Tools developed for use in tumor suppression studies.**

We have created tools that will allow us to assess the therapeutic potential of Lunasin by determining whether Lunasin has anti-tumorigenic and/or anti-metastatic effects *in vivo* by visualizing size and metastasis of tumors in *Drosophila*. One such tool is the balanced transgenic line. Balancer chromosomes allow for the maintenance of heterozygous stocks while allowing recessive lethal mutations to be preserved in a population. Transgenes inserted into the *Drosophila* genome can often be selected against and after several generations may be lost. Therefore, it is essential that these transgenes be balanced. Transgenic line GEJ1-L2, with EGFP-Lun mapped to the chromosome iii, is balanced and primed to be used in tumor suppression studies utilizing the *Drosophila* tumor invasion model.
4.1 Future Directions

The EGFP-Lun transgenic flies created in this study have the potential for great utility in understanding the effects of Lunasin in many disease backgrounds. However, in order to conclusively demonstrate this utility, we must show that our EGFP-Lun lines act in an identical manner to un-tagged Lunasin. This requires that we first show that our untagged-Lunasin transgenic lines express Lunasin. This can be accomplished the RTPCR confirmation of *lunasin* mRNA. At the time of this writing, RTPCR experiments are underway.

In addition to assessing overall tumor growth, it would be beneficial to study differences in tumor composition. Lunasin has been found to increase CDK-inhibitors (p21 and p27) in colon cancer cells and mouse embryo fibroblasts *in vitro* (DIA and GONZALEZ DE MEJIA 2011; LAM et al. 2003). Additionally, Lunasin modifies expression levels of ECM genes (Table 2) in a human colon cancer cell line (KM12L4). As these have been demonstrated *in vitro*, it is essential that the effects of Lunasin, on a molecular scale, be investigated *in vivo*. By running a microarray on tumors in animals expressing Lunasin and on tumors in those not expressing Lunasin, we can investigate how Lunasin affects protein levels (i.e. p21, p27, and ECM genes) in tumor environments.

In order to make generalizations regarding the efficacy of Lunasin as an anti-cancer therapeutic *in vivo*, it is imperative to study the effects of Lunasin in multiple *in vivo* tumor models. In addition to the *RasV12/lgI* tumor model, *scrib−/−* mutants are often employed in tumor studies as *RasV12/scrib−/−* animals develop tumors with metastatic capabilities (PAGLIARINI and XU 2003). By employing this tumor model, we can observe gross growth rates of tumors in the presence of lunasin and without, and also conduct
protein micro-arrays to compare to changes in protein levels between the tumor backgrounds.

Lunasin has also been found to inhibit inflammation by reducing expression levels of proinflammatory cytokines (LIU and PAN 2010). Inflammation has been implicated in several human ailments including Alzheimer’s disease (AD) and Parkinson’s disease (PD). There are Drosophila models for AD and PD which could be utilized in conjunction with the transgenic flies developed in this study to assess the effects of Lunasin on the disease pathology. If Lunasin reduces pathogenicity of these diseases (AD, PD, and cancer) in Drosophila then it would be valuable to assess the effects of Lunasin on disease progression in a mammalian model more closely related to humans than flies.

Cancers have been a concern for human health, but neoplasias are becoming a conservation concern as well. Tasmanian devil facial tumors, genital carcinomas in California sea lions and several dolphin species, and fibropapillomas in Green Sea Turtles are threatening the future of these species (MCALOOSE and NEWTON 2009). Lunasin also may hold some promise in combating these novel wildlife neoplasias as Lunasin works in many ways to limit tumorigenesis.
Supplementary figure 1. 1 Kb+ ladder
This DNA ladder was used throughout the cloning process to estimate fragment size of electrophoresed DNA samples. The doublet at 2 Kb and 1650 bp provide a distinct reference for enumerating the ladder bands.
Supplementary figure 2. Absorption spectrum of (A) pUAST::EGFP-lun clone (sample E9) and (B) pUAST::lun (sample 1b).

Spectroscopy readings of isolated pUAST::EGFP-lun (A) revealed an OD$_{260}$/OD$_{280}$ of 1.90 and a yield of 97 μg; while isolated pUAST::lun has an OD$_{260}$/OD$_{280}$ of 1.93 and yielded 941 μg. Absorption spectrum of (A) pUAST::EGFP-lun clone (sample E9) and (B) pUAST::lun (sample 1b).
Supplementary figure 3. Sequence data for pUAST::EGFP-lun clone, E9

EGFP-Lun begins at bp 66 and extends to bp 917. Bp 1-65 and 918-1186 represent pUAST sequence. Color and height of the bars correspond to nucleotide call confidence: blue indicates a call of high confidence, yellow of intermediate confidence, and red indicates a poor read with low confidence. Tall bars represent higher confidence than shorter bars.
Supplementary figure 4. Sequence data for pUAST::lun clone, 1b

*lunasin* begins at bp 62 (ATG) and extends to bp 196. Bp 1-61 and 197-579 represent pUAST sequence. Color and height of the bars correspond to nucleotide call confidence: blue indicates a call of high confidence, yellow of intermediate confidence, and red indicates a poor read with very low confidence. Tall bars represent higher confidence than short bars.
Protocol 1. Restriction Digest (double)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>1 µL</td>
</tr>
<tr>
<td>XhoI</td>
<td>1 µL</td>
</tr>
<tr>
<td>10X Buffer H</td>
<td>2 µL</td>
</tr>
<tr>
<td>Substrate DNA</td>
<td>≤1 µg</td>
</tr>
<tr>
<td>Sterile water</td>
<td>to 20 µL</td>
</tr>
</tbody>
</table>

1. Combine reagents in PCR tube according to table above. Add enzymes last.

2. Incubate in thermal cycler at 37 °C for 1 hr.
Protocol 2. Gel Extraction (QIAGEN; cat. no. 20021)

1. Excise DNA band from the agarose gel using a razor blade.

2. Weigh the gel slice. Add Buffer QX1 (and water) according to table below:

<table>
<thead>
<tr>
<th>DNA fragment size</th>
<th>DNA fragment:</th>
<th>Amount reagent:</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 bp – 4 kb</td>
<td>Lunasin and EGFP-Lun</td>
<td>3 volumes Buffer QX1</td>
</tr>
<tr>
<td>&gt;4 kb</td>
<td>pUAST</td>
<td>3 volumes Buffer QX1</td>
</tr>
</tbody>
</table>

   plus 2 volumes of H$_2$O

Ex. If Lunasin fragment slice weighs 150 mg, add 450 µL Buffer QX1

3. Resuspend QIAEX II by vortexing for 30 s. Add QIAEX II to the sample according to the table below and mix:

<table>
<thead>
<tr>
<th>Amount DNA:</th>
<th>Amount QIAEX II:</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 2 ug DNA</td>
<td>Add 10 µL of QIAEX II</td>
</tr>
<tr>
<td>2-10 ug DNA</td>
<td>Add 30 µL of QIAEX II</td>
</tr>
</tbody>
</table>

4. Incubate at 50 °C for 12 minutes. Mix by vortexing every 2 minutes.

5. Centrifuge the sample at 10,000 X g for 30 s and carefully remove supernatant with a pipet.

6. Wash the pellet with 500 µL Buffer QX1. Vortex the pellet, centrifuge at 10,000 x g for 30 s, and remove supernatant with pipet.

7. Wash the pellet twice with 500 µL of Buffer PE. Vortex the pellet, centrifuge for 30 s, and remove supernatant with a pipet. Repeat for second wash.

8. Air-dry the pellet for 10-15 min. To speed up drying, apply a vacuum until the pellet becomes white.
9. To elute the DNA, add 20 µL water and resuspend the pellet by vortexing. Incubate according to the table below.

<table>
<thead>
<tr>
<th>Fragment size:</th>
<th>DNA fragment:</th>
<th>Incubation directions:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA fragments ≤4 kb</td>
<td>Lunasin and EGFP-Lun</td>
<td>Incubate at room temp. (25 °C) for 5 min</td>
</tr>
<tr>
<td>DNA fragments 4-10 kb</td>
<td>pUAST</td>
<td>Incubate at 50 °C for 5 min</td>
</tr>
</tbody>
</table>

10. Centrifuge for 30 s. Pipet the supernatant into a clean microcentrifuge tube.

11. Analyze purity and concentration via NanoDrop spectrometer.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>**</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>**</td>
</tr>
<tr>
<td>10X ligase buffer</td>
<td>2 µL</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1 µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>to 20 µL</td>
</tr>
</tbody>
</table>

1. Set up ligation reactions as described in table above.

2. Incubate at 10 °C for 16 hrs.

** Determine amount of DNA to be ligated (100-300 ng), choose vector: insert ratio (1:3, 1:9, 1:20 etc.), and calculate volume of plasmid and vector DNA to ligate.
**Protocol 4. Transformation**

1. Chill ligation reaction and DH5α competent cells on ice.

2. Combine 100 µL DH5α cells with 10 µL ligation reaction.

3. Combine 100 µL DH5α cells with 10 µL water (negative control)

4. Heat shock at 42 °C for 90 s.

5. Place mixtures on ice for 2 min.

6. Add 500 µL Luria Broth

7. Incubate mixture at 37 °C for 45 min with shaking (225rpm)

8. Plate 300 µL of mixture on LB-Amp (50 µg/mL) plates.

9. Incubate at 37 °C for 16-20 hours.

Note: Resulting colonies contain the Amp<sup>r</sup> gene.
Protocol 5. Plasmid isolation – Mini (QIAGEN; cat. no. 27104)

1. Pick a single colony from a freshly streaked Ampicillin selection plate and inoculate a starter culture of 5 mL LB-Amp (50 µg/mL). Incubate for 8-12 hours at 37 °C with vigorous shaking.

2. Centrifuge cells at 3,000 x g for 5 min at 4 °C

3. Resuspend pelleted cells in 250 µL Buffer P1 and transfer to a microcentrifuge tube.

4. Add 250 µL Buffer P2 and invert tube 6 times.

5. Add 350 µL Buffer N3 and invert tube 6 times.

6. Centrifuge for 10 min at 13,000 rpm in a table-top centrifuge.

7. Apply supernatant from step 6 to QIAprep spin column.

8. Centrifuge for 60 s. Discard flowthrough.

9. Wash the QIAprep spin column by adding 0.5 mL Buffer PB and centrifuge at 13,000 rpm for 60 s.

10. Discard flowthrough and centrifuge an additional 60 s.

11. Elute DNA into clean microcentrifuge tube by adding 50 µL Buffer EB to center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.
Protocol 6. Whole colony PCR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SuperMix</td>
<td>22.5µL</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>200 nM</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>200 nM</td>
</tr>
<tr>
<td>Sterile water</td>
<td>to 25 µL</td>
</tr>
<tr>
<td>Transformed colony</td>
<td>1</td>
</tr>
</tbody>
</table>

1. Set up reactions according to the table above.

2. Load into Thermal Cycler and set the PCR parameters as follows:

   Initial denaturation: 95 °C for 3 min

   30 cycles of:

   Denaturation: 95 °C for 40 s
   Annealing: 57 °C for 30 s
   Extension: 72 °C for 30 s

   Final extension: 72 °C for 4 min

   Hold: 4 °C for ∞
Protocol 7. Plasmid Isolation – Maxi (QIAGEN; cat. no. 12963)

1. Pick a single colony from a freshly streaked Ampicillin selection plate and inoculate a starter culture of 2-5 mL LB-Amp (50 µg/mL). Incubate for 8-12 hours at 37 °C with vigorous shaking (250-300 rpm).

2. Inoculate 300-500 mL LB-Amp (50 µg/mL) with 150 µL of starter culture. Incubate for 12-16 hrs at 37 °C with vigorous shaking (250 rpm).

3. Centrifuge cells at 6,000 x g for 15 min at 4 °C

4. Discard supernatant and resuspend pellet in 10 mL buffer P1.

5. Add 10 mL Buffer P2, mix by inverting the tube 6 times and incubate at room temperature (25 °C) for 5 min.

6. Add 10 mL chilled Buffer P3, invert 6 times, and incubate on ice for 20 min.

7. Centrifuge at 20,000 x g for 30 min at 4 °C.

8. Immediately transfer supernatant and recentrifuge at 20,000 x g for 15 min at 4 °C. Immediately transfer supernatant to clean vial.

9. Equilibrate a QIAGEN-tip 500 by applying 10 mL Buffer QBT, and allow the column to empty by gravity flow.

10. Apply the supernatant from step 8 to the QIAGEN-tip and assist the flow by applying a vacuum to the tip.

11. Wash the QIAGEN-tip with 2 x 30 mL Buffer QC and assist the flow by applying a vacuum to the tip.

12. Elute the DNA with 15 mL Buffer QF. Collect elute.

13. Precipitate DNA by adding 10.5 mL room temp Isopropanol to the eluted DNA. Mix and centrifuge at 15,000 x g for 30 min at 4 °C. Decant supernatant.
14. Wash DNA pellet with 5 mL room temp 70 % ethanol. Centrifuge at 15,000 x g for 10 min. Decant supernatant, being cognizant to not disturb the pellet.

15. Air-dry the pellet for 10 min and redissolve the DNA in 800 µL water.


**LITERATURE CITED**


ADDGENE, pUAST Vector Map, pp. in Vector Database.


STOELTZING, O., W. LIU, N. REINMUTH, F. FAN, G. PARRY et al., 2003 Inhibition of integrin alpha5beta1 function with a small peptide (ATN-161) plus continuous 5-

TAMURA, M., J. GU, K. MATSUMOTO, S. AOTA, R. PARSONS et al., 1998 Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 280: 1614-1617.


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Graduation Date: May 2011
Summa Cum Laude

REPRESENTATIVE COURSEWORK

Parasitology
Human Parasitology
Immunology
Human Anatomy
Molecular Biology
Cell Biology
Genetics
Molecular Genetics

Entomology
Environmental Physiology
Limnology
Ecology & Evolutionary Biology
Organismal Biology
Biochemistry
General Chemistry I & II
Organic Chemistry I & II

RESEARCH INTERESTS

* Immunology and pathogenesis of tropical parasitic diseases

RESEARCH EXPERIENCE

Western Kentucky University
Graduate Student; Advisor: Ajay Srivastava, Ph.D.
Development of tools to assess the effects of Lunasin on normal development and tumor progression in Drosophila melanogaster.

* Created transgenic Drosophila capable of overexpressing Lunasin and Lunasin tagged with a fluorescent marker, EGFP;
* Observed and recorded developmental phenotypes due to expression of Lunasin;
* Created tools for use in tumor progression studies
**University of California, Santa Cruz**
Field Technician; Advisor: A. Marm Kilpatrick, Ph.D.
Survival and distribution of the mosquitoes, *Culex pipiens* and *Culex restuans*
*Trapped mosquitoes using CDC Light and CDC Gravid traps;
*Retrieved trapped insects and reset traps daily;
*Identified collected mosquitoes to genus (to species when possible);
*Data collected was to be used for a larger project on West Nile Virus transmission and prevalence.

**University of California, Davis**
REU Summer Intern; Advisor: Gary Cherr, Ph.D. and Jim Moore, Ph.D.
Multidrug resistance in normal and cancerous hemocytes of the bay mussel, *Mytilus trossulus*
*Performed CAM exclusion fluorescence assay to quantify fluorescence levels in Drug Transport Protein (DTP) inhibited cells which corresponds to DTP activity;
*Concluded that DTP activity differs not only between neoplastic and normal individuals, but also among neoplastic individuals

**Mercyhurst University**
Research Assistant; Advisor: Steve Mauro, Ph.D.
Elucidating factors leading to the induction of lytic phase in bacteriophage λ in *E. coli*
*Worked to elucidate factors influencing the shift from lysogenic to lytic cycle by employing PCR, spectroscopy, bioinformatics, and plating techniques

**Mercyhurst University**
Research Assistant; Advisor: John Campbell, Ph.D.
Factors influencing tumor growth in the Brown Bullhead of Presque Isle, Erie, PA
*Tested how different variables (light frequencies and intensities, water depth, and water temperature) affected the behavior of brown bullheads, *Ameiurus nebulosus*.

**TEACHING EXPERIENCE**

**Western Kentucky University**
Graduate Teaching Assistant: Cellular and Molecular Biology Lab (Bio 322).
*Taught two sections of the Cellular and Molecular Biology lab each semester;
*Prepared materials for each lab period (i.e. buffers, media plates, media, stock solutions, etc.);
*Updated course materials to match the objectives of the class

**Mercyhurst University**
Academic Tutor: Organismal Biology, Cell Biology, Ecology, Chemistry
*Clarified concepts for students in order to increase understanding;
*Developed study plans
PRACTICAL EXPERIENCE

<table>
<thead>
<tr>
<th>Experience</th>
<th>Location</th>
<th>Dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lost River Cave</td>
<td>Bowling Green, KY</td>
<td>2012</td>
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<tr>
<td>Tour Guide and Gift Shop Cashier</td>
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<tr>
<td>*Develop and lead interpretive tours incorporating the history, geology, and biology of the cave and river system.</td>
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</tbody>
</table>

| Mercyhurst University Biology Department | Erie, PA | 2010-2011 |
| Animal Caretaker                           |          |            |
| * Cleaned and maintained 1-180 gal marine tank and 1-180 gal freshwater, lake tank; |
| * Purchase supplies for maintenance of tanks and animals |

| Mercyhurst University Residence Life | Erie, PA | 2009-2011 |
| Resident Assistant                  |          |            |
| * Created a friendly, respectful and considerate environment among the residents; |
| * Met with residents expressing concern (roommate conflicts, personal issues, etc.); |
| * Completed duty rounds to promote the safety and security of the campus community |

| Mercyhurst University Forensics Department | Erie, PA | 2008-2010 |
| Student Assistant                       |          |            |
| * Processed human and animal remains; |
| * Assisted with Bomb Blast Recovery short course; |
| * Ensured sanitary conditions in laboratory for health and safety; |
| * Resident assistant for summer Forensic short courses |

PRESENTATIONS

GE Jones. 2013. “Understanding the effects of lunasin on normal development and tumor progression in Drosophila melanogaster.” Student Research Conference. Western Kentucky University. Bowling Green, KY.

GE Jones. 2013. “Understanding the effects of lunasin on normal development and tumor progression in Drosophila melanogaster.” Graduate Student Research Symposium. Western Kentucky University. Bowling Green, KY.

HONORS and AWARDS

Western KY University Outstanding Biology Graduate Student, 2013
Western KY University Graduate Student Research Grant, 2011-2012
Western KY University Graduate Assistantship, 2011-2013
Mercyhurst Dean’s List Honor Student, 2008-2011
Mercyhurst University Egan Scholarship, 2008-2011
Mercyhurst University Presidential Scholarship, 2008-2011
Springville Griffith Institute Language Scholarship, 2008-2009

ACTIVITIES and COMMUNITY SERVICE

<table>
<thead>
<tr>
<th>Activity</th>
<th>Location</th>
<th>Dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC - RAM Volunteer</td>
<td>Greenup, KY</td>
<td>June 2013</td>
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<tr>
<td>KY FIRST Lego Competition Volunteer</td>
<td>Bowling Green, KY</td>
<td>2011 &amp; 2012</td>
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<tr>
<td>Project AIMS assistant</td>
<td>Bowling Green, KY</td>
<td>April 2012</td>
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<tr>
<td>Human Dissection</td>
<td>Erie, PA</td>
<td>Fall-Winter 2010</td>
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<tr>
<td>PA Junior Academy of Science Judge</td>
<td>Erie, PA</td>
<td>2010</td>
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<tr>
<td>Emmaus Soup Kitchen Volunteer</td>
<td>Erie, PA</td>
<td>2009</td>
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<tr>
<td>Mercyhurst Forensics Club</td>
<td>Erie, PA</td>
<td>2008-2010</td>
</tr>
<tr>
<td>Mercyhurst Wind Ens. &amp; Pep Band</td>
<td>Erie, PA</td>
<td>2008-2010</td>
</tr>
<tr>
<td>NYS Envirothon participant</td>
<td>Springville, NY</td>
<td>2008</td>
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<tr>
<td>NYS Science Olympiad participant</td>
<td>Springville, NY</td>
<td>2006 &amp; 2007</td>
</tr>
<tr>
<td>Concord Veterinary Clinic Volunteer</td>
<td>Springville, NY</td>
<td>2004-2007</td>
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REFERENCES

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