Responses to ionizing radiation and translation inhibition in Drosophila melanogaster and human cancer cells

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RESPONSES TO IONIZING RADIATION AND TRANSLATION INHIBITION IN

DROSOPHILA MELANOGASTER AND HUMAN CANCER CELLS

by

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B.A., University of Pennsylvania, 2009

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This thesis entitled:

Responses to Ionizing Radiation and Translation Inhibition in *Drosophila melanogaster* and Human Cancer Cells

written by Stefanie Michaela Stickel
has been approved for the Department of Molecular, Cellular, and Developmental Biology

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Mark Winey, Committee Chair

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Date: ____________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Abstract

A common regimen for the treatment of solid tumors includes ionizing radiation (IR), chemotherapies, and targeted agents, such as kinase inhibitors. Cancer therapies have been traditionally tailored to the tissue origin of the tumor, rather than the mutation type; however, this concept has been changing with evidence that agents targeted to specific mutations are effective in many cancer types. The latter theory assumes that inhibition of the oncogenic mutation trumps differences between tissues, a concept that has never been proven in a whole organism. My results in the first half of this thesis show for the first time that isogenic mutations in separate tissues do in fact respond similarly to treatments in a Drosophila melanogaster tumor model. This indicates that mutation status takes precedence over tissue origin, suggesting that targeted therapies could be used against a specific mutation regardless of cancer type.

Our understanding of oncology has improved drastically over time; however, the identification of new effective agents is still important. Furthermore, understanding the molecular and cellular mechanisms by which these treatments act is essential to effectively combating cancer and identifying patient sub-populations that would benefit from the treatment. My results in the second half of this thesis suggest that bouvardin, a plant-derived molecule with anti-cancer activity, inhibits translation elongation by
locking elongation factor 2 (EF2) to the ribosome in human cells. We also found that bouvardin has the ability to enhance the effect of IR in head and neck cancer (HNC) and glioma cells, as well as, mouse xenografts of HNC. The effect of bouvardin and IR treatment on cell growth and proliferation, however, was different between HNC and glioma cells. In HNC cells, bouvardin and IR slows the growth of HNC cells, but does not affect the overall mitotic activity. In glioma cells, the combination treatment inhibits mitotic activity and induces senescence. These results suggest that inhibition of translation elongation is an effective modulator of IR in multiple cancer models.
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Chapter 1: Introduction

Protein synthesis and cancer

Regulation of protein synthesis is critical for cell growth, proliferation and survival and is essential in all forms of life (Pavitt and Ashe, 2008; Shahbazian et al., 2010). Translation is composed of three steps: initiation, elongation, and termination. Initiation is a highly complex process involving many eukaryotic initiation factors (eIFs) to fully assemble the 80S ribosome. *De novo* protein synthesis occurs mostly through ‘cap-dependent initiation’ (Braunstein et al., 2009; Svitkin et al., 2005; Trivigno et al., 2013), a form of translation initiation that requires the cap-binding initiation factor eIF4E to initiate protein synthesis. Under normal conditions, the rate-limiting step of translation is at the initiation step, where eIF4E controls whether translation initiation can progress (Gandin et al., 2008).

Canonical cap-dependent translation initiation begins with formation of the pre-initiation complex (PIC) and mRNP complex (Fig. 1-1) (Aitken and Lorsch, 2012; Fraser and Doudna, 2007; Trivigno et al., 2013). The PIC is composed of Met-tRNA$_i$-eIF2-GDP, eIF1, 1A, 3, and 5, and the small 40S ribosomal subunit. The mRNP complex is composed of the circularized mRNA, eIF4A, B, E, and G, as well as, the poly A-binding protein (PABP) (further details described in Fig. 1-1 legend). When the PIC and mRNP associate, this 43S-mRNA complex can then scan for the start (AUG) codon. Once the start codon is reached, the 60S subunit can join, forming the 80S ribosome complex ready for elongation.
Figure 1-1: Eukaryotic cap-dependent translation initiation

(1) Translation initiation begins with formation of the ternary complex, composed of eIF2-GTP and Met-tRNA. (2) The ternary complex then binds the 40S ribosome subunit, along with eIF1, eIF1A, eIF3, and eIF5. This complex is termed the pre-initiation complex (PIC). (3) Meanwhile, eIF4E, eIF4G, eIF4A, and eIF4B bind the 5’ UTR of the mRNA. eIF4G acts as a scaffold, while eIF4E binds the 5’ cap structure and the helicase eIF4A and eIF4B unwind the 5’ secondary mRNA structure. Circularization of the mRNA is accomplished when poly A-binding protein binds the 3’ UTR and eIF4G. This complex is now termed the mRNP. (4) Once (2) and (3) are completed, the association between the PIC and mRNP can occur through eIF3 (from the PIC) and eIF4G (from the mRNP) binding. This complex, termed the 43S-mRNA complex, then scans for the start (AUG) codon. (5) Once the start codon reached, eIF1 releases and eIF2-GTP is converted to its GDP-bound state with the aid of eIF5, leading to scanning arrest. eIF2-GDP and eIF5 then dissociate. (6) Lastly, hydrolysis of the GTPase eIF5B mediates the joining of the 60S subunit to form the 80S complex and eIF5B-GDP and eIF1A dissociate. (Image adapted from Fraser and Doudna, 2007 to include updated mechanistic information from Aitken and Lorsch, 2012; Trivigno et al., 2013)
A switch to non-canonical cap-independent initiation can occur under stress conditions. In contrast to cap-dependent translation, cap-independent translation does not require all of the same eIFs due to a structured internal ribosome entry site (IRES) located in the 5’ UTR of the mRNA that is capable of binding factors part of the canonical translation complex (Fig. 1-2) (Komar and Hatzoglou, 2011). Because the ribosome does not scan on IRES mRNAs, IRES trans-acting factors (ITAFs) help position the ribosome on the start codon. Additionally, it is important to note that IRES-mediated translation initiation does not utilize the cap-binding eIF4E initiation factor, the rate-limiting step of cap-dependent translation initiation. Inhibition of eIF4E has been shown to be involved in the switch from cap-dependent to cap-independent translation initiation, as described later in this section.

Eukaryotic cap-independent initiation has been described during physiological conditions (mitosis and differentiation); however, it is mostly studied in response to cellular stress (hypoxia, ionizing radiation, nutrient limitation, etc) (Komar and Hatzoglou, 2011). Protein synthesis is the most energy consuming process of the cell; therefore, translation regulation is tightly coupled to growth and stress stimuli (Braunstein et al., 2009; Buttgereit and Brand, 1995; Holcik and Sonenberg, 2005; Trivigno et al., 2013). The inhibition of global protein synthesis can save energy and shift resources to repair and survival. Around 10% of mRNAs contain an IRES (Holcik and Sonenberg, 2005; Trivigno et al., 2013); therefore, while global translation may be blocked, the translation of cap-independent mRNAs involved in survival can still occur.

While there are canonical and non-canonical methods of translation initiation, elongation occurs under only one known mechanism and is much less complex.
Figure 1-2: Cap-dependent versus cap-independent initiation

(A) In cap-dependent translation initiation, the cap-binding protein, eukaryotic initiation factor 4E (eIF4E), binds to the 5' cap (red) and the scaffolding protein, eIF4G. Poly A-binding protein (PABP) circularizes the mRNA by binding both the 3' UTR and eIF4G. (B) In cap-independent translation initiation, the internal ribosome entry site (IRES) binds IRES trans-acting factors (ITAFs) and proteolytic fragments of eIF4G or p97/DAP5/NAT1. PABP circularizes the mRNA by binding both the 3'UTR and proteolytic fragments of eIF4G or p97/DAP5/NAT1. In both forms of translation initiation, eIF4G binds eIF4A and eIF3, which is bound to the 40S ribosomal subunit. Only eIFs that are pertinent to this process are indicated and individual components of the translation machinery are not drawn to scale. (Image from (Holcik and Sonenberg, 2005))
Elongation begins when GTP-bound elongation factor 1 alpha (EF1a) brings an aminoacyl tRNA to the A site of the ribosome (Fig. 1-3) (Kapp and Lorsch, 2004). EF1a will then undergo GTP-hydrolysis to ensure the aminoacyl tRNA is properly deposited in the A site and then leave the ribosomes in its GDP bound state. The guanine nucleotide exchange factor (GEF) EF1b (not shown) helps to catalyze the exchange of GDP for GTP on EF1a so it may be used again. Next, the peptidyl transferase center of the large 60S ribosomal subunit catalyzes the transfer of the peptide on the peptidyl tRNA to the amino acid on the aminoacyl tRNA (now the peptidyl tRNA). This results in a shift where the acceptor sites of the deacylated tRNA and peptidyl tRNA are in the E- and P-sites, respectively, but the anticodon ends are in the P- and A-sites, respectively.

Elongation factor 2 (EF2) hydrolyzes a GTP to fully translocate the deacylated tRNA to the E-site (where it leaves the ribosome) and the peptidyl tRNA to the P-site. There is currently no known GEF of EF2. This process will continue cycling until a stop codon is reached and termination will occur with the help of release factors.

As mentioned above, protein synthesis is tightly coupled to growth and stress stimuli. The phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway controls most of the components involved in initiation of protein synthesis, including the rate-limiting factor eIF4E (Fig. 1-4) (Hay and Sonenberg, 2004). Briefly, in response to growth stimuli, PI3K becomes activated, which then subsequently activates Akt. Akt then inhibits tuberous sclerosis 1/2 (TSC1/2) complex, an inhibitor of Rheb. Rheb can then go on to activate mTOR, which regulates three components involved in translation initiation: eIF4E, eIF4G, and S6 kinase (S6K)
Figure 1-3: Eukaryotic translation elongation

(1) Elongation factor EF1A delivers the aminoacyl-tRNA into the A-site of the ribosome. (2) The 60S ribosome subunit then catalyzes the formation of the peptide bond, transferring the nascent peptide onto the A-site tRNA. (3) EF2 then translocates the peptidyl-tRNA into the P-site and the deacylated tRNA into the E-site, where it exits the ribosome. (Image adapted from (Schneider-Poetsch et al., 2010b))
Initiation of cap-dependent translation occurs through the PI3K/Akt signaling cascade. Insulin, hormones, and growth factors activate this pathway through PI3K and Akt. Akt can also receive inputs from PDK1 and the rictor/GβL/mTOR complex. Akt then inhibits TSC1/2, which inhibits Rheb, an activator of mTOR activity. mTOR can then increase translation activation through release of eIF4E from 4E-BP1 and phosphorylation of eIF4G and S6 kinase (S6K). Downregulation of cap-dependent translation initiation can also be accomplished through decreased energy signals and increased AMP/ATP ratios. These signals activate AMPK activity, which activates the TSC1/2 complex to inhibit mTOR activity through Rheb. (Image adapted from (Petroulakis et al., 2006))
(Hay and Sonenberg, 2004; Mamane et al., 2006; Petroulakis et al., 2006; Trivigno et al., 2013; Wang and Proud, 2006). eIF4E is regulated by the repressor, eIF4E-binding protein 1 (4E-BP1). In response to growth factors, nutrients, and energy, mTOR becomes activated and phosphorylates 4E-BP1. This phosphorylation inactivates 4E-BP1, releasing eIF4E to bind eIF4G and initiate cap-dependent initiation. mTOR has also been shown to stimulate the interaction between eIF4G (part of the mRNP complex) and eIF3 (part of the PIC) to enhance translation (Harris et al., 2006). Lastly, mTOR can phosphorylate S6K, which can then go on to phosphorylate eIF4B and S6 to stimulate translation. Therefore, regulation through mTOR can directly and indirectly activate or inhibit cap-dependent translation initiation. Recent studies have also indicated that the EF2 kinase (EF2K), which phosphorylates and inactivates EF2, is regulated by mTOR (Browne and Proud, 2004; Wang et al., 2001), indicating that mTOR also has the capability of regulating translation at the elongation step.

Because cancer cells have higher rates of proliferation, it suggests a requirement for higher rates of protein synthesis. In line with this, many cancers have been shown to have misregulation of factors involved in protein synthesis. Translation initiation and elongation factors are found to be overexpressed in many cancers, including tumors of the head and neck, breast, stomach, bladder, lung, prostate, colon, and kidney (Avdulov et al., 2004; De Benedetti and Graff, 2004; Joseph et al., 2004; Nakamura et al., 2009; Nathan et al., 1997; Scaggiante et al., 2012; Silvera et al., 2009; Tomlinson et al., 2005). In vitro experiments in cells have shown that increased expression of eIF3, eIF4G, or eIF4E leads cells to develop oncogenic properties and/or undergo cellular transformation (Ahlemann et al., 2006; Avdulov et al., 2004; Fukuchi-Shimogori et al.,
Furthermore, overexpression of eIF4E has even been shown to increase the translation of weakly translated mRNAs that contain a structured, G-C rich 5’ UTR, without altering global protein levels. These “weak” mRNAs encode proteins involved in growth, angiogenesis, and tumor invasion (De Benedetti and Graff, 2004; Graff et al., 2007; Mamane et al., 2004; Rosenwald et al., 1995), indicating that eIF4E overexpression has the ability to specifically increase the translation of factors involved in tumorigenesis.

Additionally, separate studies have shown that downregulation of both initiation and elongation factors eIF3, eIF4G, eIF4E, EF1a, and EF2 led to decreased oncogenic properties in various cancer cell lines (Anand, 2002; DeFatta et al., 2000; Mazan-Mamczarz et al., 2014; Nakamura et al., 2009; Savinainen et al., 2006). Suppression of eIF4E in a mouse xenograft model also showed tumor reduction without toxicity (Graff et al., 2007).

While there is a wealth of evidence that misregulation of translation and tumorigenesis may be coupled, there are very few molecules that directly target translation factors. The link between protein synthesis and cancer has only been recently uncovered; therefore, the identification or development of molecules that target translation may be in progress. The translation elongation inhibitor homoharringtonine (Synribo), however, was recently approved in 2012 for chronic myeloid leukemia (CML), potentially paving the way for other chemotherapies that target the protein synthesis.
**Ionizing radiation as a treatment for cancer**

The major effect of ionizing radiation (gamma rays, X-rays, alpha particles, beta particles) on cells is the creation of DNA double-strand breaks (DSB) (Belli et al., 2002; Jackson, 2002; Su, 2006). In response to DSBs, cells undergo cell cycle checkpoint arrest in order to allow for repair of DNA damage. From this point, cells can undergo two fates: 1) DNA damage is repaired and normal growth proceeds or 2) cells undergo permanent cell cycle arrest or apoptosis if the damage is irreparable (Belli et al., 2002; Su, 2006). More specifically, in response to DSBs, “sensors” of DSBs (DNA damage-binding factors) activate “transducers”, which amplify and diversify the DNA-damage signal by targeting various downstream “effectors” (Fig. 1-5). These “effectors” lead to the induction of cell cycle checkpoints, DNA repair, or permanent cell cycle arrest or apoptosis when repair is irreparable (Belli et al., 2002; Jackson, 2002; Su, 2006).

Ionizing radiation (IR) treatment of solid cancers has been utilized for purposes of regressing, preventing recurrence of, or palliative care of cancers (www.cancer.gov). However, a phenomenon termed “accelerated repopulation”, the measurable increase in the tumor growth rate after radiation therapy (Marcu, 2009), is a concern as it can lead to regeneration of a tumor after treatment. Accelerated repopulation was first documented in a transplantable mouse fibrosarcoma, where a dose of radiation increased the growth of the tumor compared to no treatment (Szczepanski and Trott, 1975). The single dose titration of IR in a transplantable rat rhabdomyosarcoma, showed the rate of repopulation was correlated with the dose of IR. Evidence of accelerated repopulation has also been demonstrated in head and neck, cervical, and
Ionizing radiation induces double-strand breaks (DSBs), which are recognized by “sensors” that scan chromatin for damage. When a DSB is recognized, these “sensors” signal to “transducers”, which amplify and diversify the signal to various “effectors”. These “effectors” lead to the induction of cell cycle checkpoints, DNA repair, or permanent cell cycle arrest or apoptosis when repair is irreparable. (Image from (Belli et al., 2002))
non-small cell lung cancers (Chen et al., 2011; Hall et al., 2011; Huang et al., 2012; Marcu, 2009) and been implicated in the failure of chemo- and radiation therapy combinations in patients (Kim and Tannock, 2005).

The typical scheduling regimen of IR treatment in cancer patients is generally daily on weekdays for 5-7 weeks in fractions of low doses (Kim and Tannock, 2005). Fractionated low doses of IR, rather than a single high dose, is designed to curb accelerated repopulation, give normal tissues time to recover, as well as, reoxygenate hypoxic cells, making them radiosensitive. Portions of the tumor are often hypoxic and approximately 3-fold less sensitive to IR treatment (Kim and Tannock, 2005; Marks and Dewhirst, 1991; Pires et al., 2012; Wachsberger et al., 2003). Although reoxygenating hypoxic cells makes them radiosensitive, it also allows quiescent tumor cells that were not growing to re-enter the cell cycle and proliferate. Therefore, retreatment helps curb the growth of these now actively proliferating cells. While the exact mechanisms of accelerated repopulation are not well understood, IR treatment can induce proliferation through epidermal growth factor receptor (EGFR) activation (Kim and Tannock, 2005).

A phenomenon similar to accelerated repopulation has been described in Drosophila melanogaster, however, it has been termed ‘compensatory proliferation’. The process of compensatory proliferation following ionizing radiation (IR) has been studied and well characterized in Drosophila melanogaster using the imaginal discs in the larvae. It was first discovered in wing imaginal discs where it was observed that while irradiation killed approximately 50% of the cells, proliferation of remaining cells yielded a normal wing (Haynie, 1977). Imaginal discs are a useful tissue for studying
compensatory proliferation as there is very little developmental apoptosis, yet the cells still respond to IR. Compensatory proliferation shows a requirement for factors involved in apoptosis (Dronc (caspase-9 like), JNK, p53), stress responses (mei-41 (ATR) and okra (Rad54)), and proliferation (Hh, Dpp, Wg) in imaginal eye-antennae and/or wing discs (Fan and Bergmann, 2008; Jaklevic and Su, 2004b; Martin et al., 2009; Perez-Garijo et al., 2009; Ryoo et al., 2004).

While a lot of work needs to still be accomplished in this field, the mechanisms of apoptosis-induced compensatory proliferation may give insight into how human tumors are able to undergo accelerated repopulation (Fan and Bergmann, 2008). Furthermore, the similarities between compensatory proliferation in Drosophila and accelerated repopulation in human tumors make this organism a great opportunity to screen molecules that may inhibit the regeneration process after IR treatment. Our lab has taken advantage of these similarities and performed screens to identify new molecules that enhance the effects of IR to potentially combat the regeneration process (Gladstone et al., 2012; Jaklevic et al., 2006). While the goal in radiation therapy is to decrease the time between treatments to prevent accelerated repopulation, the identification of cytostatic molecules that can inhibit this process would provide a therapeutic option to prevent tumor regeneration after IR treatment.

**Ionizing radiation and translation inhibition as a cancer therapy**

While there are currently no translation inhibitors approved for the treatment of cancers in combination with ionizing radiation (IR), many studies have suggested there is a connection between ionizing radiation and translation inhibition. As described
earlier, mTOR plays a strong role in regulating protein synthesis (Fig. 1-4). Further studies have established that this role can be extended to controlling translation in response to IR, as well. One study has shown that IR exposure inhibited cap-dependent translation through c-Abl, a protein that can bind and inhibit the activity of mTOR (Kumar et al., 2000). Further examination revealed that this was due to enhanced binding of the mTOR effector 4E-BP1 to the rate-limiting initiation factor eIF4E, inhibiting the ability of eIF4E to initiate cap-dependent initiation. Because c-Abl is activated in response to DNA damage during IR treatment, it was hypothesized that this may be a way for genotoxic stress to trigger inhibition of cap-dependent translation and induce cell cycle arrest.

Another study showed that 24 hours after treatment with IR and the translation inhibitor cycloheximide, factors involved in DNA repair and G2/M arrest were inhibited and those involved in apoptosis were increased (Braunstein et al., 2009). Further examination without cycloheximide treatment revealed that cap-dependent initiation was inhibited in the first 6 hours post-IR treatment; however, by 12 hours post-IR treatment, cap-dependent translation was restored. The mechanisms of this were due to increased protein, but not transcription levels of 4E-BP1, an inhibitor of eIF4E, early on. It was also shown that activation of 4E-BP1 after IR is p53- and ATM-dependent, indicating that the double-strand break (DSB) machinery after IR exposure may be controlling cap-dependent translation initiation inhibition. The authors hypothesized that in the early experiment with IR and cycloheximide treatment, translation of essential survival factors after IR were inhibited by cycloheximide and, therefore, lead to induction of apoptosis.
The concept of inhibiting survival factors with translation inhibition in response to IR is not a new theory. An older study found that when human lymphocytes were pre-irradiated with low doses of X-rays, cells become less sensitive to subsequent X-ray exposure (Youngblom et al., 1989). However, if cells are treated with cycloheximide 4-6 hours after initial dose, the adaptive response was inhibited. The authors also hypothesized that this was due to induction of repair enzymes after initial exposure.

Studies have also shown that p21-activated kinase 2 (Pak2, gamma-Pak) is activated by IR in mouse fibroblasts and human leukemia cells (Roig and Traugh, 1999). Expression of wild-type Pak2 in a rabbit reticulocyte lysate and cultured cells has been shown to inhibit translation, while kinase-inactive mutants have no effect. Because Pak2 has the ability to phosphorylate, bind, and inactivate eIF4G, it is hypothesized that translation inhibition is due to the decreased association between eIF4G and eIF4E required for cap-dependent translation (Ling et al., 2005). Reduced association of eIF4G with eIF4E after exposure to IR was observed in a separate study, as well (Paglin et al., 2005).

Overall, these studies indicate that after IR exposure, cap-dependent translation is largely inhibited due to inactivation of the mTOR pathway by various mechanisms, including signaling from DNA repair factors. This can lead to a switch from global cap-dependent translation to cap-independent translation of select mRNAs involved in cell survival and proliferation. Some of these factors selectively synthesized include proteins involved in proliferation, such as cyclin D1 and c-myc, angiogenesis, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), and inhibition of apoptosis, such as Bcl-2, and survivin (De Benedetti and Graff, 2004;
DeFatta et al., 2000; Graff et al., 2007). In the presence of protein synthesis inhibitors, the translation of these survival factors may be blocked, leading to cell death or growth inhibition. These mechanisms indicate that the combination of IR and translation inhibition may be a promising treatment of tumors by inhibiting survival mechanisms in response to IR.

**Bouvardin and its identification as a radiation modulator**

Combination therapies for the treatment of cancer are usually more effective than monotherapies as tumors often have mutations in multiple pathways that can lead to survival even if one pathway is blocked (Li et al., 2014). Therefore, even if one pathway is inhibited, a redundant one can often compensate to allow for cell survival. In order to identify enhancers of radiation, our lab performed a screen using a library of molecules from the National Cancer Institute Developmental Therapeutics Program (NCI-DTP) in *Drosophila melanogaster* (Gladstone et al., 2012). Briefly, 3rd instar larvae (Fly life cycle: Fig. 1-6) were irradiated and added to food containing molecules from the NCI-DTP library. Vials were incubated for 10 days and the percent eclosion (percent of adult flies that emerge from the pupal case) was measured (Fig. 1-7). Molecules that reduced the survival by two standard deviations from the mean of the population were marked as a “hit” and retested.

From this screen, we identified 16 molecules that fell into three categories according to their mode of action: 1) microtubule depolymerizing agents, 2) DNA antimetabolites (topoisomerase I inhibitors, nucleotide analogs, DNA damaging agents, etc.), and 3) protein synthesis inhibitors (Gladstone et al., 2012). Of particular
interest was the identification of multiple microtubule poisons and DNA anti-metabolites, as there are several FDA-approved chemotherapies that fall into these classes. Additionally, the topoisomerase inhibitor camptothecin and its analog topotecan identified in this screen. Topotecan is FDA-approved for the treatment of cervical, ovarian, and small cell lung cancers and has also been shown to synergize with radiation in human squamous cell carcinoma, melanoma, non-small cell lung cancer (NSCLC), and glioma cells (Boscia et al., 1993; Hennequin et al., 1994; Kohara et al., 2002; Lamond et al., 1996a; Lamond et al., 1996b, c; Marchesini et al., 1996). It has also been shown to synergize in xenografts of rhabdomyosarcoma (Chastagner et al., 2000). Identifying specific FDA-approved molecules, as well as others that fall into FDA-approved classes is a proof-of-principle that this screen has the ability to identify drugs that are effective against human cancers.

Three of the molecules discovered in this screen, streptovitacin A (NSC 39147), didemnin B (NSC 325319), and bouvardin (NSC 259968), were identified as protein synthesis inhibitors, specifically targeting translation elongation. Because there is evidence that misregulation of protein synthesis and tumorigenesis may be linked (see “Translation and Cancer”), we sought to determine if translation inhibition combined with IR was effective in human cancer cells. Of the three molecules, we chose to continue our studies with bouvardin as cycloheximide (the parent drug of streptovitacin) has problems with toxicity in humans (Emmanouil-Nikoloussi, 2007; Emmanouil-Nikoloussi et al., 2010) and didemnin B has already been extensively studied as an inhibitor of cancer cell growth in multiple tissue types (Crampton et al., 1984; Jouin et al., 1991; Lobo et al., 1997). Additionally, a derivative of didemnin B, Aplidin, is already in clinical
Figure 1-6: *Drosophila melanogaster* life cycle

The life of a fruit fly begins as an embryo in an egg, from which it will hatch as a 1\textsuperscript{st}-instar larva about 24 hours later. As the larva grows, it will molt twice producing the 2\textsuperscript{nd}- and 3\textsuperscript{rd}-instar larva. The 3\textsuperscript{rd}-instar larva then undergoes pupation, the process of forming a pupal case. Inside the pupal case, the pupa undergoes metamorphosis, from which it will eclose (emerge) as an adult fly. (Image acquired from http://www.zoology.ubc.ca/~bio463/lecture_13.htm)
Figure 1-7: Outline of drug screen to identify sensitizers of ionizing radiation (IR) in *Drosophila melanogaster*

First, 3\textsuperscript{rd} instar *Drosophila* larvae were exposed to ionizing radiation (IR; specifically, X-rays). Irradiated larvae were then added to food mixed with molecules from the NCI-DTP library. Vials were then incubated for 10 days before the percent eclosion (percent of adult flies that emerge from the pupal case) was measured. Percent eclosion was calculated as the number of filled pupal cases (dead pupae) over the total number of pupal cases (dead pupae and eclosed flies).
trials for the treatment of multiple myeloma (www.clinicaltrials.gov).

Bouvardin is a cyclic hexapeptide originally isolated from the plant *Bouvardia ternifolia* (Jolad et al., 1977) (Fig. 1-8). Previous studies in a rabbit reticulocyte lysate have suggested that bouvardin inhibits translocation of the elongating peptide during protein synthesis (Zalacain et al., 1982). Two separate studies also identified bouvardin as a selective inhibitor of both engineered breast cancer stem cells [(Sajithlal et al., 2010); published PTC application #WO 2011/130677], as well as, engineered tumor cells compared to isogenic primary human foreskin fibroblasts (Dolma et al., 2003). Our lab was also able to confirm that the effects of bouvardin and IR treatment in *Drosophila* translated to human cancer cell based assays. We showed that bouvardin was able to synergize (have a greater than additive effect) with ionizing radiation (IR) in a non-small cell lung cancer (NSCLC) cell line and with Taxol in two head and neck cancer (HNC) cell lines (Gladstone et al., 2012). We additionally reported that bouvardin and IR synergized in mouse xenografts of the NSCLC cell line.

While there is a basic understanding of the mechanism of bouvardin, a lot still remains to be determined. For example, while there is evidence that bouvardin targets translation elongation in a rabbit reticulocyte lysate, it is important to confirm this also occurs in human cells, as well as, identify which molecule(s) it is targeting. Furthermore, while our lab has previously shown that bouvardin and IR synergize in multiple cancer types (Gladstone et al., 2012), the cellular mechanisms that cause this synergy has never been examined. For example, is this combination inducing apoptosis or is there a cytostatic effect of the drug? Answers to these questions would
Figure 1-8: Molecular structure of bouvardin
Molecular structure of the cyclic hexapeptide, bouvardin. (Figure from (Dolma et al., 2003))
help elucidate as to how IR and inhibition of translation elongation target human cancer cells.
Chapter 2: Oncogenic mutations produce similar phenotypes in *Drosophila* tissues of diverse origins

**Introduction**

Traditionally, cancer therapies are tailored to the site of disease (ovary, lung, brain, etc.). For example, a typical chemotherapy regime for ovarian cancer is cis/carboplatin plus a taxane, whereas a typical chemotherapy regime for colon cancer is oxaliplatin plus 5-Fluorouracil (5-FU) (http://www.cancer.org). Development of therapies based on the disease site is also seen for targeted agents. For example, the EGFR inhibitor Erbitux is FDA-approved for colorectal and head and neck cancers, despite the fact that EGFR hyper-activation occurs in many cancer types. Recent advances in genome sequencing reveal common mutations in tumors from distinct tissue types. Consequently, an emerging interest in oncology is to not tailor the treatment to tissue origin, but to the oncogenic mutations present in the tumor. B-RAF inhibitors, for example, may be used on tumors with the cognate B-RAF mutation regardless of whether they are melanoma or colorectal cancer. Integral to such a practice is the idea that the same oncogenic mutation(s) produces similar outcomes in different organs; however, this hypothesis has never been tested experimentally. One could envision two extreme possibilities. Oncogenic mutations may exert their effect regardless of cell type and produce similar outcomes. Alternatively, underlying differences amongst tissues (e.g. epigenetic status, transcription program, or signaling pathways) could interact with the effects of oncogenic mutations in such a way to yield varying outcomes.
To compare the consequences of oncogenic mutations in different tissues, I utilized a well-characterized system to induce tissue-specific tumors in *Drosophila melanogaster* using oncogenic Ras (Brumby and Richardson, 2003; Dow et al., 2008; Humbert et al., 2008; Pagliarini and Xu, 2003). Activating mutations in the small GTPase Ras are found in 20–30% of human cancers, with *Ras*\textsuperscript{V12} being the most frequent allele. Oncogenic mutants of Ras have been expressed in mice before (reviewed in (Kamata and Pritchard, 2011)), but these experiments used different alleles and different isoforms (KRas, NRas or HRas), precluding a direct comparison.

In *Drosophila* larvae, ectopic expression of *Ras*\textsuperscript{V12} induces overgrowth (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). A screen for modifiers identified genes that normally control cellular apical–basal polarity, including *scribble*. *scrib* encodes a homolog of the human tumor suppressor hScrib (Dow et al., 2003; Humbert et al., 2008). *scrib* homozygous mutant clones in larval imaginal discs are normally eliminated (Brumby and Richardson, 2003); however, when *scrib* mutations are combined with *Ras*\textsuperscript{V12}, the resulting cells not only overgrew but also became invasive. In these studies, *eyeless* promoter-driven FLP recombinase (*eyFLP*) was used to target *Ras*\textsuperscript{V12}, a GFP marker, and loss of heterozygosity (LOH) in *scrib* by mitotic recombination in the developing eye-antennae discs and the optic lobes. The resulting overgrowth invades the adjacent ventral nerve cord. Due to their accelerated growth rate that is no longer coordinated with the developmental program, these tumors have been called ‘neoplastic’ (Brumby and Richardson, 2003). Neoplastic tumors prolong the larval state for up to 13 days after egg deposition (AED), instead of the normal 5 days and tumor-bearing larvae die without forming pupae. When transplanted into the abdomen of wild-
type adult females hosts, neoplasms remained not only proliferative but also invasive, spreading into the intestines and ovaries (Pagliarini and Xu, 2003).

To study neoplasms induced by the \( Ras^{V12}/scrib^1 \) combination in different tissues, I took advantage of observations made in earlier studies that in addition to neoplasms in the eye-antennae disc and the optic lobe of the brain (collectively referred to as cephalic neoplasms), some \( eyFLP \) drivers produced additional GFP-positive growths (Pagliarini and Xu, 2003). I identified a mesoderm-derived cell population in the gonad as progenitors of secondary neoplasms and compared the characteristics of cephalic and gonadal tumors (Stickel and Su, 2014). These results support my hypothesis that oncogenic mutations can exert similar effects in diverse organs.

**Results**

**Over-growth in the gonad is sex-limited**

I generated larvae with GFP-positive cephalic and gonadal tumors using the \( eyFLP1 \) line (Fig. 2-1 A-B). Quantification of tumor incidence over time showed that gonadal neoplasms were at apparent d7 (day 7) after egg deposition (AED) and plateaued in ~40% of larvae (Fig. 2-1 C). Because gonadal neoplasms occurred in approximately half of the larvae, I investigated whether this was a sex-specific phenomenon.

*Drosophila* gonad development begins when germ cells and somatic gonadal precursors (SGPs) coalesce in the embryo (Casper and Van Doren, 2006). By late 3rd larval instar (the final larval stage), sexual dimorphism in gonads is clear (Brown and King, 1961); male gonads are larger and have a more advanced program of
Figure 2-1: Gonad tumors occur in about forty percent of *Drosophila* larvae

(A,B) GFP-positive growth appears in the cephalic region of all larvae and in the gonads of some larvae (arrowhead in panel B). Larvae with both uni- and bi-lateral gonadal neoplasms were observed. (C) Quantification of larvae with gonadal GFP. 25 to 149 larvae were removed and counted per day. The average of two independent experiments is shown.
gametogenesis. Images taken by Dr. Tin Tin Su verified that female gonads of d7 and older larvae were in fact smaller and appeared homogenous by DNA stain (Fig. 2-2 A-B). In the male gonads, we observed sperm heads with elongated nuclei (Fig. 2-2 C-C’) and male-specific differentiated cells (Fig. 2-2 D-D’). 3rd instar larvae normally do not display sperm heads; however, prolonged larval life in the presence of neoplastic tumors allowed the gonads to mature and helped us differentiate between male and female gonads unequivocally. Cell types present in male gonads alone were also revealed with DNA stain. These include: hub cells/germline stem cells (GSCs) at the anterior pole, somatic gonadal precursors (SGPs) interspersed with germ cells in the middle, and closely-packed cells of the ‘terminal body’ (TB) at the posterior pole (Fig. 2-2 D-D’) (Casper and Van Doren, 2006; Renault, 2012). The identity of the hub/GSCs, SGPs and TB were confirmed by staining for Eya and Fas3 proteins. Eya staining marks the TB (Fig. 2-3), whereas Fas3 staining marks the hub/GSCs (Fig. 2-4). Using these criteria, I found that all GFP-positive gonads examined were male (n>200), while all female gonads examined were GFP-negative (n>80). I conclude that gonadal overgrowth due to RasV12/scrib1 mutations is sex-limited, occurring only in males.

**Gonadal overgrowth begins in the terminal body and spreads to the anterior**

The simplest explanation for male-specific gonadal overgrowth is that gonadal eyFLP1 expression occurs in a cell type(s) present only in male gonads. To test this hypothesis, I examined which gonadal cell type(s) eyFLP1 is active in. In whole live larvae, there were signs of initiation at the posterior pole as a crescent of GFP facing the anterior (Fig. 2-5 A). In gonads from eyFLP1 only controls (without
Figure 2-2: Male and female gonads are sexually dimorphic

(A,B) Female gonads are smaller than male gonads. The male gonad shown has a GFP-positive neoplasm. (C,C’) Male gonads show signs of spermatogenesis (arrowheads in C,C’) and have male-specific cell types: hub cells/GSC at the anterior pole (D’) and terminal body (TB) at the posterior pole (D). TB cells are more closely packed than hub cells/GSC and can be distinguished by DNA stain. Polyploid fat cells that surround the gonads are also visible (arrowhead in panel G). (D,D’) are two focal planes of the same gonad. Scale bar: 50 μm (A,B,C,D,D’), 20 μm (C’). (Images taken by Tin Tin Su)
Figure 2-3: Closely packed terminal body (TB) cells confirmed with Eya staining

Gonads were dissected from male wandering 3rd instar y1w1118 larvae, fixed and stained with antibodies against Eya (B,D,F) and for DNA (A,C,E). (B) Somatic Gonadal Precursors (SGPs), including a subset that forms the terminal body (yellow bracket), stain for Eya (B, magnified in D). (E) Hub cells and germline stem cells (GSCs) at the anterior pole do not stain for Eya (E,F). Scale bar: 50 μm (B, also applies to A), 5.5 μm (C, also applies to D–F). (Images taken by Tin Tin Su)
Figure 2-4: Loosely packed hub/GSC cells confirmed with Fas3 staining

Gonads were dissected from male wandering 3rd instar y1w1118 larvae, fixed and stained with antibodies against Fas3 (B) and for DNA (A). (B) Hub cells stain strongly for Fas3 (B, arrow). Scale bar: 50 μm (A, also applies to B). (Images taken by Tin Tin Su)
Figure 2-5: Ras\textsuperscript{V12}/scrib\textsuperscript{1} neoplasms originate in the posterior end of male gonads

Larvae were (A) imaged live for GFP or (B-F) dissected, fixed and stained with an antibody to Eya and/or DNA dye before imaging (as indicated). (A) A GFP crescent in the posterior end of a larval gonad. (B) A male gonad from a control (non-tumor-bearing) larva expressing eyFLP without UAS-Ras or scrib\textsuperscript{1} mutation; arrowhead points to densely-packed cells of the TB, some of which show GFP. (C–F) show the magnified posterior region of a similar gonad. TB cells are stained with an antibody to Eya in panels D and F. GFP-positive Ras\textsuperscript{V12}/scrib\textsuperscript{1} cells appear interspersed with Eya-positive cells, indicating a site of origin. Scale bar: 50 \( \mu \text{m} \) (B), 10 \( \mu \text{m} \) (C, also applies to D-F). (Control and Eya staining images taken by Tin Tin Su)
Ras$^{V12/scrib^1}$, GFP-positive cells appeared at the posterior pole where the TB is located (Fig. 2-5 B). Co-staining for Eya confirmed that GFP-positive cells were indeed a subset of TB cells (Fig. 2-5 C-F). The TB is derived from posterior SGPs, termed male-specific somatic gonadal precursors (ms-SGPs), which are of mesoderm origin; equivalent cells in females are eliminated by apoptosis during embryogenesis (Casper and Van Doren, 2006; Renault, 2012). I conclude that eyFLP1 is active in a cell type present in male gonads but not female gonads, explaining the observed sex-limited overgrowth.

UAS-GFP appeared in only a subset of TB cells in eyFLP1 only controls. This was expected because only cells that lost GAL80 through mitotic recombination would be capable of doing so (see Materials and Methods). Likewise, GFP was expressed only in a subset of cells in the eye-antennae disc and the optic lobes in the same larva (Fig. 2-6). At the points examined, Tin Tin observed GFP-positive cells only in the neuroectoderm and male gonads using this driver. If eyFLP1 was active among cells at the posterior pole, I would expect overgrowth to initiate at the same site. Initiation of overgrowth at the posterior and spread to the anterior was also evident in gonads dissected from larvae at d7 and d10 AED (Fig. 2-7). GFP-positive cells in these gonads expanded beyond the normal location of the TB (Fig. 2-7 A versus Fig. 2-2 D), and engulfed the gonad in a posterior-to-anterior fashion (Fig. 2-7 A versus B).

Ras$^{V12/scrib^1}$ cells in the optic lobe show actin-based cytoplasmic extensions with a proposed role in invasion (Pagliarini and Xu, 2003). These extensions were detected in GFP images (Fig. 2-8 A,B, arrowhead). Cytoplasmic extensions have also been observed in human cells expressing Ras$^{V12}$ and dominant negative scrib$^{KD}$ grown in
**Figure 2-6: Cephalic tissues that express GFP from eyFLP1**

Cephalic tissues that express GFP from eyFLP1. Tissues shown were dissected from a 3rd instar larva from a control eyFLP1 only cross (see Materials and Methods), fixed, and stained for DNA. Bilateral expression of GFP is seen in eye antennae discs and the optic lobes, but not the ventral nerve cord. Note the patchy expression of GFP as expected from clonal induction through mitotic recombination. Scale bar: 100 μm.

(Image taken by Tin Tin Su)
Figure 2-7: \( \text{Ras}^{\text{V}12}/\text{scrib}^{1} \) gonad neoplasms originate in the posterior end and spread anteriorly

(A,B) Gonads from tumor-bearing larvae at d7 and d10 AED. The growth of GFP-positive cells is concentrated at the posterior end in both gonads, but anterior advancement is greater in the latter. Scale bar: 50 \( \mu \text{m} \). (Images taken by Tin Tin Su)
Figure 2-8: Cephalic and gonad neoplasms display cytoplasmic extensions

(A-D) Cytoplasmic extensions in cephalic and gonad neoplasms. (A) GFP-positive cells originating in the optic lobe (OL) invade the ventral nerve cord (VNC). The boxed area is magnified in panel B. Arrowheads indicate cytoplasmic extensions. (C) GFP-positive cells in the gonad also display cytoplasmic extensions. The boxed area is magnified in panel D. We counted over 20 cells in greater than 5 cephalic and 5 gonad neoplasms. Virtually all GFP-positive neoplastic cells neighboring wild-type tissue displayed this phenotype. Scale bar: 50 μm (A), 20 μm (C), 10 μm (B, D). (Images taken by Tin Tin Su)
Matrigel (Dow et al., 2008). Tin Tin observed similar extensions in GFP-positive cells of the gonad (Fig. 2-8 C,D, arrowhead).

Previous authors noted gonadal GFP in some but not all eyFLP lines (Pagliarini and Xu, 2003). This makes it unlikely that gonadal GFP were metastases from cephalic tumors, which would be expected in all eyFLP lines that produced cephalic tumors. In wild-type gonads, the TB remains a dense pack of cells at the posterior pole during larval stages (e.g. Fig. 2-2 D). In contrast, GFP-positive over-growth exceeded what was seen for the TB in wild-type gonads and appeared to be unrelated to the normal developmental program. Therefore, I refer to gonadal overgrowth also as ‘neoplasia’. Because TB cells are of mesodermal origin and eye discs/optic lobes are of neuroectodermal origin, neoplasia in these larvae occurred in cells that originated from different germ layers and were differentiating into different fates. The incidence of neoplastic tumors in different cell types in different organs in the same animal offers a unique opportunity to compare their cell biological characteristics. I examined characteristics that are relevant to oncology: cell cycle checkpoints, radiation-induced apoptosis, aneuploidy and response to the chemotherapeutic drug Taxol.

**Cephalic and gonad neoplasms maintain intact DNA-damage and spindle checkpoints**

In order to examine the DNA damage checkpoint, I exposed larvae to 4000 R of X-rays and assayed for mitotic activity one hour later. Irradiated cells with an intact DNA damage checkpoint will halt at G2/M, resulting in a decreased mitotic index compared to unirradiated controls. 4000 R is typically used in *Drosophila* larvae
because it is the LD50 for wild-type strains. I used the same dose to facilitate comparison to other studies. Without irradiation, I measured similar mitotic indices in control (non-tumor) eye-antennae imaginal discs and cephalic neoplasms as identified by the expression of the GFP marker (Fig. 2-9 A,C,I). This suggests that neoplastic transformation of cephalic tissues did not speed up the cell cycle. Rather, it may be the prolonged period of proliferation (i.e. the failure to exit the cell cycle and differentiate) that resulted in the overgrowth of cephalic tumors. In contrast, cells of the terminal body of the gonad in control larvae show little or no mitotic activity (Fig. 2-9 B,I). Upon neoplastic transformation, however, mitotic activity in these cells increased to reach the level found in cephalic tumors (Fig. 2-9 D,I). Thus, in the case of TB cells, neoplastic transformation did increase the rate of proliferation. At one hour after irradiation, I saw significant reduction in the mitotic indices of control cephalic tissues (eye-antennae discs), cephalic tumors and gonadal tumors (Fig. 2-9 E, G-I). I conclude that the DNA damage checkpoint that arrest cells in G2/M is active in cephalic and gonadal tumors alike.

In order to examine the spindle assembly checkpoint, tissues were incubated with the microtubule depolymerizer colchicine for 2 hours. Without microtubules, cells with an intact spindle checkpoint will accumulate in mitosis, increasing the mitotic index. Mitotic indices in GFP-positive cephalic and gonad neoplasms showed a similar increase of about 3-fold compared to controls without colchicine (Fig. 2-10 C,D,G-I). This is in agreement with the increase in mitotic activity of about 2-fold I saw in control brains and gonads (Fig. 2-10 A,B,E,F,I). I note that basal mitotic indices here (without colchicine) differed from those in radiation experiments (without IR), possibly because
Figure 2-9: Cephalic and gonad neoplasms maintain an intact DNA damage checkpoint

(A–H) Larvae were irradiated with 4000 R of X-rays 1 hour before dissection, fixed, and stained with an antibody to phospho-histone H3 (pH3, red) and for DNA (blue). (A,B,E,F) from control cephalic and gonad tissues. (C,D,G,H) from cephalic and gonad neoplasms. Insets in panels A–H show pH3 only images. (I) Mitotic indices were computed as the number of pH3-positive cells over the total in panels such as those in A–H. d7 and d10 indicate days AED. -IR = 0 R; +IR = 4000 R of X-rays. Error represented as standard error of the mean. Statistical significance between +/-IR pairs was computed using unpaired 2-tailed t-test. *P<0.05; **P<0.001. Scale bar: 10 µm (A, also applies to B–H).
Figure 2-10: Cephalic and gonad neoplasms maintain an intact mitotic spindle checkpoint

(A–H) Larvae were dissected, incubated in colchicine for 2 hours, fixed, and stained with an antibody to phosphohistone H3 (pH3, red) and for DNA (blue). (A,B,E,F) from cephalic and gonad control tissues. (C,D,G,H) from cephalic and gonad neoplasms. Insets in panels A–H show pH3 only images. (I) Mitotic indices were computed as the number of pH3-positive cells over the total in panels such as those in A–H. -C = PBS only; +C = 125 mM colchicine. Error represented as standard error of the mean. Statistical significance between -/+C pairs was computed using unpaired 2-tailed t-test. *P<0.05. Scale bar: 10 µm (A, also applies to B–H).
the samples had been incubating in medium for 2 hours before fixing, whereas they were fixed immediately after dissection in radiation experiments. I conclude that the spindle assembly checkpoint that arrests cells in mitosis is intact in cephalic and gonad neoplasms.

**Cephalic and gonad neoplasms are resistant to IR-induced apoptosis**

Apoptosis is a major response to ionizing radiation (IR) and accounts in part for the therapeutic effect of radiation therapy in cancer. To examine whether cephalic and gonad neoplasms can undergo IR-induced apoptosis, I irradiated the larvae with 4000 R of X-rays, fixed, and stained the tissues for cleaved (active) caspase-3. Wing imaginal discs from tumor-bearing larvae served as a control in these experiments and showed very little activated caspase-3 staining without irradiation (Fig. 2-11 A). At 4 hours after irradiation, wing imaginal discs showed increased activated caspase-3 staining as expected (Fig. 2-11 D). This increase was confirmed by *in vitro* caspase-3 activity assays (Fig. 2-11 G). Without irradiation, GFP-positive cephalic neoplasms showed elevated basal levels of activated caspase-3 staining (Fig. 2-11 B) and caspase-3 activity in *in vitro* activity assays (Fig. 2-11 G). Interestingly, after irradiation there was no significant increase in caspase-3 staining (Fig. 2-11 E) or activity *in vitro* (Fig. 2-11 G) in cephalic neoplasms. I conclude that cephalic neoplasms are defective for IR-induced apoptosis. The gonad neoplasms showed no constitutive activated caspase-3 staining and no increase in this signal after IR (Fig. 2-11 C,F). The neoplastic tissue in the gonads was of lower abundance and harder to isolate cleanly; therefore, I was unable to obtain sufficient material for *in vitro* caspase-3 activity assays. I conclude that
Figure 2-11: Cephalic and gonad neoplasms show resistance to IR-induced apoptosis

(A–F) Larvae were dissected 4 hours after irradiation with 4000 R of X-rays, fixed, and stained with an antibody to activated caspase-3 (C3, red) and for DNA (blue). (A,D) from wild-type imaginal wing discs in larvae with cephalic neoplasms. (B,C,E,F) from cephalic and gonad neoplasms. We examined between 5 and 10 cephalic and gonad neoplasms with the same trend of high and low basal apoptosis, respectively. Insets in panels A–F show caspase-3 only images. (G) Activated caspase-3 levels were measured in control tissues and cephalic neoplasms using an in vitro caspase-3 activity assay. Units are in fluorescence and normalized to the protein levels in each lysate. −IR = 0 R; +IR = 4000 R of X-rays. Error represented as standard error of the mean. Scale bar: 10 μm (A, also applies to B–F).
even though cephalic neoplasms appear to have a high basal rate of apoptosis and gonad neoplasms have virtually none before treatment, neither responded to IR.

**Cephalic and gonad neoplasms have increased aneuploidy**

Using mitotic chromosome spreads (Fig. 2-12 A,B), I detected a low but significant level of aneuploidy, a hallmark of human cancers, in both cephalic and gonad neoplasms (Fig. 2-12 C). All instances of aneuploidy involved chromosome loss, not gain. Sex chromosomes were most frequently affected; 92% of aneuploid cells in cephalic tumors and 85% of aneuploid cells in gonad tumors had a single X without an accompanying X or Y. Wild-type brains, in contrast, have an undetectable level of aneuploidy in these assays (Fig. 2-12 C; (Stumpff et al., 2004)). This indicates the cephalic and gonad neoplasms are both susceptible to aneuploidy.

**Cephalic and gonad neoplasms are resistant to IR and bouvardin, but sensitive to Taxol.**

*Ras*<sup>V12</sup>*scrib<sup>1</sup>* larvae never make it to pupae or adult flies. Rather, they die as large, bloated larvae. I wanted to examine if larvae bearing these tumors responded to IR, bouvardin, or Taxol treatment by monitoring increased larval survival, progression to pupation, or reduction of cephalic and gonad neoplasms. I chose these treatments because IR and Taxol are standard cancer therapies and bouvardin, a protein synthesis inhibitor, was identified as a molecule that sensitized wild-type and mutant larvae to IR (Gladstone et al., 2012). I utilized a protocol similar to that which was used to
Figure 2-12: Cephalic and gonad neoplasms exhibit increased aneuploidy
(A,B) Examples of chromosomes from gonads stained for DNA. Four pairs of Drosophila chromosomes are indicated in panel A. Panel B is missing a sex chromosome. (C) Aneuploid mitoses are expressed as percent of total. The difference between cephalic and gonad neoplasms is not significant (P<0.12, 2-tailed Fisher’s exact test). Scale bar: 10 μm (A, also applies to B). (Quantification performed by Tin Tin Su)
successfully to identify small molecules with therapeutic potential in this tumor model (Willoughby et al., 2013).

First I examined the ability for IR to increase the lifespan of larvae. I treated d4 larvae and examined increased survival at d14. I chose a d14 timepoint because the survival of Ras$^{V12/\text{scrib}^1}$ larvae without treatment at d14 is less than 15% (Fig. 2-1 C). I titrated radiation in larvae using a range of doses used in Drosophila (Fig. 2-13 A). My results indicate that IR does not have the ability to increase survival of tumor-bearing larvae. This effect was reproduced three more times with 2000R and 4000R doses (Fig. 2-13 B). I did notice increased survival of d14 control larvae compared to d12 larvae in Fig. 2-1 C. I attribute this to treatment with DMSO in these experiments, which was done as a control for potential future IR-drug combination treatments. This observation suggests that DMSO has the ability to slightly increase larval survival. I also observed differences in the percent survival for control larvae in each experiment (Fig. 2-13 A-B). This may be due to differences in food quality as the cornmeal can dry out over long incubation periods. Regardless, the trend I see between experiments is consistent.

Because bouvardin has been shown to sensitize WT and mutant strains ((Gladstone et al., 2012); data not shown), I wanted to examine if it could increase the survival of larvae with Ras$^{V12/\text{scrib}^1}$ neoplasms. Following a similar protocol as with radiation, d4 larvae were added to cornmeal with bouvardin. After examining the vials at d14, it appeared that the tumors were not reduced and the percent survival was the same with and without treatment. Therefore, I decided to leave the vials to incubate and see if treated larvae were able to pupate. Interestingly, I observed that
Figure 2-13: Ionizing radiation does not increase the survival of $Ras^{V12/scrib}$ larvae

$d4$ $Ras^{V12/scrib}$ larvae were exposed to different doses of IR and incubated in cornmeal with DMSO for 10 days. (A) The percent survival was measured at d14, which indicated that IR treatment had no effect on percent survival. This was reproduced three more times with 2000 R and 4000 R in panel B.
DMSO (control) and bouvardin treatment was able to induce pupariation in a small percentage of larvae and even allow for the eclosion of one fly in the 30 uM bouvardin treatment (Fig. 2-14 A). However, when this was repeated with higher concentrations of bouvardin, I found that DMSO (control) treatment alone also allowed for the survival of some larvae to adulthood. The survival-enhancing effects I see with DMSO alone are consistent with the increased percent survival I see in Fig. 2-13, as described above. These results indicate that IR and bouvardin alone were unable to reduce tumor growth and/or increase survival.

Lastly, I wanted to examine if the chemotherapeutic agent Taxol was able to reduce growth of cephalic and gonad neoplasms. Tin Tin administered Taxol to d4 larvae and examined the larvae daily up to d10. Taxol-treated larvae were similar in size to untreated controls, suggesting that concentrations used here did not prevent growth generally. Unlike with IR or bouvardin, Tin Tin found that Taxol was able to reduce GFP in the cephalic region (Fig. 2-15 A; see figure legend for quantification). Treated animals also appeared healthier and more active; of all larvae that were briefly chilled to immobilize and lined up for imaging in Fig. 2-15 A, untreated larvae remained flaccid whereas treated larvae recovered mobility and were crawling away by the time the image was acquired. Treated larvae, however, were unable to be rescued to the pupa or adult stages.

Taxol also reduced or eliminated gonadal GFP (Fig. 2-15 A, arrowheads, Fig. 2-15 B; see figure legend for quantifications). In this protocol, Taxol was added before gonadal GFP appeared (Fig. 2-1 C). Therefore, Taxol may have been able to prevent new growth or growth at early stages in the gonad. Cephalic GFP was already visible at
Figure 2-14: Bouvardin does not increase the survival of $Ras^{V12/scrib^1}$ larvae
d4 $Ras^{V12/scrib^1}$ larvae were exposed to different doses of bouvardin and
incubated in cornmeal until flies eclosed (~ 2 weeks). (A) The number of dead larvae, dead pupae, and eclosed flies were measured at different doses of bouvardin and the percent of total was calculated. This was reproduced with higher concentrations in panel B. Bouvardin treatment does not appear to increase the survival of larvae to adulthood.
Figure 2-15: Cephalic and gonad neoplasms are reduced after treatment to Taxol

(A) d9 larvae were imaged live for GFP. Arrowheads indicate GFP-positive gonads. Quantification of the GFP fluorescence using Image J software showed that Taxol treatment reduced the mean cephalic GFP from 1266±12 (mean fluorescence signal arbitrary units, -Taxol) to 816±8 (+Taxol). The difference was significant (P<0.01, 2-tailed Student’s t-test). Similarly, Taxol treatment reduced the mean gonadal GFP from 2467 (-Taxol) to 863 (+Taxol). The difference was also significant (P<0.05, 2-tailed Student’s t-test).

(C) Western blots of extracts from (C) anterior or (D) posterior halves of Taxol-treated larvae compared to control vehicle-treated larvae as in panel A. (C) Tubulin signal is the same in -/+ Taxol lane, but GFP is reduced in the +Taxol lane. (D) The reduction of gonadal GFP by Taxol was significant (P<10^{-5}, Fisher’s exact test, 2-tail).

(C,D) Western blots of extracts from (C) anterior or (D) posterior halves of Taxol-treated larvae compared to control vehicle-treated larvae as in panel A. (C) Tubulin signal is the same in -/+ Taxol lane, but GFP is reduced in the +Taxol lane. Two different exposures are shown. In panel D, GFP signal is much weaker than the tubulin signal, therefore we loaded ‘larval equivalents’ (see Materials and Methods) and used the tubulin signal to confirm equal loading. For example, 2.5 = extracts from 2.5 larvae; 1.3 = extracts from 1.3 larvae. Molecular weight markers are shown along the side. Similar data were obtained in two independent experiments. (Tin Tin Su)
d4, which may have allowed Taxol to reduce but not completely eliminate the tumors. Tin Tin then quantified the reduction in tumor growth by examining the GFP (tumor) signal by Western blot in the anterior and posterior halves of the larvae found in Fig. 2-15 A. She found that Taxol treatment decreased the GFP signal in both cephalic and gonad neoplasms (Fig. 2-15 C,D). Therefore, we conclude that Taxol as the ability to reduce tumor growth of both cephalic and gonad neoplasms.

**Discussion**

In conclusion, I found that neoplasms induced by Ras\(^{V12/scrib^1}\) mutations in tissues of neural ectodermal and mesodermal origins behaved similarly in every manner examined: cellular morphology, cell cycle checkpoints, radiation-induced apoptosis, aneuploidy and response to Taxol. As stated above, oncogenic mutations in different tissues could produce one of two outcomes. Oncogenic mutations may exert their effect regardless of cell type differences. Alternatively, underlying differences among tissues could interact with oncogenic mutations to produce different outcomes. My results indicate that both cephalic and gonad neoplasms responded similarly in every way tested.

First, I found that both cephalic and gonad neoplasms displayed cytoplasmic extensions. This invasive phenotype has also been found in human cells transformed with Ras\(^{V12}\) and knockdown of hScrib (Dow et al., 2008). Next, I found that cephalic and gonad neoplasms were both able to maintain intact DNA damage and spindle checkpoints when treated with the IR and colchicine, respectively. These results are not surprising as a previous study has shown that human cells with oncogenic Ras have
normal or even enhanced spindle and mitotic checkpoints (Luo et al., 2009). When I examined the response of cephalic and gonad neoplasms to IR, I found that both lacked the ability to undergo IR-induced apoptosis. I believe this is due to the nature of oncogenic Ras$^{\text{V12}}$. The proapoptotic protein Hid is a homolog of mammalian SMAC/DIABLO proteins and plays an essential role in IR-induced apoptosis in Drosophila. Reduction of hid gene dosage by half is enough to prevent IR-induced apoptosis (Brodsky et al., 2004). Because Hid has been shown to be inhibited by Ras (Bergmann et al., 1998), I speculate that oncogenic Ras$^{\text{V12}}$ is inhibiting Hid and preventing IR-induced apoptosis.

I also observed that both cephalic and gonad neoplasms have increased levels of aneuploidy. Constitutively active Ras can induce aneuploidy and chromosomal instability in cultured cells within as few as one cell cycle (Denko et al., 1994; Guerra et al., 2003; Woo and Poon, 2004). The mechanism for Ras-induced aneuploidy remains elusive, but my results indicate that this activity of oncogenic Ras is conserved in Drosophila. Lastly, while IR and bouvardin treatments neither increased survival of larvae, nor reduced neoplastic growth, Taxol treatment significantly reduced both cephalic and gonad neoplasms. A previous study showed that colon cancer cells expressing oncogenic KRas were hypersensitive to Taxol compared to isogenic wild-type cells (Luo et al., 2009). My results indicate that this characteristic of Ras-driven cancer is also conserved in Drosophila.

I conclude that, at least for Ras$^{\text{V12}}$/scrib$^1$ and the tissues examined here, oncogenic effects appear to override tissue-specific differences to produce neoplasms with similar morphology, mitotic indices, resistance to IR-induced apoptosis, aneuploidy,
and sensitivity to a chemotherapeutic agent. Furthermore, these responses are identical to what is seen in human cells with oncogenic Ras, further substantiating the use of this model to study human tumors in Drosophila.

Materials & Methods

Drosophila

All stocks used here have been described before (Pagliarini and Xu, 2003): eyFLP1; Act>y⁺>Gal4, UAS-GFP; P[FRT82B], Tub-GAL80 virgin females were crossed to w; UAS-Ras⁶¹²; P[FRT82B], scrib¹/TM6B males to generate tumors and to P{ry[+t7.2]=neoFRT}82B ry[506] males to generate controls that express GFP in the gonad (Fig. 2-5 B). y¹w¹¹¹⁸ served as wild type. Embryos were collected on Nutri-fly (Bloomington Formula), and cultured at 25°C.

Staining

Larvae were dissected in PBS and fixed in 10% formaldehyde in PBT (PBS + 0.2% Tween-20) for 10 minutes at room temperature (RT; for pH 3) or in 4% paraformaldehyde in PBTx (PBS + 0.1% Triton X-100) for 30 minutes at RT (for Caspase-3, Eya, and Fas3). For antibody staining, samples were blocked in 3% normal goat serum (NGS) in PBT (for pH 3) or 5% NGS in PBTx (for Caspase-3, Eya, and Fas3) for at least 1 hour before incubation with primary antibodies: rabbit anti-pH 3 Ser10 (1:1000, Upstate Biotech), rabbit anti-Caspase-3 (1:100, Cell Signaling cat. no. 9661 lot 32), mouse monoclonal anti-Eya and anti-Fas3 (1:25, Developmental Hybridoma Bank), in block for at least 1 hour at RT. Secondary antibodies anti-rabbit rhodamine, anti-mouse FITC or anti-mouse Rhodamine Red-X (Jackson) diluted 1:500
in block. Samples were stained with 10 µg/mL Hoechst33258 (Sigma) in PBT or PBTx and mounted in Fluoromount G (Southern Biotech).

**in vitro Caspase-3 assay**

Caspase-3 activity was measured using the Caspase-3/CPP32 Fluorometric Assay Kit (BioVision) according to the manufacturer's protocol. Tissues were dissected in chilled PBS and stored at −80°C. Fluorescent readings were adjusted based on the lysate protein concentration, which was measured using a DC Protein Assay (Bio-Rad).

**Chromosome squashes**

Chromosome squashes were performed as described before (Pimpinelli et al., 2010), with a 1.5 hour incubation in saline (0.7% w/v NaCl in water) containing 1.5 mM colchicine (Sigma) to trap mitotic cells. The samples were stained with Hoechst33258 as described.

**Imaging**

Whole larvae were imaged for GFP using a Nikon SMZ 1500 stereomicroscope. Fixed tissues were imaged using a Leica DMR compound fluorescence microscope. All images were acquired at room temperature. Objective lenses used were 5×air/NA 0.15, 10×air/NA0.30, 20×air/NA0.50, 40×air/NA0.74 and 100×oil/NA1.30. Images were collected using a SensiCam CCD camera and Slidebook software (Intelligent Imaging). Slidebook images were exported as TIFF documents, processed in Photoshop (Adobe) and assembled in Illustrator.

**IR and bouvardin treatment**

3 µl DMSO or bouvardin was added to 3 mL of cornmeal (IR) or Nutri-fly (bouvardin) food. About 50 d4 larvae were added per vial and were incubated or irradiated and then
incubated at 25 °C for the indicated number of days. Larvae were removed from food and the percent survival was measured. Larvae were marked as alive if they were breathing (tracheal movement) and still possessed strong GFP-positive tumors.

**Taxol treatment**

200 µl of water containing 2 µl of DMSO (control) or 2 µl of 100 mM Taxol (Paclitaxel, Sigma) in DMSO was added per culture vial, and gently mixed into the top layer of food containing larvae using a pipet tip. Each vial contained approximately 5 ml of food. Lower Taxol concentrations administered in this manner did not change tumor size.

**Western blotting**

Larvae were bisected to separate the head and gonad tumors and flash frozen in liquid nitrogen. Larvae were homogenized in PBS, an equal volume of 2× SDS loading buffer was added to the extract, and boiled to denature proteins. The extracts were separated on 10% polyacrylamide gels and Western blotted using standard protocols. Primary antibodies were 1:500 rabbit anti-GFP (Life Technologies cat. no. A11122) and 1:100 mouse anti-tubulin (Developmental Hybridoma Bank) in block (PBT with 0.2% Tween-20, 5% milk). HRP-conjugated secondary antibodies (Amersham) were used at 1:2500 in block. Western blots were developed using ECL (Thermo Scientific). From the known larval equivalent in the final sample (e.g. 1 larva/4 µl), various amounts of the sample were loaded to give the larval equivalents shown in the figures (e.g. loading 2 µl would give 0.5 larva equivalent).
Chapter 3: Bouvardin inhibits translation elongation and enhances the effect of ionizing radiation in human cancer cells

Introduction

Protein synthesis is required for growth, yet it remains underutilized as a drug target in diseases of unregulated growth, such as cancer. In recent years, the concept of inhibiting the ribosome to target cancers has become more appealing as the evidence that tumors utilize protein synthesis for oncogenesis has increased. For example, translation initiation and elongation factors are found to be overexpressed in many cancers, including tumors of the head and neck, breast, stomach, bladder, lung, prostate, colon, and kidney (Avdulov et al., 2004; De Benedetti and Graff, 2004; Joseph et al., 2004; Nakamura et al., 2009; Nathan et al., 1997; Scaggiante et al., 2012; Silvera et al., 2009; Tomlinson et al., 2005). Additionally, a number of initiation and elongation factors have been shown to transform normal cells when overexpressed (Ahlemann et al., 2006; Avdulov et al., 2004; Fukuchi-Shimogori et al., 1997; Lazaris-Karatzas et al., 1990; Savinainen et al., 2006; Zhang et al., 2007). Conversely, downregulation of translation factors has been shown to inhibit cancer cell growth (Anand, 2002; DeFatta et al., 2000; Graff et al., 2007; Mazan-Mamczarz et al., 2014; Nakamura et al., 2009; Savinainen et al., 2006).

Despite evidence that inhibition of translation may be a promising therapeutic in oncology, drugs that target translation at the ribosome directly remain limited. Denileukin diftitox, under the brand name Ontak, is a fusion between Interleukin-2 and diptheria toxin (DT) and was approved in 1999 for the treatment of Cutaneous T-Cell Lymphoma (CTCL). DT covalently modifies elongation factor 2 (EF2) to inhibit protein
The translation elongation inhibitor homoharringtonine, under the brand name Synribo, was approved in 2012 for chronic myelogenous leukemia (CML). Identification of new translation inhibitors that can target cancer cells would increase therapeutic options.

Our lab identified the protein synthesis inhibitor bouvardin in a screen for molecules that enhanced the effects of ionizing radiation (IR) in *Drosophila melanogaster* (Gladstone et al., 2012). This screen takes advantage of similarities in radiation biology of tumors and *Drosophila* larvae. Both systems are capable of regeneration through ‘accelerated repopulation’ in which surviving cells after radiation treatment proliferate even faster than before (Hall, 2004; Jaklevic and Su, 2004a). The screen allows for the identification of small molecules that inhibit this or any other process that confers radioresistance. Independently, bouvardin was also identified in a screen for selective inhibitors of engineered breast cancer stem cells [(Sajithlal et al., 2010); published PCT application #WO 2011/130677)]. Given the proposed role of cancer stem cells in regeneration after therapy, these data support the idea that bouvardin can interfere with the regrowth of tumors following standard therapy. In agreement, our subsequent studies found that bouvardin synergizes with ionizing radiation (IR) and Taxol in several human cancer cell lines (Gladstone et al., 2012).

Here, I addressed the mechanism of action of bouvardin and investigated whether bouvardin can enhance the effect of radiation in models of human cancers for which radiation is a major therapeutic option. My data suggest that bouvardin blocks protein synthesis by disrupting the dissociation of EF2 from the 80S ribosome, thereby disrupting translation elongation. I also found that bouvardin is efficacious in targeting
head and neck cancer (HNC) and glioma cells \textit{in vitro}, as well as, in a mouse xenograft model of HNC. Interestingly, although bouvardin enhanced the effect of radiation in cell-based assays, the data suggests different cellular mechanisms are responsible for the enhancement in HNC and glioma cells.

\textbf{Results}

\textbf{Bouvardin inhibits translation in human cells}

Previous studies on bouvardin found that it has the ability to inhibit eukaryotic, but not prokaryotic, protein synthesis (Basrur et al., 1986; Tobey et al., 1978; Zalacain et al., 1982). Using a mammalian \textit{in vitro} translation system, I confirmed that bouvardin and the known translation inhibitor cycloheximide were able to inhibit protein synthesis in a rabbit reticulocyte lysate (RRL) (Fig. 3-1; (Gladstone et al., 2012)). A follow-up experiment allowed me to determine that bouvardin inhibited translation \textit{in vitro} with an IC50 of about 10 nM within 15 minutes of drug addition (Fig. 3-2). While inhibition of translation has been performed previously in a RRL, I wanted to test for the first time if bouvardin can inhibit protein synthesis in a human cell lysate. Using an \textit{in vitro} HeLa lysate kit similar to that with RRL, I found that bouvardin also has the ability to inhibit translation of a GFP mRNA with an IC50 of approximately 500 nM within 5 hours of drug addition (Fig. 3-3).

While the inhibition of translation by bouvardin in a human cell lysate has provided the first piece of evidence that bouvardin can target translation in human cells, I wanted to confirm that bouvardin also causes translation inhibition in live cells. In the Detroit 562 (Det562) head and neck cancer (HNC) cell line, I monitored new protein
Figure 3-1: Cycloheximide and bouvardin inhibit translation *in vitro* in a rabbit reticulocyte lysate (RRL)

(A) Cycloheximide and (B) bouvardin inhibit *in vitro* translation in rabbit reticulocyte lysate (RRL) in the nanomolar range. Inhibition of protein synthesis was monitored using a luciferase mRNA and reading luciferase activity output in relative light units (RLUs). Percent inhibition of luciferase activity was calculated by dividing the RLUs from each concentration over control RLUs. Error represented as standard error of the mean. Bottom panel A and B show Western blots probing with anti-luciferase antibodies. Top band ran at approximately 61 kDa, the molecular weight of luciferase. CTRL = RRL minus luciferase mRNA.
Figure 3-2: Bouvardin inhibits *in vitro* translation with an IC50 of ~10 nM

Bouvardin inhibits translation *in vitro* in a rabbit reticulocyte lysate with an IC50 of 2.356 nM. IC50 calculated using Prism software. Error bar = 1 STD.
Figure 3-3: Bouvardin inhibits translation *in vitro* in a human cell lysate

Bouvardin inhibits translation *in vitro* in a HeLa cell lysate in the high nanomolar range. Protein synthesis inhibition was monitored using a GFP mRNA and reading the fluorescence of translated GFP protein. Error represented as standard error of the mean.
synthesis in live cells by quantifying the rate of incorporation of an amino acid analog, which was detected using a method based on Click chemistry (Fig. 3-4). I found that bouvardin blocked new protein synthesis in these cells in a dose dependent manner, with an IC50 of approximately 100 nM within 2.5 hours after drug addition. I observed a considerable discrepancy in IC50 values between the rabbit reticulocyte lysate, HeLa cell lysate, and Det562 cells. This could be due to translation optimization of the RRL kit assay, differences in the ribosome concentrations between experimental systems, and/or species-specific sensitivity to bouvardin. Regardless, these data indicate, for the first time, that bouvardin inhibits translation in human cells.

Previous studies using yeast and rabbit ribosomes have indicated that bouvardin inhibits the elongation step of translation (Zalacain et al., 1982). To investigate whether this was also the case for human ribosomes, I performed polysome profiling in HeLa cells, which is the cell line commonly used for this assay (Fig. 3-5 A). Cells were treated with DMSO (control) or drug for 30 min, harvested, and fractionated on sucrose density gradients. I monitored 40S (small) and 60S (large) ribosomal subunits, the 80S ribosome (elongating complex), and polysomes (multiple 80S ribosomes per mRNA). The identity of each peak in the polysome profile was confirmed by assaying for 18S (40S ribosomal subunit) and 28S (60S ribosomal subunit) rRNA in each fraction (Fig. 3-5 B).

Inhibition of elongation typically increases the relative abundance of 80S ribosomes as these become unable to proceed through the elongation cycle. Cycloheximide, a known inhibitor of translation elongation, increased the 80S peak but
Figure 3-4: Bouvardin inhibits translation in live human cells

(A) Bouvardin inhibits translation in a dose-response manner in live Det562 cells. Briefly, DMSO control (CTRL), 50 uM cycloheximide (CHX), or bouvardin (BVD) was incubated with L-azidohomoalanine (L-AHA) for 2.5 hours with cells before harvesting. Newly synthesized proteins were monitored by running purified cellular lysates on a 10% acrylamide gel and visualizing with a 300 nm excitation source. Coomassie stain shows bulk protein translation was not affected. Quantification of the Click-iT assay was performed by measuring the mean grey value of each lane in Image J. The signal compared to control is listed below each lane.
Figure 3-5: Bouvardin inhibits translation elongation in live human HeLa cells

(A) DMSO (control), 100 ug/mL cycloheximide, or 10 uM bouvardin was added to HeLa cells and incubated for 30 minutes. Cells were harvested and fractionated on a 10-60% sucrose gradient. The absorbance at 254 nm was monitored. Accumulation of an 80S ribosomal peak indicates a block in translation elongation. (B) rRNA was extracted from each fraction and analyzed on a 1% agarose gel to confirm 40S, 60S, and 80S ribosome peaks in the corresponding polysome profile fractions.
did not change the polysomes levels (Fig. 3-5 A, CHX). These results are expected based on the literature. I found that bouvardin (BVD) also increased the 80S peak without altering the polysome fraction (Fig. 3-5 A, BVD). These data indicate that bouvardin inhibits translation elongation on human ribosomes.

To address the mechanism by which bouvardin blocks translation elongation, I analyzed the 80S fractions for elongation factor 1 alpha (EF1a) and elongation factor 2 (EF2) by Western blotting (Fig. 3-6 A). After normalizing for ribosome levels using RPL13a, I found that bouvardin dramatically increased the level of EF2 but not EF1a in the 80S fractions compared to DMSO controls (CTRL) (Fig. 3-6 B). This data suggests that bouvardin locked EF2 on the ribosome. EF2 must dissociate and re-associate with the 80S ribosome to add each amino acid to the polypeptide chain. Therefore, stabilization of an EF2-80S complex by bouvardin could explain how the latter can block translation elongation. This idea that bouvardin stabilized EF2-80S complexes is consistent with the finding that bouvardin did not change the level of polysomes, for example, by dissociating them. The accumulation of EF2 was not seen in extracts from cycloheximide-treated cells, which is consistent with the literature (Nolan et al., 1975). This suggests that bouvardin and cycloheximide block elongation by different mechanisms.

**Bouvardin inhibits the clonogenic growth of HNC and glioma cells**

Previous studies by the National Cancer Institute (NCI) examined the ability for bouvardin to inhibit the growth of NCI-60 human cancer cell lines.
Figure 3-6: Bouvardin likely locks elongation factor 2 (EF2) to the ribosome to inhibit translation elongation

(A) The 80S peak fraction from Figure 3-5 was analyzed by Western blotting against eukaryotic elongation factor 1a (eEF1a), eukaryotic elongation factor 2 (eEF2), and ribosomal protein L13a (RPL13a; protein from the 60S subunit). (B) The mean gray value for the bands was quantified in ImageJ, normalized to RPL13a, and shown in the box compared to control treatments. Bouvardin increased the amount of EF2 that co-fractionated with the 80S complex. Similar data was obtained in two independent polysome profiling experiments.
It was found to be particularly efficacious on central nervous system (CNS) cancers and melanoma cells (Fig. 3-7). Most of the CNS cell lines were glioma lines and because radiation is a standard therapy for gliomas, we chose to ask if bouvardin can enhance the effect of IR in this cell type. The NCI-60 collection did not include HNC cell lines; therefore, I chose to examine the effect of bouvardin on HNC cells as IR is also a standard therapy for HNCs. Dr. Nathan Gomes, a post-doc in the Su lab, found that bouvardin had GI50s in the nM range on both cancer cell types (Table 3-1). Of significance is the finding that sensitivity to bouvardin is seen in HPV-negative HNC cells (both HNC cell lines in Table 3-1) and high O-6-methylguanine-DNA methyltransferase (MGMT)-expressing glioma cell lines (SNB-19 and SF-295; unpublished data). HPV-negative HNC has a poor prognosis and is refractory to radiation therapy (www.cancer.gov). Gliomas with high levels of MGMT, a repair enzyme that reverses alkylation damage to DNA, are resistant to temozolomide (TMZ), a lead DNA damaging chemotherapy for gliomas (Kitange et al., 2009). The efficacy of bouvardin in such cell lines bodes well for its potential as a viable therapeutic option.

Our lab identified bouvardin in a screen for small molecule inhibitors of tissue regeneration after ionizing radiation (IR) damage in *Drosophila* (Gladstone et al., 2012). We predicted that such molecules would enhance the effect of IR in human cancer cells, as well. Consistent, we found that bouvardin synergizes with IR in a non-small cell lung cancer (NSCLC) line, H157, and enhances the effect of IR on H157 xenografts in mice (Gladstone et al., 2012). Radiation is not the therapy of choice for NSCLC, but is for HNC and glioma. To determine whether bouvardin could enhance the effect of IR on these cancer types, I assessed the effect of bouvardin and IR using clonogenic
Figure 3-7: NCI-60 DTP Human Tumor Cell Line Screen Mean Graph

NCI-60 panel obtained from: http://dtp.nci.nih.gov/. Briefly, the sensitivity and resistance to bouvardin was tested in 60 cell lines from 9 cancer cell types. The vertical line represents the mean GI50 of all cell lines in the panel to bouvardin. Positive values (red) project to the right of the vertical line and represent cellular sensitivities to bouvardin that exceed the mean. Negative values (blue) project to the left and represent cell line sensitivities to bouvardin that were less than the mean. The positive and negative values, called deltas, are generated from the GI50 data by a three-step calculation. The GI50 value for each cell line tested against bouvardin is converted to its log10 GI50 value. These log10 GI50 values are then averaged. Each log10 GI50 value is subtracted from the average to create the delta. Thus, a bar projecting 3 units to the right denotes that the GI50 for that cell line occurs at a concentration 1000 times less than the average concentration required for all the cell lines used in the experiment.
Table 3-1: Bouvardin inhibits the viability of various head and neck cancer (HNC) and glioma cell lines

Growth Inhibition 50% (GI50) values of head and neck cancer (HNC) and central nervous system (CNA) cell lines treated with bouvardin (nM) for 5-days. MGMT levels were determined by Western blot. - = undetectable, + = low, ++ = medium, +++ = high, ND = not performed. HPV status received from the literature. (Nathan Gomes)

<table>
<thead>
<tr>
<th>Bvd GI50 (nM)</th>
<th>U87MG</th>
<th>U251</th>
<th>SF-268</th>
<th>SNB-19</th>
<th>SNB-78</th>
<th>SF-295</th>
<th>T96G</th>
<th>FaDu</th>
<th>Det562</th>
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<td></td>
<td>2.9</td>
<td>3.3</td>
<td>4.7</td>
<td>6.6</td>
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<td>159.7</td>
<td>515.7</td>
<td>4.6</td>
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</tr>
<tr>
<td>MGMT status</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>N.D.</td>
</tr>
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</table>

Table 3-1: Bouvardin inhibits the viability of various head and neck cancer (HNC) and glioma cell lines

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assays, a common assay used in radiation biology. I used two HNC cell lines, Det562 and FaDu (Fig. 3-8), as well as, two glioma cell lines, SNB-19 and U-251 (Fig. 3-9). I used doses of bouvardin and IR that produced partial effects in each cell line so I may readily detect additive or greater than additive effects of the combination. These doses were determined by performing titrations of bouvardin and IR in each cell line (data not shown).

In all four lines, bouvardin enhanced the effect of IR (Fig. 3-8, -9). In Det562 and FaDu HNC cells, bouvardin and IR treatment had a greater than additive effect, meaning the combination exceeded the simple sum expected if the treatments were additive. For example, on Det562 cells, 6 nM Bouvardin reduced the signal to 0.64 ± 0.04% of controls and 6 Gy of IR reduced the signal to 0.84 ± 0.04% of controls (Fig. 3-8 A). For an additive effect, I would expect the combination to reduce the signal to 0.53 ± 0.042%. Instead, the combination reduced the signal to 0.30 ± 0.06. In SNB-19 and U-251 glioma cells, the combined effect of bouvardin and IR was additive, meaning the reduction in clonogenic growth in the combination was about equal to the sum of the single treatments.

**Bouvardin and IR prolong the doubling time of Det562 HNC cells**

I next wanted to examine whether the growth inhibitory effects I saw in the clonogenics assays were due to cytotoxic or cytostatic effects. To address the first possibility, I asked if bouvardin and IR could induce apoptosis in Det562 HNC cells. Cells were treated with bouvardin, IR or the combination at doses that gave ~25%
Figure 3-8: Bouvardin and irradiation inhibits the growth of HNC cells in clonogenic assays

Clonogenic assays of HNC cell lines treated with bouvardin and ionizing radiation (IR). (A) Det562 HNC cells were seeded at a single cell density. The following day, cells were treated with vehicle (DMSO), bouvardin, IR, or the combination. After 24 hours, drug was removed and cells were allowed to grow for 9 days. Colonies were fixed and stained with sulforhodamine B (SRB). After imaging, SRB was solubilized and quantified using a plate reader. (B) Clonogenic assays of FaDu HNC cells performed as for Det562 cells with the indicated bouvardin and IR doses.

Representative images are shown. The signal expected if bouvardin and IR acted in an additive manner is shown as a horizontal line above each combination treatment. SRB quantification is of three biological replicates each of which consisted of at least three technical replicates. Data is normalized to the untreated controls. Error represented as standard error of the mean. (FaDu assay performed by Nathan Gomes)
Figure 3-9: Bouvardin and irradiation inhibits the growth of glioma cells in clonogenic assays

Clonogenic assays of (A) SNB-19 and (B) U-251 glioma cells treated with bouvardin and ionizing radiation. Assays were performed as for Det562 HNC cells in Fig. 3-8. Representative images are shown. The signal expected if bouvardin and IR acted in an additive manner is shown as a horizontal line above each combination treatment. SRB quantification is of three biological replicates each of which consisted of at least three technical replicates. Data is normalized to the untreated controls. Error represented as standard error of the mean. (Nathan Gomes)
fraction affected and stained 48 hours later for cleaved-caspase 3, a marker for apoptosis (Fig. 3-10). In cell lines that undergo apoptosis in response to radiation or drug, apoptosis is detectable by this time point (e.g. Fig. 3-12 shows induction of apoptosis at 48 hours in colorectal cancer cells treated with 5-Fluorouracil (5-FU)). I found that bouvardin and IR as single agents induced low levels of apoptosis and that the combination further increased the level of apoptosis. However, even at the highest levels seen, no more than 5% of cells were apoptotic. I reason that this low level of apoptosis cannot account for the level of reduced clonogenicity I observed (Fig. 3-8 A).

To identify other mechanisms that contribute to reduced clonogenicity, I addressed the possibility that the bouvardin and IR were having a cytostatic effect on Det562 HNC cells. Cells were treated with bouvardin, IR or the combination and the cell number was quantified at 24 and 72 hours after treatment (Fig. 3-11). During this 48 hour period, control cells grew about 3.5-fold, translating to a doubling time of 26.6 hours. During the same time period, bouvardin treated cells grew 1.8-fold, translating to a doubling time of 56.6 hours. In irradiated samples, cell numbers were lower than in the control at the 24 hour timepoint, presumably due to cell cycle arrest by IR. Between 24 and 72 hour timepoints, irradiated cells increased by 5 fold, translating to a doubling time of 20.7 hours. The faster proliferation of irradiated cells compared to the controls during this timepoint may reflect accelerated repopulation. Lastly, combination treatment resulted in a 2-fold increase in cell number, translating into a doubling time of 48 hours. Because the starting cell numbers in the combination treatment were as low as in the irradiated sample, and the doubling times were longer than in irradiated samples, the final cell counts were the lowest for the combination treatment.
Figure 3-10: Bouvardin and IR did not significantly increase apoptosis in Det562 HNC cells

(A-D) Det562 HNC cells were seeded in 12-well plates (20,000 cells) and treated 24 hours later with DMSO (control), bouvardin (BVD, 3 nM), IR (5 Gy) or both. At 48 hours after treatment, cells were fixed and stained for an antibody against cleaved-caspase 3 (red) and DNA (blue). (E) Percent apoptosis was calculated by counting the number of caspase-3-positive cells within the first 300 cells encountered per slide. Error bar = 1 STD.
Figure 3-11: Bouvardin and IR treatment increases the doubling time of Det562 HNC cells

(A) Det562 HNC cells were seeded in 12-well plates (20,000 cells) and treated 24 hours later with control, bouvardin (BVD; 3 nM), IR (5 Gy) or both. The number of cells in three representative clones were counted and averaged between two slides 24 and 72 hours after treatment. Error represented as standard error of the mean.
The cell numbers in combination-treated samples were about 30% of controls at 72 hours post treatment (Figure 3-11). This difference is similar to the difference in the final clonogenic growth quantified 9 days after treatment (Figure 3-8 A). These data suggest that once initial differences were established at 72 hours, subsequent proliferation ensued at similar rates in control and treated samples for the next 6 days. Staining for phosphorylated histone H3 (pH3), a marker for mitosis, supports this idea. I performed pH3 staining 1, 3, and 10 days after treatment to address if bouvardin and IR have short- or long-term effects on the mitotic activity of Det562 cells. Not surprisingly, I found the mitotic indices were similar among the four treatment groups at all timepoints examined (Fig. 3-12,-13). I did notice a decrease in mitotic activity after IR alone treatment at 24 hours (Fig. 3-12); however, this is consistent with cell cycle arrest after IR exposure. Interestingly, the combination treatment of IR and bouvardin at 24 hours does not arrest cells in mitosis. I hypothesize that bouvardin may be inhibiting the translation of essential factors involved in cell cycle arrest after IR treatment.

**Bouvardin and IR induces senescence in SNB-19 glioma cells**

As in the case of Det562 HNC cells, Nathan observed very little signs of apoptosis in SNB-19 glioma cells treated with bouvardin, IR or the combination (Fig. 3-14). However, unlike in Det562 HNC cells, bouvardin and IR treatment altered the mitotic activity of SNB-19 glioma cells 9 days after treatment (Fig. 3-15). Nathan observed that the mitotic indices were slightly reduced by treatments with bouvardin or IR; however, for the combination he observed a differential effect depending on the colony size. For large colonies, which composed of about 25% of the total, the mitotic
Figure 3-12: Bouvardin and IR treatment does not alter the mitotic activity Det562 cells 24 or 72 hours after treatment

Det562 cells were plated as in 12-well plates (20,000 cells/well) and treated 24 hours later with control (DMSO), bouvardin (BVD, 3 nM), IR (5 Gy) or both. Cells were then incubated for 24 or 72 hours, fixed, and stained for an antibody against phospho histone H3 (pH3, red) and for DNA (blue). The percent of cells in mitosis was calculated from three representative clones and averaged between two slides. Error represented as standard error of the mean.
Figure 3-13: Bouvardin and IR treatment does not alter the mitotic activity of Det562 HNC cells in clonogenic format

(A-D) Det562 HNC cells were plated as in 6-well plates (300 cells/well) and treated 24 hours later with control (DMSO), bouvardin (BVD, 6 nM), IR (6 Gy) or both. Cells were replaced with fresh media 24 hours later, incubated for 9 days, and then fixed and stained for an antibody against phospho histone H3 (pH3, red) and for DNA (blue). (F) Quantification reveals that the mitotic activity of Det562 cells is unaffected by bouvardin and IR treatment. Error is represented as standard error of the mean.
**Figure 3-14: Bouvardin and IR do not induce apoptosis in SNB-19 glioma cells**

HCT116 and SNB-19 cells were plated as in clonogenic experiments and treated with 5-Fluorouracil (5-FU; 375 uM), bouvardin (BVD; 5 nM), and/or IR (8 Gy) 24 hours after plating. Cells were harvested at 48 hours after treatment and processed for Western blot analysis using an antibody against PARP. HCT116 cells treated with 5-FU served as a positive control and showed robust PARP cleavage 48 hours after drug treatment. (Nathan Gomes)
Figure 3-15: Bouvardin and IR treatment decreases the mitotic activity of SNB-19 glioma cells

(A-D) SNB-19 cells were plated in 6-well plates (300 cells) and treated 24 hours later with control (DMSO), bouvardin (BVD, 3 nM), IR (2 Gy) or both. Cells were replaced with fresh media 24 hours later, incubated for 9 days, and then fixed and stained for an antibody against phospho histone H3 (pH3, red) and DNA (blue). (E) Quantification reveals that the mitotic activity of SNB-19 cells is decreased with bouvardin and IR treatment. Error is represented as standard error of the mean. (Nathan Gomes)
index was further reduced by the combination treatment. However, for small colonies, which composed of about 75% of the total, the mitotic index was almost completely absent. This indicates that while a portion of the population has reduced mitotic activity, the majority of colonies are not undergoing mitosis. I do not know the reason as to why a small population of these cells are less affected; however, it could be due to resistance to the treatment(s) at certain stages of the cell cycle.

To account for this observation, Nathan addressed the possibility that the small colonies of SNB-19 cells treated with the combination of bouvardin and IR have entered senescence. Staining for senescence-associated β-galactosidase revealed no change in the number of β-galactosidase-positive cells after single treatments with bouvardin or IR compared to control untreated cells (Fig. 3-16 A). However, for the combination he again observed a differential effect depending on colony size. For the large colonies, he observed a very small number of β-galactosidase-positive cells, similar to the control and single treatments. Converesely, for the small colonies almost 80% of the cells were β-galactosidase-positive, suggesting many cells in this group were in a senescence-like state. Det562 HNC cells showed no signs of increased β-galactosidase staining after single or combination treatments (Fig. 3-16 B), which is consistent with continued mitotic activity (Figure 3-13). I conclude that even though the combination of bouvardin and IR significantly reduced the clonogenic growth of Det562 and SNB-19 cells, the underlying mechanisms were different.
**Figure 3-16: Bouvardin and IR treatment induces senescence in a subset of SNB-19 cells**

(A) SNB-19 cells were plated as in 6-well plates (300 cells) and treated 24 hours later with control (DMSO), bouvardin (BVD, 3 nM), IR (2 Gy) or both. Cells were replaced with fresh media 24 hours later, incubated for 9 days, and then stained with the senescence marker, β-galactosidase (blue). (B) Det562 cells were seeded, treated (BVD = 6 nM; IR = 6 Gy), and stained as in (A). (SNB-19 and imaging performed by Nathan Gomes)
Bouvardin and IR inhibit tumor growth in mouse xenografts

To investigate whether growth inhibition by bouvardin observed in cells was conserved in vivo, our collaborator Dr. Barbara Frederick examined the effect of bouvardin on xenografts in mice. Det562 HNC cells were grown in the flank of nude mice. Once the tumor volume reached approximately 200 mm$^3$, mice were randomized into treatment groups and treated with vehicle, 2 Gy of IR, bouvardin at two different concentrations, or the combinations (Fig. 3-17 A; arrows indicate days irradiated, bouvardin was administered 24 hours prior to IR). Radiation was directed to the tumor alone by using a shield to protect the mouse from whole-body radiation. In the first lag of the experiment, mice were subjected to 4 doses of treatment over a two-week period ending at day 20 after implantation. The dose and schedule of IR (4 fractions of 2 Gy each) represent clinically relevant conditions.

Barbara found that bouvardin or IR alone showed partial control of tumor growth at the doses used. The combination of bouvardin with IR further decreased the single agent effect and enhanced radiation in mouse xenografts, which is similar to what I observed in Det562 and SNB-19 clonogenic assays. Similar results were observed in a second independent xenograft experiment.

After the conclusion of the first lag of treatment, the tumors resumed growth. Soon after, tumors in mice treated with vehicle, bouvardin or IR alone were large enough to require the sacrifice of the animals. Tumors in mice treated with the combination also resumed growth, but did so more slowly (between days 20 and 30 for the bottom two lines in Fig. 3-17 A). Measurement of tumor growth delay supports this conclusion (Fig. 3-17 B).
Figure 3-17: Bouvardin and IR reduce tumor growth of Det562 HNC mouse xenografts

(A) Tumor volumes were plotted against treatment days. Arrows indicate days on which mice were irradiated. Drug was administered 24 hours prior to IR. Bouvardin was administered intraperitoneally by injection. Student's t-test analysis shows the differences in tumor volumes between the following treatment arms were statistically significant: radiation vs. controls on d20 (p<0.05) and d23 (p<0.001); radiation vs. radiation + low dose drug on d27 (p<0.01) and d30 (p<0.05); radiation vs. radiation + high dose drug on d16 (p<0.05) and on d20, d27 and d30 (p<0.01); control vs. radiation + low dose drug on d13 (p<0.05), d16 (p<0.01) and on d20 and d23 (p<0.001); control vs. radiation + high dose drug on d16 (p<0.01) and on d20 and d23 (p<0.001). For the re-treatments, the difference between with and without retreatment with the high dose drug was significant at d37 (p<0.05). The difference between high and low drug doses was not significant. n=9 for the radiation only arm and 10 for each of the other arms.

(B) Tumor growth delay (TGD) was computed by determining the number of days from the start of treatment (day 9) to when tumors reached 1000 mm$^3$ for each mouse. Bouvardin and IR treatment increased the TGD. Error represented as standard error of the mean. (Barbara Frederick; tumor delay graph compiled by Tin Tin Su)
To investigate whether these tumors were still responsive to the treatment, Barbara randomly split each group into two arms (solid and dashed lines) (Fig. 3-17 A). One of these arms was left untreated (solid lines) while the other received the same combination of drug and IR as in the first lag of treatment (dashed lines). Arrows indicate the days which mice were irradiated during the second lag of treatment. Barbara found that these tumors remained responsive to treatment; the tumor volumes in re-treated mice were significantly less than the tumor volumes in 'control' mice that were not re-treated. I interpret these data to mean that the first lag of treatment did not select for cells resistant to treatment and that the survivors remained responsive to retreatment. The experiment was terminated when control animals that did not receive the retreatment had to be sacrificed due to large tumor volumes. Treatment toxicity was also measured by examining behavioral signs of the mice (e.g. the ability to eat). It was determined that the concentrations of bouvardin and IR used in this xenograft did not have a toxic effect in the mice.

Tumor growth delay (TGD) was computed by determining the number of days from the start of treatment (day 9) to when tumors reached 1000 mm$^3$ for each mouse (Fig. 3-17 B). IR alone resulted in a significant increase in TGD compared to controls. The addition of bouvardin further increased the TGD compared to IR alone. In a few treatment groups, some members of the group were terminated before the end of the experiment because tumors exceeded the allowable size (IR or drug alone). Conversely, some members had tumors smaller than 1000 mm$^3$ on the day of sacrifice. In those cases, tumor growth was extrapolated based on the slope of the line between the last two measurements. TGD by combination treatments is likely to be an
underestimate because many mice in these groups had tumors smaller than 1000 mm$^3$ when the experiment was terminated and the maximum delay to this time point was used.

**Discussion**

Previous studies demonstrated the ability for bouvardin to target protein synthesis in *in vitro* yeast and rabbit systems. I report here that bouvardin can also inhibit human ribosomes and block translation elongation. Furthermore, analysis of polysome profile fractions has revealed that bouvardin increases the presence of EF2 in the 80S complex peak, indicating that it has the potential to lock EF2 on the ribosome. I also observed no change in the level of polysomes after bouvardin treatment, further substantiating a locking or stalling mechanism. Other elongation inhibitors such as fusidic acid and sordarin have also been shown to lock EF2 to the ribosome (Justice et al., 1998; Nolan et al., 1976). Conversely, the elongation inhibitor puromycin targets translation elongation by inducing premature peptide release and subsequent ribosome subunit dissociation, which causes an accumulation of 80S complexes but a decreased polysome population (Chan et al., 2004; Chung et al., 2013). Additionally, cycloheximide did not increase the level of EF2 in the 80S peak, suggesting that bouvardin and cycloheximide block translation elongation by different mechanisms. Cycloheximide did increase the amount of EF1a in the 80S fraction. This would be consistent with cycloheximide stabilizing an EF1a-80S complex to block translation elongation.
While our lab has previously reported that bouvardin combined with IR has the ability to inhibit the growth of various cell lines (Gladstone et al., 2012), this study examined the cellular mechanisms for growth inhibition of HNC and glioma cells. The ability of bouvardin to induce apoptosis, alone or in combination with IR, is modest in both cell lines studied. In Det562 HNC cells, the large reduction in cell growth in the clonogenic assays may be attributed to increased doubling time of bouvardin-treated cells. In clonogenic assays, cells were exposed to bouvardin for only 24 hours. This may explain why, after an initial increase in doubling times, cells resumed proliferation to display mitotic indices that are similar to controls at 9 days after treatment. The combination of reduced cell numbers by IR and increased doubling time by bouvardin made the combination treatment most efficacious in this setting.

SNB-19 glioma cells exhibited an even more drastic response to the combination treatment by showing signs of senescence. This is remarkable given that cells were exposed to bouvardin for only 24 hours as in the case of Det562 HNC cells. Yet, the effect of the combination of bouvardin and radiation appears to last as long as 9 days post-treatment in these cells. From this data, I conclude that while the combination of bouvardin and IR slowed down but did not stop the growth of Det562 HNC cells, the same treatment in SNB-19 glioma cells retarded growth in all cells and induced a senescence-like state in about 75% of the colonies. I do not know the basis for the differential response of these two cell lines to bouvardin and IR. In the case of Det562 HNC cells, prolonged doubling times caused by bouvardin alone (56.6 hours vs. 26.6 hours in controls) may be sufficient to prevent accelerated repopulation that normally occurs after irradiation. In SNB-19 glioma cells, I speculate that following radiation
exposure, these cells may need to translate a special subset of proteins in order to recover from radiation-induced damage. Failure to synthesize these proteins (e.g. in the presence of bouvardin) would then prevent optimal recovery, thereby ultimately channeling these cells in a senescence-like state several days later.

In conclusion, I found that bouvardin inhibits translation on human ribosome and in human cells. I propose that the mechanism of action is stabilization of 80S-EF2 complex, thereby interrupting the elongation cycle. I also found that bouvardin inhibited the growth of HNC and glioma cells despite being HPV-negative and having high MGMT levels, respectively. This provides a potential therapeutic option for the corresponding hard-to-treat patient populations. In support of this idea, bouvardin enhanced the effect of radiation in both cell types and in mouse xenografts of HNC. Finally, cellular mechanisms by which bouvardin enhanced the effect of radiation may be different for the two cell lines studied despite similar outcomes on clonogenic growth. Based on these results, I propose that bouvardin offers a viable potential therapeutic option to improve the standard of care in HNC and glioma.

**Materials & Methods**

**Cell culture**

Glioma cell lines were purchased from ATCC. Cells were maintained in either Dulbecco’s modified Eagle medium (Det562, FaDu; Gibco) or RPMI medium (SNB-19, U-251; Gibco) with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals). All lines were maintained in a humidified incubator with 5% CO₂.
**in vitro translation assays**

Translation inhibition in a rabbit reticulocyte lysate (Promega) was performed according to the manufacturer’s instructions. Luciferase mRNA provided in the kit was used at a final concentration of 1 µg/µl. The reactions were incubated for 15 minutes at 37°C and quenched by 1:50 dilution with water. Luciferase activity was measured using a Multi-Mode Microplate Reader (Synergy 2 by BioTek) immediately after luciferase substrate addition. IC50 determination was performed using Prism. For Western blot analysis, samples were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was then blocked with 5% milk in PBT (PBS with 0.2% Tween) for 1 hour and incubated with polyclonal α-luciferase primary antibodies (goat, 1:1000; Promega) in block overnight. Membranes were washed 3 times with PBT, incubated with HRP-conjugated anti-goat IgG secondary antibodies (donkey, 1:5000; Promega) in 2.5% milk for 2 hours, and visualized using SuperSignal West Pico Solutions (Thermo Scientific).

Translation inhibition in a HeLa cell lysate was performed using the 1-Step Human Coupled IVT Kit (Pierce) and GFP mRNA (Pierce). Briefly, the reaction mix was made according to the manufacturer’s instructions along with 0.75 µg/µL GFP mRNA. Various concentrations of drug were then added to the reaction mixture in a 96-well plate. The plate was then incubated at 30 °C for 5 hours and GFP signal was measured by reading the fluorescence (excitation: 485 nm; emission: 525 nm) in a Multi-Mode Microplate Reader (Synergy 2 by BioTek).

Translation inhibition in Det562 cells was monitored using Click-iT Metabolic Labeling Reagent (L-azidohomoalanine; Invitrogen) and the Click-iT Protein Reaction
Buffer Kit (Invitrogen), according to the manufacturer’s instructions. Briefly, cells were incubated with methionine-free DMEM (Invitrogen Cat# 21013-024) for 30 minutes to deplete methionine reserves. L-azidohomoalanine was added to the media to a final concentration of 50 μM, along with 0.05% DMSO (control), cycloheximide (50 μM), or bouvardin. Cells were incubated for 2.5 hours before harvesting and processing according to instructions. The precipitated sample was separated on a 10% polyacrylamide gel and imaged on a 300 nm excitation source (Syngene GVM20). Quantification was performed by measuring the mean gray value of each lane in Image J. The gel was stained with Coomassie to confirm similar total protein levels among different samples.

**Polysome profiling**

HeLa cells were grown in 2 x 15 cm (diameter) culture dishes in log-phase up to harvesting. Cells were treated with 0.1% DMSO, cycloheximide (100 μg/mL), or bouvardin (10 μM) for 30 minutes prior to harvest. All subsequent steps were performed at 4 °C. Cells were washed in PBS supplemented with 100 μg/mL cycloheximide, trypsinized using standard protocols, and pelleted by centrifugation. The medium was removed and cells were washed with PBS/cycloheximide and lysed in 900 μL TMK100 buffer (10 mM TrisHCl (pH 7.4), 5 mM MgCl₂, 100 mM KCl, 1% Triton X-100, 0.5% deoxycholate, 5U/mL RNasin Ribonuclease inhibitor (Promega), 2 mM dithiothreitol). Prior to loading, the extracts were centrifuged at ~15,000 x g for 5 minutes to clear cellular debris. 300 μL of supernatant was loaded onto a 10%-60% sucrose gradient (100 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 20 mM HEPESxKOH (pH 7.4)), which was prepared in Seton SW41 tubes using a gradient station (BioComp instruments).
Samples were centrifuged for 3 hours and 10 minutes in an SW41 rotor at 36,000 rpms at 4 °C (Beckman-Coulter Optima L-90K). The fractions were collected and the OD$_{254}$ was monitored (BioComp Instruments). rRNA was extracted from peak fractions using an RNeasy Mini Kit (Qiagen) and analyzed by electrophoresis using 1% agarose gels.

**Western blot analysis**

For Western blot analysis, samples were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was then blocked with 5% milk in PBT (PBS with 0.2% Tween) for 1 hour and incubated with primary antibodies against either PARP (1:750; Cell Signaling #9542) and gamma-Tubulin (1:1000; Sigma T-5192) or eEF1A, eEF2, and RPL13a (rabbit, 1:1000; Cell Signaling) in block overnight. Membranes were washed 3 times with PBT, incubated with HRP-conjugated secondary antibodies (1:2,500; Santa Cruz) in 2.5% milk for 2 hours, and visualized using SuperSignal West Pico Solutions (Thermo Scientific).

**Cell growth assays**

GI50 for bouvardin was determined with cell growth assays utilizing CellTiter-Glo Luminescent Cell Viability Assay (Promega). In brief, cells were seeded in 96-well plates (4,000 cells per well) and grown overnight. Bouvardin was then added to the cells at concentrations ranging from 0.003 to 10µM. Cells were allowed to grow for five days and then quantified using CellTiter-Glo according to the manufacturer’s instructions. Data was compiled and GI50’s were calculated using Prism software (nonlinear regression). Shown GI50’s are the average of at least two biological replicates, consisting of six technical replicates per bouvardin concentration.
For cell doubling times, cells were seeded on cover slips (Fisher) in 12-well plates (20,000 cells per well) and treated 24 hours later with DMSO (control) or bouvardin and -/+ IR. Plates were then incubated for 24 or 72 hours. Cover slips were removed and fixed in 10% formaldehyde in PBS with 0.2% Tween-20 (PBT) for 10 minutes, stained for 5 minutes with 10 µg/mL Hoechst33258 (Sigma) in PBS, and washed twice with PBS. Lastly, coverslips were mounted in Fluoromount G (Southern Biotech). Cell doubling times were computed using the online application:
http://www.doubling-time.com/compute.php

**Clonogenic assays**

Cells were seeded in 6-well plates (300 cells per well) and grown overnight. Plates were then irradiated and DMSO (control) or bouvardin in fresh media was added. The amount of DMSO added was equal to the volume of drug. After a 24 hour incubation period, fresh media without DMSO/drug was added and the plates were incubated for 8 more days. Cells were fixed with ice cold 10% trichloroacetic acid for 30 minutes and washed 5 times with distilled water. Cells were then stained in 0.4% Sulforhodamine B (SRB) in 1% acetic acid for 30 minutes at room temperature, washed 5 times with 1% acetic acid, air-dried and imaged on an Olympus SZX12 Stereomicroscope. To solubilize the SRB, 10 nM unbuffered Tris Base (pH 10.5) was added and the plates were shaken on a platform for 5 minutes. SRB levels were then measured by reading fluorescence (440 nm excitation/600 nm emission) with a Multi-Mode Microplate Reader (Synergy 2 by BioTek).

Standard deviation for expected fraction survival for co-treatment of drug and radiation is computed according to the formula for the standard deviation of a product of...
two normally distributed variables. For two normally distributed variables with means $m_1$ and $m_2$ and standard deviations of $s_1$ and $s_2$, the product will have mean $m_1m_2$ and the standard deviation $= \sqrt{(m_1^2s_2^2 + m_2^2s_1^2 + s_1^2s_2^2)}$ [page 140 of Menzel (Menzel, 1960)].

**Immunohistochemistry**

For caspase 3 staining, cells were seeded on cover slips (Fisher) in 12-well plates (20,000 cells per well) and treated 24 hours later with DMSO (control) or bouvardin and -/-+ IR, and incubated for 48 hours. Cover slips were removed and fixed in 4% formaldehyde in PBS for 30 minutes, washed for 5 minutes twice with PBS and once with PBS with 0.5% Triton X-100 (PBTx), and blocked for 1 hour with 0.1% PBTx plus 5% normal goat serum (NGS). Cells were then incubated overnight at 4 °C with rabbit anti-caspase-3 (1:100, Cell Signaling Cat#9661 Lot 37) primary antibody. Cells were then washed three times with block and incubated for 2 hours with anti-rabbit rhodamine secondary antibodies (1:500, Jackson Immuno#711-025-152). Lastly, samples were stained for 5 minutes once with 10 µg/mL Hoechst33258 (Sigma), washed in 0.1% PBTx (caspase-3), and mounted in Fluoromount G (Southern Biotech).

For phospho histone H3 (pH3) staining, cells were seeded on cover slips in 6-well plates (300 cells per well) and treated 24 hours later with DMSO (control) or bouvardin and -/-+ IR. Fresh media was added 24 hours after treatment and cells were incubated for 9 days. Cover slips were removed and fixed in 10% formaldehyde in PBS with 0.2% Tween-20 (PBT) for 10 minutes, washed three times with PBS, and blocked for 1 hour with PBT with 3% NGS. Cells were then incubated overnight at 4 °C with rabbit anti-phospho Histone H3 (Ser10) (1:1000, Millipore) primary antibody. Cells were
then washed three times with block and incubated for 2 hours with anti-rabbit rhodamine secondary antibodies (1:500, Jackson Immuno#711-025-152). Lastly, samples were stained for 5 minutes once with 10 µg/mL Hoechst33258 (Sigma), washed in PBT (pH3), and mounted in Fluoromount G (Southern Biotech).

Cells were imaged using a Leica DMR compound fluorescence microscope at room temperature. Objective lenses used were 5Xair/NA 0.15, 10Xair/NA0.30, 20Xair/NA0.50, 40Xair/NA0.74 and 100Xoil/NA1.30. Images were collected using a SensiCam CCD camera and Slidebook software (Intelligent Imaging). Slidebook images were exported as TIFF documents and processed/assembled in Photoshop CS3 (Adobe).

**Senescence Assays**

Senescence staining was performed using the Senescence β-Galactosidase Staining Kit (Cell Signaling #9860), according to the manufacturer’s instructions. Briefly, cells were treated, fixed, and then stained overnight with X-gal to detect β-galactosidase. Stained coverslips were kept in 70% glycerol at 4 °C until imaging.

**Xenografts**

For the flank HNC model, female nude athymic mice that were 6- to 8-weeks old were purchased from Harlan Laboratories and housed in a pathogen-free facility approved by the American Association for the Accreditation of Laboratory Animal Care and met all current regulations and standards of the US Department of Agriculture, the US Department of Health and Human Services, and the National Institutes of Health. Animal procedures were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Colorado. Det562 cells
were grown to 80% confluence and harvested by trypsinization. Trypsin was neutralized with complete medium containing FBS. Cells were washed 3x with RPMI (no FBS) and resuspended in unsupplemented RPMI to a concentration of 2x10^6 per 100 μL. Cells were mixed 1:2 with Matrigel (BD Biosciences #354234) and 1x10^6 cells/mouse (100 μL) were injected subcutaneously into the rear flanks of athymic nude mice. Mice bearing tumors with a volume of ~200 mm^3 were randomly assigned to treatment groups of ten animals each (control, drug alone, radiation alone or the combination). The animals received drug diluted in saline by intraperitoneal injection. Drug stocks were prepared by dissolving 2 mg of Bouvardin (NCI DTP) into 50 μL DMSO and stored frozen in aliquots. 5 μL of the frozen DMSO stock was diluted first with 45 μL 0.5% carboxymethylcellulose (Sigma) to produce a 4 mg/ml working stock. 3 μL of the working stock was diluted into 100 μL saline to generate the working solution for injection. Mice were anesthetized with ketamine/xylazine before irradiation with X-rays and positioned under a lead shield such that only the tumor-bearing leg was exposed. Tumor volume was calculated using the formula \( V = \frac{a^2 \times b}{2} \), where \( a \) and \( b \) are the smallest and largest tumor diameters, respectively, as determined using calipers. Animals were euthanized when tumor volume exceeded 2 cm^3.
Chapter 4: Conclusions and Future Directions

In this thesis I have described my efforts to 1) determine if isogenic mutations behave the same or differently in different tissues of *Drosophila melanogaster* and 2) determine the effect of the protein synthesis inhibitor bouvardin and ionizing radiation (IR) in human cancer cells. In the first part of my thesis, I tested the theory as to whether isogenic mutations in separate tissues are morphologically distinct and if they behave the same or differently in response to various treatments. Because cancer therapies are normally targeted to the tissue origin and not the mutation, this model could be used to justify utilizing treatments approved for one tissue in another if they have identical mutations. In order to test this theory, I utilized a system in *Drosophila melanogaster* to induce ectoderm-derived cephalic (eye disc and optic lobe) and mesoderm-derived gonad tumors using Ras$^{V12}$ and scrib$^{1}$ mutations. Cells with these mutations are marked by GFP, allowing us to easily visualize and isolate tumors. While the cephalic and gonad neoplasms were previously characterized (Pagliarini and Xu, 2003), I was able to determine that gonad neoplasms were sex-specific, only occurring in males and originating in posterior terminal body cells. When I examined the morphological similarities between cephalic and gonad tumors, I found they both displayed cytoplasmic extensions, which were previously characterized as invasive in both this model and in cultured human cells engineered with identical mutations (Dow et al., 2008; Pagliarini and Xu, 2003). I next examined intact checkpoints and responses to IR. While the DNA-damage and spindle checkpoints were intact, both cephalic and gonad neoplasms were resistant to IR-induced apoptosis. Lastly, I found that treatment
with the protein synthesis inhibitor bouvardin or IR had no effect on survival or tumor reduction in either tumor type; however, treatment with Taxol significantly reduced the growth of both cephalic and gonad neoplasms. This was the first study ever performed in a whole organism where isogenic tumors in separate tissue types responded identically in every way examined.

In the second part of my thesis, I examined the molecular mechanism of a translation inhibitor and its ability to enhance the effects of IR in human cancer cells. Targeting protein synthesis has recently become a topic of interest in cancer therapy with emerging evidence that many cancer types are mutant for factors involved in translation. Furthermore, other studies have shown that certain translation factors have the ability to transform cultured cells into an oncogenic-like state and downregulation of these factors in cancer cells inhibits growth. We previously identified the protein synthesis inhibitor bouvardin as a sensitizer of ionizing radiation (IR) in Drosophila melanogaster and human cancer cells (Gladstone et al., 2012). While previous studies have indicated that bouvardin targets translation elongation in an in vitro system (Zalacain et al., 1982), my results indicate for the first time that bouvardin targets translation elongation in human cells and preliminary evidence indicates this may be due to locking elongation factor 2 (EF2) to the ribosome. I have also shown that bouvardin has the ability to enhance the effects of IR in head and neck cancer (HNC) and glioma cells, as well as, a mouse xenograft of HNC. Further examination of the cellular mechanism in HNC and glioma cells revealed a differential response. I found that in Det562 HNC cells, bouvardin and IR had no affect on the mitotic index; however, the growth of cells was slowed. In contrast, bouvardin and IR treatment decreased the
mitotic index of SNB-19 glioma cells and induced senescence in a subpopulation of colonies.

While the results in this thesis indicate that chemotherapy drugs effective against a particular mutation in one tissue may be as effective in another and translation elongation inhibition and IR are effective in inhibiting the growth of both HNC and glioma cells, a number of questions still remain. I would like to address those concerns here, as well as, discuss potential projects that could arise from these results.

**Does the response of isogenic mutations in different tissue origins apply for other mutations?**

When the Ras\textsuperscript{V12}/scrib\textsuperscript{1} tumor model was first identified in a screen for mutations that caused metastatic behavior, the authors also found that Ras\textsuperscript{V12} cooperated with \textit{lethal giant larvae (lgl)}, \textit{discs large (dlg)}, \textit{bazooka}, \textit{stardust}, and \textit{cdc42} to induce cephalic tumors (Pagliarini and Xu, 2003). These proteins all play a role in cell polarity, cell shape, and/or epithelial morphology. Furthermore, a separate study was able to generate tumors by combining scrib mutations with oncogenic Notch or Abrupt transcription factor (Brumby et al., 2011; Brumby and Richardson, 2003; Turkel et al., 2013). It would be interesting to repeat the experiments reported here in other tissues and oncogenic mutant combinations using various drivers. This would allow us to confirm if the override of tissue-specific differences by oncogenic transformation is universal.

**Do isogenic mutations respond similarly to all forms for treatments?**

While cephalic and gonad neoplasms behaved and responded the same in every way examined (cellular morphology, DNA damage and mitotic spindle checkpoints, IR-
induced apoptosis, and response to IR/chemotherapies), it would be interesting to test other molecules and determine if we see a divergent response. It would especially be worthwhile to examine the response to targeted agents. For example, in the Ras$^{V12}$/scrib$^1$ model, an inhibitor of oncogenic Ras (Mackenzie et al., 2013) could be used to determine if both cephalic and gonad tumors respond similarly to the treatment. This would not only be more clinically relevant, but would ensure the similar response we see in tumor tissues is related to targeting the mutation and not just a response to an agent that targets invasive, rapidly proliferating cells.

**Accelerated repopulation in response ionizing radiation (IR) and screening for radiation sensitizers**

When examining the response to IR, I found that treatment neither reduced tumors, nor increased the survival of larvae. As described in Chapter 1, compensatory proliferation or accelerated repopulation can cause the regrowth of tumors after a single exposure to IR. Therefore, the resistance of these tumors to IR may be due to accelerated tumor growth after IR exposure. Further studies using fractionated low doses of IR should be performed to determine more definitively if the tumors are responsive to IR. If this can be achieved, we could design a screen to identify additional radiation sensitizers, similar to the study we have previously performed in larvae without tumors (Gladstone et al., 2012). Screening for molecules that reduce tumor size has been previously conducted and a protocol was established to identify molecules that reduced GFP-positive Ras$^{V12}$/scrib$^1$ tumors (Willoughby et al., 2013). This proof-of-principal screen identified acivicin, a known inhibitor of tumor cell growth (Griffiths and Keast, 1991; Roy et al., 2008; Roy and Maity, 2005, 2007), further indicating that the
model used here may well represent human tumors. In addition to quantifying GFP reduction, we could also measure increased lifespan or even the ability for larvae to pupate or survive to adulthood. A screen to identify sensitizers of IR may even prove more fruitful than the search for single-agents as described in (Willoughby et al., 2013) since many chemotherapy molecules are not as effective when administered as a single agent (Li et al., 2014).

**Why are Drosophila tumors resistant to IR-induced apoptosis?**

I observed in Chapter 2 that Ras^{V12}/scrib^{1} cephalic and gonad neoplasms were resistant to IR-induced apoptosis. I have a number of hypotheses for why this is the case. My first theory involves the role of oncogenic Ras. The proapoptotic protein Hid, a homolog of mammalian SMAC/DIABLO proteins, plays an essential role in IR-induced apoptosis as reduction of hid gene dosage by half is enough to prevent IR-induced apoptosis (Brodsky et al., 2004). Because Hid is inhibited by Ras through phosphorylation by the MAPK pathway (Bergmann et al., 1998), I speculate that oncogenic Ras^{V12} is inhibiting Hid and preventing IR-induced apoptosis. To test this theory, we could examine the levels of phosphorylated Hid in the tumor using mass spectrometry. This would indicate whether oncogenic Ras is inhibiting Hid through MAPK. Additionally, we could mutate hid and examine if we get a reduction in IR-induced apoptosis.

A second hypothesis I have is that the high levels of basal apoptosis in the tumors are protecting the cells from undergoing further IR-induced apoptosis. Our lab has recently found that induction of apoptosis in a strip of cells in the wing imaginal disc protects the neighbors from undergoing IR-induced apoptosis through the receptor
tyrosine kinase Tie and microRNA bantam (Bilak, in press). Therefore, high levels of basal apoptosis in the tumors may be preventing the induction of further apoptosis after IR exposure. To test this theory, we could examine protection from IR-induced apoptosis by mutating tie and examine if high basal levels of apoptosis do or do not protect cells from death after IR exposure.

**Why is bouvardin an effective agent in human cancer cells, but ineffective against tumors in Drosophila?**

Bouvardin has the ability to target multiple cancer types; however, I found that it was unable to reduce the growth of tumors in the Ras\(^{V12}/\text{scrib}^1\) Drosophila model. While there is no obvious reason as to why these tumors are resistant to bouvardin, there may be a few explanations. First, while there is much evidence that various cancers are mutated for proteins part of the translation apparatus and cancer cell growth can be inhibited with downregulation of these factors (reviewed in Introduction), it is possible that Ras\(^{V12}/\text{scrib}^1\) cells are not relying on enhanced translation rates for survival as some other cancers may be.

The second explanation could be due to fact that we see the greatest effects of bouvardin when it is combined with IR. If we are able to see an effect with fractionated IR as described above, it would be interesting to use the combination with bouvardin and examine if tumor reduction is observed. Additionally, bouvardin has been able to synergize with Taxol in human cancer cells (Gladstone et al., 2012). It would be worthwhile to combine bouvardin with Taxol in this model and examine tumor reduction, especially because we were able to demonstrate that Taxol inhibited both cephalic and gonad growth.
**What is the exact molecular target(s) of bouvardin?**

Prescribing chemotherapies based on the mutation responsible for tumorigenesis rather than the tissue origin has becoming increasingly popular with advances in sequencing technology. Understanding the exact molecular target of bouvardin would not only help clinicians if this molecule made it to the clinic, but to also encourage the identification of other elongation inhibitors as potential chemotherapies. Our preliminary results indicate that bouvardin may be locking EF2 to the ribosome; however, this needs to be confirmed before a definitive conclusion can be reached. In order to test this, binding experiments should be performed by titrating bouvardin in a cell lysate and immunoprecipitating the ribosomes. We can then immunoblot for EF2 and examine if the levels bound to the ribosome increase with bouvardin treatment.

Furthermore, when the mechanism of an elongation inhibitor is examined, their ability to target elongation factor 1 alpha (EF1a), the peptidyl transferase center, and EF2 is usually analyzed in order to have a complete understanding of the molecular mechanism (Dang et al., 2011; Schneider-Poetsch et al., 2010a). A previous study that examined the mechanism of bouvardin indicated that peptidyl transferase activity was unaffected, while EF2 was potentially targeted (Zalacain et al., 1982). Because these studies were performed *in vitro* in a rabbit reticulocyte and the results weren’t completely reported, it would be useful to repeat these mechanistic experiments in a human cell context, as well as, fully assess the effect of bouvardin on all steps of translation elongation.
Why is there a differential response to bouvardin and IR in Det562 HNC and SNB-19 glioma cells?

My results on the response of head and neck cancer (HNC) and glioma cells to bouvardin and IR treatment have revealed a differential response to the combination treatment. While it may indicate the underlying cause may be the tissue origin, these studies must be repeated in multiple HNC and glioma lines to make a definitive conclusion that the cellular mechanism is connected to the tissue origin. If these results do lead to a conclusion that the growth of HNC cells is slowed and glioma cells undergo both growth inhibition, as well as, senescence induction, we would need to differentiate between whether the effect we see is caused by underlying differences in the tissue type or different mutations common to each cancer type. Screening for mutations and single-nucleotide polymorphisms (SNPs) between the lines may reveal whether this is the case.

The differential response may also be due to differences between the two cancer cell types. Brain tumor cell lines, including gliomas, tend to have higher levels of aneuploidy (El-Zein et al., 1999). Because there is evidence that aneuploidy can induce senescence (Bernard and Augert, 2010; Estrada et al., 2013), it is possible that bouvardin and IR treatment are further increasing aneuploidy in the glioma cell lines and inducing a senescent-like state. In order to test if glioma cells have increased aneuploidy with the combination, I could examine chromosome spreads of mitotic cells before and after treatment and calculate if the percent of aneuploidy increases.
Why is targeting translation elongation effective in combination with IR?

As reviewed in the Introduction, treatment with IR can inhibit cap-dependent translation initiation, but allow for the translation of cap-independent IRES mRNAs involved in survival. Previous studies have hypothesized that treatment with the translation elongation inhibitor cycloheximide and IR is effective due to blocking the translation of IR-induced cap-independent survival factors (Braunstein et al., 2009; Youngblom et al., 1989). This hypothesis also fits in line with our results. In our clonogenic assays (Figs. 3-8 and 3-9), bouvardin was only added for the first 24 hours of treatment; however, we see a dramatic inhibition of cell growth 9 days later. If bouvardin is left on the full 9 days, the results are the same (data not shown). This indicates that the effect of bouvardin occurs within the first 24 hours of treatment and may be potentially blocking the translation of factors necessary for survival. To test this theory, I want to measure the levels of survival factors (proteins involved in proliferation, inhibition of apoptosis, and repair) in control, single, and combination treatments. Specifically, I will measure the levels of two proliferation markers, cyclin D1 and c-myc, as well as two antiapoptotic factors, Bcl-2 and survivin, by Western blot 6, 12, and 24 hours after treatment. These factors have been shown previously to be upregulated in response to IR (De Benedetti and Graff, 2004; Graff et al., 2007). If I can determine that bouvardin is inhibiting the synthesis of these proteins after IR treatment, it may indicate a mechanism for why HNC and glioma cell lines are inhibited (Chapter 3).

I would also like to measure the levels of markers that indicate increased translation rates in these cell lines. As I described in the Introduction, the regulation of translation initiation occurs through mTOR. Therefore, measuring the downstream
effectors of mTOR would be useful in determining if the HNC and glioma cell lines have increased translation rates. To do this I can probe for S6 Kinase (S6K) phosphorylation and 4E-BP phosphorylation, as well as, measure increased eIF4E-el4G association through immunoprecipitation assays. I would like to compare the levels in the HNC and glioma cell lines to 'wild-type' fibroblast cells, as well as, a few cancer cell lines that are resistant to bouvardin treatment. Increased levels of mTOR effectors in the HNC and glioma cell lines compared to wild-type and resistant cancer lines may indicate that these cells required increased protein synthesis for survival and inhibition by bouvardin sensitizes them.

Lastly, we believe that elongation is a more effective target when used in combination with IR rather than initiation inhibitors. eIF4E has been described as the potential ‘Achilles’ heel’ of cancer cells and inhibition could selectively target tumor cells (De Benedetti and Graff, 2004; Graff and Zimmer, 2003). However, if an eIF4E inhibitor was combined with IR, cap-dependent translation would be inhibited and the drug would presumably have no effect. Therefore, we believe targeting elongation would be more effective if combined with IR as a cancer therapy.

**Final comments**

My results that indicate that 1) isogenic mutations in diverse tissue origins behave similarly in *Drosophila melanogaster* and 2) the translation elongation inhibitor bouvardin can enhance the effects of ionizing radiation (IR) differentially in head and neck cancer (HNC) and glioma cells. While these results may impact how chemotherapies are used one day, there is still much work that needs to be
accomplished to completely understand whether isogenic tumors in different organs behave similarly in a human and the mechanisms behind translation inhibition and IR in various cancer types.
Bibliography


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Appendix 1: Streptovitacin A inhibits *in vitro* translation in a dose dependent manner

*in vitro* translation protocol in a rabbit reticulocyte lysate was performed as described in the Materials & Methods section of Chapter 3.
Appendix 2: Select molecules from the ChemDiv library do not inhibit translation \textit{in vitro}

I screened select molecules from the Chemical Diversity (ChemDiv) library to determine if they have the ability to inhibit \textit{in vitro} translation. The \textit{in vitro} translation protocol in a rabbit reticulocyte lysate (RRL) was performed as described in the Materials & Methods section of Chapter 3. Because relevant physiological concentrations of protein synthesis inhibitors are in the nanomolar range, I used 100 $\mu$M concentrations of each molecule to easily identify any hits.

(A) First set of molecules tested were compared to DMSO (control). Cycloheximide was used as a positive control for translation inhibition. (B) Re-testing of molecules from panel A that showed modest inhibition were performed in triplicate. Error is represented as standard error of the mean. My results indicate that the ChemDiv molecules tested did not inhibit \textit{in vitro} translation in a rabbit reticulocyte lysate.
Appendix 3: Select indole molecules do not inhibit translation *in vitro*

I screened indole molecules developed in the laboratory of Dr. Xiang Wang (University of Colorado at Boulder, Department of Chemistry & Biochemistry) to determine if they have the ability to inhibit *in vitro* translation. The *in vitro* translation protocol in a rabbit reticulocyte lysate (RRL) was performed as described in the Materials & Methods section of Chapter 3. I used each molecule at a concentration of 200 μM.

(A) First set of molecules tested were compared to DMSO (control). Cycloheximide was used as a positive control for translation inhibition. (B) Re-testing of molecules from panel A that showed modest inhibition. My results indicate that the indole molecules provided to me did not inhibit *in vitro* translation in a rabbit reticulocyte lysate.
Appendix 4: Bouvardin and ionizing radiation (IR) treatment does not significantly reduce the growth of Det562 HNC cells within 3 days of treatment

I tested the ability for bouvardin and ionizing radiation (IR) to inhibit the growth of Det562 HNC cells within 3 days of treatment. Det562 cells were seeded in 12-well plates (20,000 cells), treated 24 hours later with DMSO (control), bouvardin (BVD), IR, or the combination, and incubated for 3 days. Cell viability was measured using a CellTiter-Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer’s instructions. Percent survival was calculated by measuring the percent max signal of controls. Error bar = 1 STD. I conclude that bouvardin and IR treatment does not have the ability to inhibit the growth of Det562 HNC cells within 3 days of treatment.
Appendix 5: Nucleolar size does not change with bouvardin and/or ionizing radiation (IR) treatment in Detroit 562 head and neck cancer (HNC) cells

It has been shown previously that changes in the nucleolus are associated with neoplastic transformation (Montanaro et al., 2008). Because the nucleolus is the site of ribosome biogenesis, I hypothesized that inhibition of translation by bouvardin (BVD) may affect nucleolar size. I added radiation to this experiment as I see a greater than additive effect on nucleolar size when combined with bouvardin. To test this, I treated cells with bouvardin and ionizing radiation (IR) and stained with the nucleolar marker, fibrillarin, using concentrations that were effective in inhibiting the growth of Det56 head and neck cancer (HNC) cells.

Detroit 562 (Det562) head and neck cancer (HNC) cells were seeded on coverslips (Fisher) in 12-well plates (20,000 cells/well). 24 hour later, cells were treated with DMSO (control), 3 nM BVD, 5 Gy of X-rays, or both. 24 hours later, I fixed cells with 4% formaldehyde in PBS for 10 minutes, washed with PBS plus 0.1% Tween-20 (PBT), and blocked for 1 hour with PBT + 3% normal goat serum (NGS). Cells were then stained with the nucleolar marker fibrillarin (mouse, 1:100) for 1 hour. Cells were then washed once for 5 minutes with 10 μg/mL Hoechst33258 in block and then twice with block only. Cells were then stained with anti-mouse rhodamine secondary antibodies (1:500) overnight, washed three times with PBT only, and mounted in Fluoromount for imaging.

My results indicate that single and combination treatments of bouvardin and/or IR did not affect the size of nucleoli in Det562 HNC cells. This should be repeated at longer timepoints and in other cancer cell lines to confirm bouvardin and/or IR does not have an effect on nucleolar size.
Appendix 6: The effect of bouvardin and ionizing radiation (IR) on imaginal wing disc size in wild-type and p53 mutant larvae

As described in the Introduction section of this thesis, tissues or tumors exposed to ionizing radiation (IR) can lead to compensatory proliferation (*Drosophila melanogaster*) or accelerated repopulation (tumors). This can lead to complete regeneration of the tissue or tumor. We sought to identify molecules that could inhibit tissue regeneration after IR in *Drosophila* and discovered the protein synthesis inhibitor bouvardin (Gladstone et al., 2012). In order to test if bouvardin inhibits the regeneration process post-IR treatment, I examined how imaginal wing disc size was affected by bouvardin and IR treatment in wild-type (Sev) and p53 mutant larvae.

Bouvardin and IR treatment was performed as described in (Gladstone et al., 2012). In these experiments, the doses of IR used were 2000 R and 4000 R for p53 mutant and wild-type larvae, respectively. IR dose used was based on the LD50 found in separate eclosion experiments. At the indicated day, up to 10 larvae were dissected, fixed in 0.4% formaldehyde in PBS + 0.2% Tween-20 (PBT), washed once for 5 minutes with 10 μg/mL Hoechst33258 in PBT, washed twice in PBT-only, and mounted on slides using Fluoromount. Images were captured according to the “Imaging” Material & Methods section in Chapter 2. Image J was used to measure the area of each wing disc. Error represented as standard error of the mean.

In wild-type larvae, the size of the wing disc increased with age in both single and combination treatments overall (A). The wing disc size did decrease after treatment with IR at 24 hours post-treatment; however, this timeline is consistent with induction of the G2/M DNA damage checkpoint after IR exposure. Wing disc size does recover around 48 hours, most likely due to compensatory proliferation. In the combination treatment, the wing disc size initially appears to be stunted; however, they appear to recover by 63 hours. The absence of a bar for certain treatments at 63 hours indicates all larvae had already pupariated. In p53 mutant larvae, it appears that control and single treatments behave like wild-type larvae; however, the combination actually decreases the size of wing discs with time. These experiments should be repeated to make any sound conclusions.
Appendix 7: The effects of bouvardin and Taxol treatment on the survival of wild-type and p53 mutant larvae

I examined the effects of bouvardin and Taxol treatment on wild-type and p53 mutant larvae, as performed in (Gladstone et al., 2012). Briefly, day 4 old larvae were added to cornmeal that contained DMSO (control), bouvardin (BVD), Taxol, or both. The fraction of flies that eclosed from the pupal case was calculated (A,B). Error represented as standard error of the mean.

Interestingly, I found that the combination of bouvardin and Taxol had an antagonistic effect in wild-type larvae (Sev, A), but a greater than additive effect in p53 mutant larvae (B). This data is reproducible; however, these experiments should be repeated using the wild-type background of p53 mutant flies (yw1118) to ensure the effect I see is due to the p53 mutation and not the background genotype.