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Characterization of regulators of blood cell number identified in a Drosophila genome-wide RNAi screen.

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Characterization of regulators of blood cell number identified in a *Drosophila* genome-wide RNAi screen

by

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This dissertation is dedicated to my Lord, Jesus Christ, who led me into the wilderness called grad school, to be trained and perfected, so that I can be a better worker, husband, father and servant.

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The genome-wide RNAi screen was developed, conducted and analyzed by Katja Brückner. The other studies presented in this thesis were performed under the guidance of Dr. Katja Brückner. Much of the work and its accompanying figures will be submitted for publication. The current co-authors for the manuscript are Kalpana Makhijani, Richelle Sopko, Norbert Perrimon and Katja Brückner.

Characterization of regulators of blood cell number identified in a *Drosophila* genome-wide RNAi screen

You Bin Lin

Abstract

The control of cell number depends on the balance between pro- and anti-death signals. The *Drosophila* PDGF/VEGF Receptor (PVR) provides an anti-apoptotic signal that is necessary for the survival of both embryonic hemocytes and the hemocyte-like Kc cell line, thus providing a model system for uncovering other mammalian PDGF/VEGF receptors regulated signaling pathways for hematopoietic cell survival. RNAi silencing of PVR in Kc cell leads to inactivation of both Ras/Erk and Akt/Tor, and reactivation of one of these pathways or inhibiting a pro-death regulator, leads to restoration of cell numbers. To identify other pathways, which involved in hemocyte survival, a genome-wide RNAi screen to identify suppressors and enhancers of the PVR RNAi-mediated cell number reduction had been conducted previously. The screen provided the basis for this thesis project, as we subsequently characterized the biological and molecular roles of genes identified in the screen. Among the suppressors identified are many known tumor suppressors and negative regulators of the Akt/Tor and Ras/Erk pathways, and as well as heterodimeric nuclear hormone receptor- ecdysone receptor (EcR) and ultraspiracle (usp). Loss of EcR or usp suppresses cell death in a PVR-silenced background, and expression of dominant negative EcR rescues hemocyte survival in PVR null mutant embryos. Ecdysone stimulation in Kc cells triggers cell death, possibly by the induction of reaper and E93. When trying to assess the relationship of EcR/usp or ecdysone signaling with

the PVR signaling pathway, biochemistry data suggest that both function in a parallel fashion. Interestingly, PVR is required for ecdysone-induced upregulation of EcR, suggesting a regulatory balance between pro-survival PVR- and pro-death ecdysone signaling that stabilizes cell lifespan. These studies suggest a novel role of ecdysone signaling in *Drosophila* embryonic development and blood cell survival, and emphasize parallels with the homologous vertebrate rexinoid receptor (RXR) that acts in pro-death signaling and tumor suppression. In addition, novel PVR suppressors identified in the screen, and their mammalian counterparts, may play a role in cell number control and tumor suppression.

Table of contents

List of Tables

Chapter 2

List of Figures

Chapter 1: General Introduction

Homeostatic control of cell number is critical for the normal physiology of all multicellular organisms. By contrast, if cellular homeostasis is violated, pathogenesis ensues. Control of cell number results from a dynamic balance between cell proliferation/survival and programmed cell death (PCD) ((Guo and Hay, 1999;Thompson, 1995;Vermeulen et al., 2003). In order to understand more about the mechanism of cell number control we need to study the signals that feed into the apoptotic and proliferation machinery which control cell survival and cell division.

The primary goal of my thesis project is to characterize the functional role of novel regulators of cell number with respect to known signaling pathways. To conduct this research, I utilized *Drosophila melanogaster* embryonic blood cells, and a related *Drosophila* cell line, as models, based on the simplicity of the system, the high degree of conservation with vertebrate systems and the wide range of genetic tools available.

1.1 Cell number control in development and disease

In the human body, roughly 100,000 cells are produced each second by mitosis, and concurrently a similar number of cells undergo PCD (Jacobson et al., 1997;Thompson, 1995). Homeostatic control of cell division and death is therefore critical to regulate cell number in humans. Homeostasis ensures the viability of the organism as different cell lineages and tissues are maintained and renewed over the life of the organism.

In multicellular organisms, cell number is controlled by balancing the rates of cell proliferation, cell survival and PCD (Guo and Hay, 1999;Thompson, 1995;Vermeulen et al., 2003). Almost half of the serious medical aliments for which treatments or preventative measures are still lacking can be attributed to malfunction in PCD (Reed, 2002). Here, I present examples in the vertebrates and *Drosophila* regarding the homeostatic control of cell number in development, followed by examples of diseases which arise when the regular checks and balances of cell number control fail.

Cell Proliferation in Development

The early development of a multicellular organism is characterized by the rapid proliferation and differentiation of the embryonic cells into more specialized types of cells, which become tissues and organs of the organism. In adults, many of these tissues still possess the capacity to proliferate in the event there is a need to replace the differentiated cells. Depending on the system, differentiated cells are replenished from two sources – (1) undifferentiated stem or progenitor cells or (2) existing differentiated

cells as known in ‘self-duplicating’ or ‘static’ tissues (Cooper, 2009;Dor et al., 2004;Duncan et al., 2009;Geissmann et al., 2010). For example blood cells, intestinal cells, and epidermal cells in the outer layer of the skin experience high turnover and are replenished by continual cell proliferation from undifferentiated stem or progenitor cells. Certain blood lineages, pancreatic β-cells and hepatocyte can be replenished by the duplication of differentiated cells.

Blood cells exist in many differentiated lineages (e.g. erythrocytes, granulocytes, moncytes and lymphocytes) with specialized functions. Because these cells are predominantly short lived and often have no or limited proliferative capacity, hematopoietic stem cells (HSCs) are required throughout life to replenish them (Orkin, 2000;Orkin and Zon, 2008). Studies in mice showed that HSCs are found in various locations throughout development from yolk sac, placenta, AGM (aorta, gonad and mesonephros) and fetal liver in the embryo to predominantly the adult bone marrow (BM) HSCs (Adams and Scadden, 2006;Dzierzak and Speck, 2008). The BM HSCs are found in two niches- endosteal niche or in perivascular niche (Adams and Scadden, 2006;Ehninger and Trumpp, 2011). HSCs in these niches were hypothesized to be either dormant (endosteal niche) or actively dividing (perivascular niche) to form more specialized daughter cells and concurrently also self-renew during homeostasis (Ehninger and Trumpp, 2011;Orkin and Zon, 2008). HSCs undergo differentiation (as regulated by transcription factors) to become progenitors that are progressively restricted to several lineages (e.g. common myeloid progenitor and common lymphoid progenitor). Eventually, these progenitors become precursors committed to unilineage differentiation

and production of mature blood cells, thus replenishing the blood cells lost (Orkin, 2000;Orkin and Zon, 2008). In the intestinal tract, the epithelial cell layer has a high turnover as the cells are constantly lost, due to toxic or pathogenic stresses as well as chemical and mechanical damage, and replenished by the underlying intestinal stem cell (ISC) population (Casali and Batlle, 2009). Likewise, in the epidermis, stem cells in the underlying basal layer, maintain the self-renewal capability of the epidermis as they replace the epidermal cells in the outer layer of the skin (Watt, 2001;Watt et al., 2006).

Tissue turnover can occur by duplication of existing differentiated cells, e.g. blood cells, pancreatic β-cells and hepatocytes. Certain differentiated blood cell populations have the ability to expand, albeit a limited capacity. Macrophages and dendritic cells are able to renew their individual subsets by self-renewal of resident differentiated cells (Geissmann et al., 2010). Langerhans cells (LCs) of the epidermis are differentiated cells able to undergo proliferation (Geissmann et al., 2010). The LC precursors, upon migrating to the epidermis, undergo a massive burst of proliferation in a newborn. The resident epidermal LC can also expand upon host encountering inflammation (Chorro et al., 2009). Also, differentiated microglia can undergo proliferation in the central nervous system when demyelination or autoimmune disorder occurs (Remington et al., 2007). Early studies suggest the mechanism for β-cell homeostasis occurs by the proliferation of existing differentiated β-cells (Like and Chick, 1969;MESSIER and LEBLOND, 1960;Tsubouchi et al., 1987). Subsequently, a lineage tracing experiment convincingly showed new β-cells can be derived from terminally differentiated β-cells which retained a significant proliferative capacity (Dor et al., 2004). In liver regeneration, the removal or damage of a

subsection of the liver will trigger a compensatory proliferation response from the existing differentiated hepatocytes to replace the exact amount of the liver lost (Duncan et al., 2009).

In *Drosophila* there are also examples which differentiated cells are replenished by undifferentiated cells. The *Drosophila* adult midgut and hindgut bears similarity to the mammalian intestine as ISCs have been identified (Micchelli and Perrimon, 2006;Ohlstein and Spradling, 2006;Takashima et al., 2008). These ISCs undergo proliferation to replace the damaged gut epithelia (Amcheslavsky et al., 2009;Buchon et al., 2009;Jiang et al., 2009). In the *Drosophila* ovary, follicle stem cells (FSCs) differentiate to form the follicular epithelium that encapsulate the 16-cell germline cyst and define the polarity of the developing oocyte (Margolis and Spradling, 1995). As cysts continually bud off from the germarium, FSCs need to replenish the follicle epithelial for generating new follicles (Nystul and Spradling, 2007;Nystul and Spradling, 2010). *Drosophila* neural stem cells, known as neuroblasts (NBs), undergoes asymmetric division to produce two daughter cells- one an undifferentiated self-renewing NB and the other ganglion mother cell (GMC) which is committed to differentiation. In *Drosophila* brain development, in order to ensure a correct balance of early versus late neural cell fates, NB proliferation has to be terminated by either exiting the cell cycle and enter quiescence, or through apoptosis (Reichert, 2011).

Cell proliferation by self-renewing differentiated cells also occurs in *Drosophila.* Recently, it has been shown that embryonic differentiated hemocytes persist into the

larval stages to be the founders of the larval hematopoietic system. These differentiated hemocytes proliferate to constitute the population of larval hemocytes (Makhijani et al., In Press). Cell proliferation by differentiated cells also occurs in *Drosophila* tracheal development as the postmitotic differentiated Tr2 tracheal cells in young larvae re-enter the cell cycle to generate the dorsal air sacs during third larval instar (Guha et al., 2008;Weaver and Krasnow, 2008).

Cell Death in Development

PCD plays an essential role in regulating cell number in animal development by counteracting cell proliferation and/or cell survival (Jacobson et al., 1997;Raff et al., 1994) and through this PCD helps to eliminate unneeded structures, excess or abnormal cells, and also to sculpt structures (Jacobson et al., 1997;Salas-Vidal et al., 2001).

PCD removes unneeded or excess immune cells which many potentially cause autoimmunity if left unattended. PCD is seen as a basic mechanism of central tolerance in the immune system where the negative selection of self-reactive T and B clones (in the thymus and bone marrow/spleen respectively) are eliminated by apoptosis through the lifetime of the mammals (Feig and Peter, 2007;Jacobi and Diamond, 2005). In response to pathogens, the immune cells usually undergo a rapid activation-induced expansion, followed by a contraction phase as infection resolves (Feig and Peter, 2007). This downmodulation of immune response in the peripheral immune system is an apoptotic process called activation-induced cell death, which is a mechanism to remove excess immune cells to prevent autoimmunity (Hildeman et al., 2002;Pellegrini et al., 2003).

PCD also shapes the nervous system by eliminating excess cells. In the developing nervous system, target-derived survival factors called neurotrophic factors regulate the balance between cell proliferation and cell death (Raff et al., 1993;Voyvodic, 1996). Both neurons and oligodendrocytes are produced in excess during neurogenesis. As many as half of the neurons created during embryogenesis will be eliminated during development as they fail to innervate their target cells, ensuring that the number of neurons does not exceed the number of their target cells (Barde, 1989;Oppenheim, 1991). A similar culling process also occurs with oligodendrocytes so that their number matches the number of axons they myelinate (Barres et al., 1992). PCD also plays a role in sculpting structures. For example, digits of the higher vertebrates are formed by eliminating the cells between developing digits through a process known as interdigital cell death (ICD) (Garcia-Martinez et al., 1993;Salas-Vidal et al., 2001).

In *Drosophila*, PCD has a similar role in development to remove unneeded or excess cells. In the central nervous system (CNS), the NBs in the developing ventral nerve cord (VNC) undergo apoptosis in late embryogenesis (Buss et al., 2006;Truman and Bate, 1988;White et al., 1994). Of the 30 NBs originally present in the VNC, only 3 remained at the end of embryogenesis (Truman and Bate, 1988). PCD occurs in oogenesis to limit the number of eggs, particularly under poor developmental or environmental conditions (McCall, 2004). In metamorphosis, ecdysone signaling induces the histolysis of unneeded larval tissues, such as salivary glands (Jiang et al., 1997) and neurons (Schubiger et al., 1998;Truman et al., 1994;Winbush and Weeks, 2011). PCD also plays a role in sculpting structures in *Drosophila*, for example in morphogenesis of structures such as leg and

head (Lohmann et al., 2002;Manjon et al., 2007), and during eye development to remove surplus cells so as to achieve a precise retinal lattice (Wolff and Ready, 1991)

Cell Proliferation and Cell Death in Cancer and Disease

The dysregulation of cell number control is observed in many diseases (Reed, 2002;Thompson, 1995). Cancer arises from genetic alterations in normal cells that lead to the activation of oncogenes and/or the inactivation of tumor suppressor genes. Because of these mutations, over-proliferation of cells, caused by the activated oncogenes is not being counteracted by the mutated tumor suppressor genes or anti-apoptotic genes (Evan and Littlewood, 1998). The resulting earmark of the mutated cells is self-sufficiency in proliferation signals and resistance to both anti-proliferative signals and apoptosis (Hanahan and Weinberg, 2000;Hanahan and Weinberg, 2011). Because of these selective advantages, the neoplastic cancer cells eventually outgrow their normal counterparts.

Oncogenes promote cell proliferation and cell survival by various mechanisms and typically fall into 4 classes (Urbain, 1999)- (1) growth factor, e.g. platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), (2) growth factor receptor, e.g. epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR), (3) Signal transduction protein and (4) Transcription factor.

Growth factor/growth factor receptor signaling such as PDGF/PDGFR and VEGF/VEGFR signaling can be oncogenic and are implicated in numerous human

cancers (see below). Examples of oncogenic signal transduction proteins are PI3K, Akt and Ras. Class I phosphoinositide 3-kinase (PI3K) is crucial in cancer as it promotes cell survival and growth (Engelman et al., 2006;Engelman, 2009;Vivanco and Sawyers, 2002). PIK3CA, the gene encoding PI3K catalytic subunit, undergoes gain of function mutation and the mutation is found frequently in common cancers such as colorectal cancer, glioblastomas, gastric cancers, hepatocellular carcinomas, breast cancers, etc (Samuels et al., 2004;Samuels and Ericson, 2006). Akt plays a central role in mediating cell survival and cell proliferation that. when dysregulated. can contribute to the development of cancer. Human tumors (e.g. ovarian carcinomas) exhibit an amplification and overexpression of Akt (Bellacosa et al., 1995;Cheng et al., 1992). Hyperactivation of all Akt isoforms (i.e. Akt1, Akt2 and Akt3) has been shown to correlate with cancers in the ovary, breast and prostate (Nakatani et al., 1999;Sun et al., 2001;Yuan et al., 2000). Mutated Ras gene remains in a constitutively active state. As Ras interacts with several families of effector proteins, mutant Ras promotes incessant downstream signals that drive both continuous cell proliferation and cell survival (Downward, 2003) which promotes the pathogenesis of many human cancers in thyroid gland, pancreas, lung, colon, myeloid cells, etc (Bos, 1989;Downward, 2003). c-Myc, an oncogenic transcription factor, is an important positive regulator of cell cycle (Amati et al., 1998) and when gene expression is dysregulated, cell cycle continues to progress in the absence of growth factor (Lutz et al., 2002), thus causing malignancies such as Burkitt's lymphoma, medulloblastoma, breast cancer and lung cancer (Albihn et al., 2010;Erikson et al., 1983).

In contrary to oncogenes, tumor suppressor genes have roles in restricting cell proliferation or promoting apoptosis during normal development (Sherr, 2004), and their loss-of-function initiates or contributes to tumorigenesis. Examples of well-characterized tumor suppressors are phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and Tuberous sclerosis complex1/2 (TSC1/2), p53 and retinoblastoma tumor suppressor protein (RB). PTEN is a suppressor of the PI3K/Akt signaling cascade (Vivanco and Sawyers, 2002). Somatic deletions and mutations of PTEN have been identified in many tumors such as prostate carcinoma and ovarian cancer (Di Cristofano and Pandolfi, 2000). TSC is a suppressor of TOR/S6K regulated growth (Saucedo et al., 2003;Stocker et al., 2003). TSC autosomal dominant mutation causes giant cells and hamartomas (i.e. benign tumors) to grow in the brain and other vital organs such as eyes and lungs (Hengstschlager et al., 2001;Ito and Rubin, 1999;Onda et al., 2002;Potter et al., 2001). p53 is a cell cycle 'checkpoint' that induces cell cycle arrest in the G1 phase or caspase-dependent apoptosis upon DNA damage or stress (Schuler and Green, 2001). Inactivation of tumor suppressor gene, p53, is an almost universal step in the development of human cancers in colon, lung, breast, hematopoietic tissues, etc (Hollstein et al., 1991;Lane, 1992;Malkin et al., 1990). RB suppressed the cell cycle progression at the G1 phase by inhibiting transcription factor E2F which regulates various downstream cell cycle genes (Leone et al., 1998). RB loss-of-function has been identified in a wide spectrum of tumors such as retinoblastoma, osteosarcomas, lung carcinomas, breast carcinomas, etc (Nevins, 2001).

Another example of where elevated cell numbers cause disease is autoimmune disease,

which arises when there is a failure to eliminate autoreactive T, and B cells during negative selection, in the thymus and bone marrow/spleen respectively, of an adult mammal (Bouillet et al., 2002;Enders et al., 2003;Nossal, 1994). These autoreactive cells target self-antigens, which allow them to survive and mistakenly attack and destroy healthy tissue (Marrack et al., 2001).

Disease also arises when there are too few cells or cells die too early. Myelodysplastic syndromes are hematologic diseases involving the ineffective production of myeloid class of blood cells (Raza.A et al., 2010;Sugimori.C et al., 2010). The cause of this is suggested to be due to premature apoptotic death of hematopoietic cells induced by extrinsic factor (i.e. pro-apoptotic cytokines) or intrinsic factor (deregulated ribosomal biosynthesis leading to activation of p53) (Barlow et al., 2010;Pellagatti et al., 2010;Raza.A et al., 2010). Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) result from a progressive degeneration and death of the nerve cells by apoptosis (Gervais et al., 1999;Gervais et al., 2002;Inoue et al., 2003). Since neurons do not usually regenerate and replace themselves, neurologic symptoms ensue as cell numbers decrease.

Drosophila has been used as a model organism in studying the function of genes involved in various human diseases (Bier, 2005). *Drosophila* has also emerged as a model for analyzing human cancers as the model system reflects the overproliferation and aberrant developmental characteristics of human cancer (Brumby and Richardson, 2005;Brumby et al., 2011;Halder and Mills, 2011;Potter et al., 2000).

Drosophila has been instrumental in unveiling numerous oncogenic signal transducers in the Ras/MAPK pathway, through the study of *Drosophila* sevenless (sev) RTK signaling. By using sensitized genetic screens, epistasis and biochemical analysis, many of the downstream proteins in sev signaling have been identified and ordered. Examples include drk/Grb2 (Olivier et al., 1993;Simon et al., 1993), Sos (Bonfini et al., 1992;Rogge et al., 1991;Simon et al., 1991), Gap1 (Gaul et al., 1992), Ras (Simon et al., 1991), phl/Raf (Dickson et al., 1992), dsor/Mek (Hsu and Perrimon, 1994;Tsuda et al., 1993), rl/MAPK (Biggs et al., 1994;Brunner et al., 1994), dMkp3/Mkp3 (Kim et al., 2002;Rintelen et al., 2003), PTP-ER/HE-PTP (Karim and Rubin, 1999). The Ras/MAPK pathway is conserved as complementary biochemical analysis done in mammalian cell lines and two-hybrid system show similar interactions and order amongst the mammalian counterparts (Gronda et al., 2001;Howe et al., 1992;Kolch et al., 1991;Moodie et al., 1993;Thomas et al., 1992;Vojtek et al., 1993;Zhang et al., 1993).

Drosophila is also used to study oncogenic RTK mediated cancers. Gliomas is caused by gain of function mutations that activate epidermal growth factor receptor (EGFR) and PI3K signaling pathway (Furnari et al., 2007). By coactivating EGFR-Ras and PI3K in *Drosophila* glia, Read et al created a *Drosophila* model to study human glioma (Read et al., 2009). Using clonal analysis in *Drosophila*, researchers are able to understand how juxtaposed wild type and mutant cells behave. This was tested in the studies on *Drosophila* c-Myc homolog, dMyc, which showed cells expressing higher levels of dMyc have a competitive advantage and induced the nearby wild type cells or cells with lower

levels of dMyc to undergo apoptosis (de la Cova et al., 2004;Moreno and Basler, 2004). These studies suggest that potentially malignant cells may have a growth advantage to create or expand tumors (Moreno and Basler, 2004). *Drosophila* possesses a homolog of mammalian oncogene cyclin D1 (also know as PRAD1) (Finley et al., 1996;Motokura et al., 1991;Wang et al., 1994). In trying to understand the role of cyclin D1 in cancer, studies in *Drosophila* suggest it is involved in promoting growth via the accumulation of cellular mass, which is consistent with the findings in mammals (Datar et al., 2000;Meyer et al., 2000).

Drosophila model system has increased our understanding of oncogenes, and has also been successfully exploited in the study of tumor suppressors. The characterization of fly homologs of TSC2 (i.e. gigas) and PTEN showed that these genes display a similar role as their mammalian homologs (Huang et al., 1999;Ito and Rubin, 1999) in negatively regulating cell proliferation, thus underlining the conserved molecular and biochemical properties of cancer-causing genes across model organisms. The RB signaling network in *Drosophila* appears as a simplified version of the mammalian RB network with all essential genes in place. However, functionally, *Drosophila* RB displays a function similar to its mammalian homolog since it negatively regulates the entry from G1 to S phase (Du, 1999). *Drosophila* p53 (dmp53) shows similar function to its mammalian counterpart by stimulating apoptosis upon genotoxic stress (Brodsky et al., 2000;Jin et al., 2000) but it lacks the cell cycle arrest function (Ollmann et al., 2000).

Drosophila is also used to study the cooperation between oncogenes and cell polarity

genes in promoting tumorigenesis and driving metastasis. In an oncogenic background, loss of cell polarity genes, such as tumor suppressor scribbled, leads to the activation and switch of JNK signaling- from proapoptotic to progrowth, which together in cooperation with the activated oncogenic Ras or Notch, drives metastasis and tumor growth (Brumby and Richardson, 2003;Igaki et al., 2006;Pagliarini and Xu, 2003;Wu et al., 2010). These studies suggest that similar cooperative mechanisms could have a role in the development of human cancers.

In investigating other human diseases such as human neurodegenerative diseases, fly models are useful as they showed similar phenotypes and symptoms to the human (Bilen and Bonini, 2005), such as Iijima et al, showed *Drosophila* to be a potential model for understanding the molecular mechanism of Alzheimer's disease (AD) as they are able to recapitulate the essential features of AD by expressing human amyloid-β peptides (a peptide suggested to cause AD pathogenesis) in the *Drosophila* brain (Iijima et al., 2004).

The role of PDGF/VEGF receptors in development and disease

PDGF/VEGF receptors in development

In mammals, the members of the PDGFR subfamily are- PDGFRα, PDGFRβ, CSF1R/c-Fms, c-Kit and Flt3. The VEGFR subfamiliy consist of three members- VEGFR1, VEGFR2 and VEGFR3 (Lemmon and Schlessinger, 2010).

PDGFR signaling is involved in hematopoiesis, as well as numerous processes such as embryogenesis and organogenesis (Andrae et al., 2008). In PDGF-B and PDGFR-β

knockout mice hematological defects such as severe anemia (Leveen et al., 1994;Soriano, 1997) occurs. However, it has also been suggested that the hematological defects could be due to impaired liver growth which leads to decreased production of blood cells (Kaminski et al., 2001). PDGFR-β is expressed in early hematopoietic/endothelial (hemangio) precursors and regulates vascular/hematopoietic fate (Rolny et al., 2006). The loss of PDGFR-α in the neural crest leads to drastic developmental defects in the mouse tissues originating from the neural crest mesenchyme due to cell death and defective cell migration (Morrison-Graham et al., 1992;Richarte et al., 2007;Soriano, 1997). For its involvement in organogenesis, PDGFR signaling plays a role in alveogenesis (Bostrom et al., 2002;Noguchi et al., 1989), neurogenesis (Calver et al., 1998) and vasculogenesis (Hellstrom et al., 1999;Lindahl et al., 1997).

FLT3 is expressed in human/mouse HSCs and early progenitors of both myeloid and B-lymphoid lineage (Buza-Vidas et al., 2011;Kikushige et al., 2008;Rosnet et al., 1996;Turner et al., 1996). FLT3 signaling is suggested to enhance the survival, regulate the expansion and self-renewal capacity of the hematopoietic progenitor cells (Kikushige et al., 2008;Rasko et al., 1995;Weisel et al., 2007), and in B lymphoid differentiation (Mackarehtschian et al., 1995).

c-kit is expressed in HSCs and more committed progenitors, including dendritic, erythroid, megakaryotic, myeloid, natural killer progenitor cells, and pro-B and pro-T cells, and mature mast cells (Lennartsson et al., 2005;Lyman and Jacobsen, 1998). The complete loss of c-kit expression leads to utero or perinatal lethality due to severe anemia

(Ronnstrand, 2004). c-Kit signaling alone or in combination with other factors (e.g. cytokines) regulates BM HSCs survival, self-renewal, growth, differentiation and mobilization (Ema et al., 2000;Heissig et al., 2002;Papayannopoulou et al., 1998;Roskoski, 2005;Simmons et al., 1994).C-kit signaling promotes the survival of the committed progenitors (Lennartsson et al., 2005).

Expression of colony stimulating factor-1 receptor (CSF-1R) or c-FMS appears in monocyte/macrophage (Stanley et al., 1997). CSF-1 stimulates proliferation, differentiation and survival of the monocytes-macrophages lineage (Sherr, 1990;Stanley et al., 1997). CSF-1 also enhances the ability of macrophages to perform their differentiated functions by potentiating their ability to eliminate pathogens.

VEGF/VEGFR signaling plays a role in the following developmental processes- hematopoiesis, vasculogenesis, angiogenesis and lymphangiogenesis. In early hematopoietic development, VEGFR-2 regulates the migration of precursor cells into a microenvironment that forms the blood island, thus allowing hematopoiesis to take place (Shalaby et al., 1997). In adult hematopoiesis, VEGFA/VEGFR forms an autocrine signaling loop that controls the survival of HSCs (Gerber et al., 2002). During transplantation of bone marrow (BM) HSCs, VEGFR1 expressed on the HSCs convey chemotactic signals for recruitment of HSCs to BM, thus reconstituting hematopoiesis (Hattori et al., 2002).

Heterozygous VEGF deficient ($VEGF^{+/-}$) mice display impairment in early vascular

development such as differentiation of blood islands, angiogenesis and lumen formation. (Carmeliet et al., 1996;Ferrara et al., 1996). Inhibiting VEGFR signaling in adult mice causes capillary regression in various organs to occur (Kamba et al., 2006), thus VEGFR signaling regulates the survival of capillaries. VEGF is pivotal in organ development due to its role in angiogenesis, such as glomerulogenesis during kidney development. Loss of VEGFR signaling induces glomerular defects (Eremina et al., 2003;Kitamoto et al., 1997;Sison et al., 2010). VEGF-C/VEGFR3 signaling is suggested to be specifically required for lymphangiogenesis- the formation of first lymphatic-vessel sprouts from embryonic veins (Karkkainen et al., 2004).

The *Drosophila* genome encodes only one homologue of the vertebrate PDGF and VEGF receptor families, known as PDGF/VEGF Receptor (PVR) (Duchek et al., 2001;Heino et al., 2001). PVR has three ligands- PDGF/VEGF factor 1 (PVF1), PDGF/VEGF factor 2 (PVF2) and PDGF/VEGF factor 3 (PVF3) (Duchek et al., 2001;Heino et al., 2001). PVR signaling is involved in hematopoiesis and is required for embryonic hemocyte survival (Brückner et al., 2004), hemocyte proliferation (Munier et al., 2002, migration (Brückner et al., 2004 ;Cho et al., 2002 ;Heino et al., 2001;Wood et al., 2006) and differentiation (Jung et al., 2005a) (see below).

PDGF/VEGF receptors in cancer

Studies show that self-sufficiency in growth signals generated by PDGFR/VEGFR family can lead to blood cancers, as well as other malignant cancers.

Gain-of-function mutations in PDGFR family genes induce self-sufficient growth signals that drive acute myeloid leukemia (AML). First, mutations in the tyrosine kinase (TK) domain or juxtamembrane region are suggested to relieve the cis-autoinhibitory interactions in the TK domains which then allow the constitutive activation of the c-Kit receptor (Corless and Heinrich, 2008), PDGFR-α (Heinrich et al., 2003;Hirota et al., 2003) or FLT3 (Yamamoto et al., 2001). Also, mutations in the extracellular region of c-Kit and in the juxtamembrane of FLT3 are suggested to stabilize the receptor dimerization in the absence of their ligand, leading to a constitutively active receptor (Corless and Heinrich, 2008;Kiyoi et al., 2002). These PDGFR mutations are also highlighted in gastrointestinal-stromal tumors (GIST) and melanoma.

Translocations of PDGFR gene also drive blood cancers. TEL/PDGFR-β fusion gene, found in chronic myeloid leukemia (CML) patients, is a chimeric protein with an active TEL promoter driving the expression of the juxtaposed truncated PDGFR-β, causing it to be constitutively active (Dash and Gilliland, 2001;Golub et al., 1994). Fusion of the FIP1L1 and PDGFR-α genes is found in a subset of patients with hypereosinophilic syndrome (Cools et al., 2003;Griffin et al., 2003). The mechanism of activation for FIP1L1- PDGFR-α tyrosine kinase activity is suggested to be FIP1L1 mediated homodimerization which serves to constitutively activate the PDGFR-α (Gotlib et al., 2004) or the deletion of an autoinhibitory PDGFR-α juxtamembrane domain (Stover et al., 2006).

Translocations of PDGF gene also drive cancers such as dermatofibrosarcoma

protuberans where collagen type 1 α1 chain (COL1A1) and PDGF-B genes fused (Simon et al., 1997), such that PDGF-B gene is overexpressed as driven by COL1A1 promoter which creates an autocrine stimulatory loop that promotes cell proliferation (Sirvent et al., 2003). The overexpression of PDGFs (Dai et al., 2001;Lindberg et al., 2009;Uhrbom et al., 1998) and PDGFR-α (Hermanson et al., 1996;Smith et al., 2000) has been implicated to drive the formation of glioma-like tumors. Inhibition of PDGFR slows down tumor growth (Servidei et al., 2006).

VEGF/VEGFR signaling is also associated with hematopoietic malignancies such as AML, CML, T cell lymphoma, Burkitt's lymphoma, etc (Bellamy et al., 1999). In the case of AML, the co-expression of VEGF and VEGFR(s) by the malignant myeloid precursors forms an autocrine and a paracrine signal that drives the malignancy (Bellamy et al., 2001;Fiedler et al., 1997). VEGF/VEGFR signaling has also been associated with other cancers (e.g. skin, colon, breast, lung, etc), implied by the upregulation of VEGF mRNA expression in their tumors (Berger et al., 1995;Ho and Kuo, 2007). Since VEGF is a survival factor (Harmey and Bouchier-Hayes, 2002), under stressed conditions (e.g. hypoxia, radiotherapy or chemotherapy), tumor cells secrete elevated levels of VEGF to enhance their own survival (Katoh et al., 1998;Riedel et al., 2004).

VEGFR signaling drives angiogenesis. As tumors require a vascular supply to provide nutrients and oxygen, they secrete VEGF to promote tumor angiogenesis and vasculogenesis forming new tumor vasculature (Dvorak et al., 1995;Gerber and Ferrara, 2003). The newly formed vasculature allows metastases by providing a route for tumor

cells to enter the blood circulation. In addition, VEGF-C/VEGFR-3 signaling promotes tumor lymphangiogenesis which facilitates lymphatic metastasis (Saharinen et al., 2004). Blocking VEGF or VEGFR is sufficient to prevent tumor angiogenesis and growth (Ferrara et al., 2003;Holash et al., 2002).

1.2 Mechanisms of cell number control

Mechanism of cell apoptosis in vertebrates and *Drosophila*

There are at least three main forms of programmed cell death: apoptosis, autophagic cell death (ACD) and necrosis/necroptosis, which can be defined by their morphologically distinct cell death modalities (Galluzzi et al., 2007;Kroemer et al., 2009). Apoptosis (Type 1 cell death) display the following morphological features: rounding-up of the cell, retraction of pseudopods, pyknosis, chromatin condensation, nuclear fragmentation, minor modification of cytoplasmic organelles and plasma membrane blebbing. ACD (Type 2 cell death) displays morphology with a lack of chromatin condensation, large-scale autophagic vacuolization of the cytoplasm and accumulation of autophagic vacuoles. Necrosis (Type 3 cell death) is traditionally considered as an accidental, uncontrolled form of cell death. However, mounting evidences show that necrosis can occur in a regulated manner (thus termed necroptosis), and that it has a role in physiological and pathological situations (Vandenabeele et al., 2010). Necrosis is morphologically defined by cytoplasmic swelling, rapture of plasma and swelling of cytoplasmic organelles.

Among all forms of PCD, the subject of apoptosis has been the most extensively studied (Lockshin and Zakeri, 2001) and regarded as the principal cell death mechanism following the initial description (Wyllie et al., 1980) and discovery of the apoptotic machinery (Nicholson et al., 1995;Yuan and Horvitz, 2004). In vertebrates, cells undergo apoptosis by two major pathways - the extrinsic pathway (death receptor pathway) and

the intrinsic pathway (the mitochondrial pathway) (Jin and El-Deiry, 2005).

The extrinsic apoptotic pathway can be triggered by the ligation of the Fas or TNFR1 death receptors. Upon ligand binding, these two receptors recruit an adaptor protein (i.e., FADD or TRADD) via their intracellular death domain. The adaptor proteins then associate directly or indirectly with initiator caspase, caspase-8, to activate the apoptotic caspase cascade (Chinnaiyan et al., 1995;Micheau and Tschopp, 2003).

The intrinsic apoptotic pathway is initiated inside the cells and determined by an event in the mitochondria called mitochondrial outer membrane permeabilization (MOMP) (Green and Kroemer, 2004). MOMP is controlled by the members of the Bcl-2 family of proteins (Newmeyer and Ferguson-Miller, 2003). Bcl-2 superfamily consists of pro-apoptotic (i.e. Bax, Bak, Bim and Bid) and anti-apoptotic members (i.e. Bcl-2 and Bcl-X_L). The anti-apoptotic Bcl-2 proteins inhibit cell death by sequestering pro-apoptotic members- Bax and Bak (Cheng et al., 2001). Upon receiving death signals, (1) BH3-only derepressors such as NOXA and PUMA help to release Bax and Bak from anti-apoptotic Bcl-2 and Bcl-X_L (Bredesen et al., 2006), (2) BH3-only proteins such as Bim and Bid activate Bax and Bak (Youle and Strasser, 2008) and (3) Bax and Bak, insert into the outer mitochondrial membrane to permeabilize the membrane (Kuwana et al., 2002). During MOMP, molecules that induce apoptosis are released. One of these is cytochrome c which induces conformational changes and heptamerization of the cytosolic protein apoptosis-promoting factor 1 (APAF-1). Heptamerized APAF-1 acts as an adaptor that binds procaspase-9, forming the apoptosome which causes caspase-9 activation (Li et al.,

1997). Subsequently, caspase-9 leads to the activation of effector caspases-3 and -7 by cleavage. The effector caspase however will still be inhibited by the inhibitor of apoptosis proteins (IAPs) (Deveraux et al., 1997) if not for the IAP antagonists (i.e., SMAC and HTRA2) released also during MOMP. The IAP antagonists released IAPs inhibition on the caspases (Du et al., 2000;Verhagen et al., 2000;Verhagen et al., 2002).

As in vertebrates, apoptosis in *Drosophila* involves the activation of the caspase cascade. *Drosophila* has an IAP known as DIAP, which inhibits caspase activity by (1) sequestering the caspase from its substrates or (2) promoting the degradation of caspases (Salvesen and Duckett, 2002;Tenev et al., 2005). DIAP's role in apoptosis is in turn regulated in many ways by the pro-apoptotic IBM (IAP binding domain) proteins- reaper, grim, head involution defect (hid) and sickle (Goyal et al., 2000). One mode of regulation occurs by competing with the binding of caspases to DIAP, thus releasing the caspases to promote apoptosis (Chai et al., 2003). IBM proteins are also able to promote DIAP degradation by ubiquitination (Ryoo et al., 2002) or inhibit the translation of DIAP (Holley et al., 2002). The activation of IBM proteins is the major mechanism for apoptosis where different signaling pathways that trigger apoptosis converge (Steller, 2008). Upon downregulating DIAP, the suppression on initiator caspase such as Dronc is lifted. Free Dronc associates with adaptor protein Dark (orthologue of mammalian APAF-1) to become activated. Active Dronc triggers the effector caspase cascade thus leading to apoptosis. *Drosophila* genes Debcl and Buffy are orthologues of the pore-forming group of proapoptotic Bcl-2 family members (Sevrioukov et al., 2007) and their discovery suggest the contribution of mitochondria in cell death in *Drosophila* (Igaki and

Miura, 2004), although it still remains to be characterized (Krieser and White, 2009). Expression of the pan-caspase inhibitor baculovirus gene, p35, can prevent apoptosis in *Drosophila* (Hay et al., 1994) cells. As this phenomenon can be observed in mammals as well (Rabizadeh et al., 1993), this suggests that the caspase cascade apoptotic pathway is evolutionarily conserved between vertebrate and *Drosophila*.

Mechanism of cell proliferation in vertebrates and *Drosophila*

Cell proliferation in both *Drosophila* and vertebrate are regulated primarily by growth factors, integrin mediated adhesion and contact inhibition.

The binding of growth factors to their respective growth factor receptors initiates downstream signaling events that eventually lead to a diverse array of cellular response, including cell proliferation (Fantl et al., 1993). Two of the downstream signaling pathways that regulate cell proliferation are the PI3K/Akt and Ras/MAPK signaling pathways (Blume-Jensen and Hunter, 2001;Zhang and Liu, 2002) and are discussed in detail below.

Integrin mediated adhesion drives cell cycle progression, sometimes in cooperation with growth factor receptors. Integrin adhesion promotes proliferation by a few mechanisms- (1) preventing the degradation of RTK PDGFR (Baron and Schwartz, 2000), (2) potentiating RTK PDGFβR's activation (Moro et al., 1998;Schneller et al., 1997), (3) activating growth promoting Ras-Erk signaling pathways (Lin et al., 1997;Renshaw et al., 1997) and lastly (4) promoting the activation of genes necessary for cell cycle

progression (Roovers et al., 1999;Walker and Assoian, 2005).

Contact inhibition of proliferation is regulated by cadherin-mediated cell-to-cell adhesion (Fagotto and Gumbiner, 1996). This mechanism regulates proliferation by controlling the levels of cytosolic β-catenin; since β-catenin and its *Drosophila* homologue, Armadillo, upon translocating into the nucleus from the cytosol is able to form complexes with transcription factors to drive the expression of cell cycle genes (e.g., cyclin D) (Tetsu and McCormick, 1999).

All the mechanisms that regulate cell proliferation eventually converge at the cell cycle machinery that drive cell division. The archetypal cell cycle typically consists of four stages- G1 (gap phase before DNA replication), S (DNA replication), G2 (gap phase before mitosis) and M phases (mitosis). The cyclin dependent kinases (CDKs) and their binding partners, cyclins, control the progression of cells through the stages of the cell cycle (Nigg, 1995). CDKs are activated by forming complex with a cyclin, and this complex phosphorylates specific proteins that regulate events (e.g. DNA synthesis, chomosome condensation, spindle assembly, etc) necessary for stage-by-stage progression through the cell cycle (Nigg, 1995).

In order to manipulate cell numbers for the purpose of treating or managing diseases, we need to understand more about the mechanisms of cell number control in terms- cell apoptosis and cell proliferation. However, it is also essential that we look further upstream, at the signals that feed into the apoptotic and proliferation machinery. For that,

the remaining of this section focuses on receptor tyrosine kinase signaling and two of downstream signaling cascades which are known to regulate cell survival and cell proliferation.

Receptor Tyrosine Kinase Signaling

Receptor tyrosine kinases (RTKs) play an important role in regulating many different processes in the cell including cell survival, cell proliferation, differentiation and cell migration (Schlessinger, 2000). Despite the existing knowledge, much remains to be learned about the complex signaling networks downstream of RTKs and how these networks regulate different cellular responses such as apoptosis and cell proliferation.

In humans, to date, there are 58 known RTKs divided into 20 subfamilies (Lemmon and Schlessinger, 2010). All RTKs are made up of a similar structural framework but different functional domains, i.e. a ligand-binding domain in the extracellular region, a transmembrane helix and a cytoplasmic region consisting of the tyrosine kinase (TK) domain and carboxy terminal and juxtamembrane regulatory regions (Lemmon and Schlessinger, 2010;Ullrich and Schlessinger, 1990). For example, platelet derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR) have an extracellur ligand-binding domain made up of Ig domains (5 for PDGFR and 7 for VEGFR), a transmembrane helix and a split TK domain (Lemmon and Schlessinger, 2010).

Upon binding of ligand, RTK is activated. The classical view of RTK activation is that

the ligand itself is bivalent (i.e. dimer), thus it binds simultaneously with two RTKs "crosslinking" them to facilitate receptor dimerization (Lemmon and Schlessinger, 2010;Schlessinger, 2000). After RTK dimerization, the activation of the TK domains takes place. Unliganded RTKs adopt a cis-autoinhibitory interaction called juxtamembrane autoinhibition (Griffith et al., 2004;Mol et al., 2004). Receptor dimerization induces the 'first phase' of trans-phosphorylation of key tyrosines in the juxtamembrane region which interferes the autoinhibition, thus permitting RTK activation (Hubbard, 2004). The second phase of transphosphorylation in TK domains creates phosphotyrosine-based docking sites which recruit downstream signaling molecules that bind directly (via their SH2 or PTB domains) or indirectly (via docking proteins) (Heldin, 1995;Pawson, 1995).

RTKs regulates a plethora of downstream signaling pathways (e.g. PI(3)K/Akt , Ras/MAPK, JAK/STAT, Ca^{2+} signaling) (Lemmon and Schlessinger, 2010;Schlessinger, 2000). Below I describe in detail the PI(3)K/Akt and Ras/MAPK signaling pathways that are conserved between mammals and *Drosophila*.

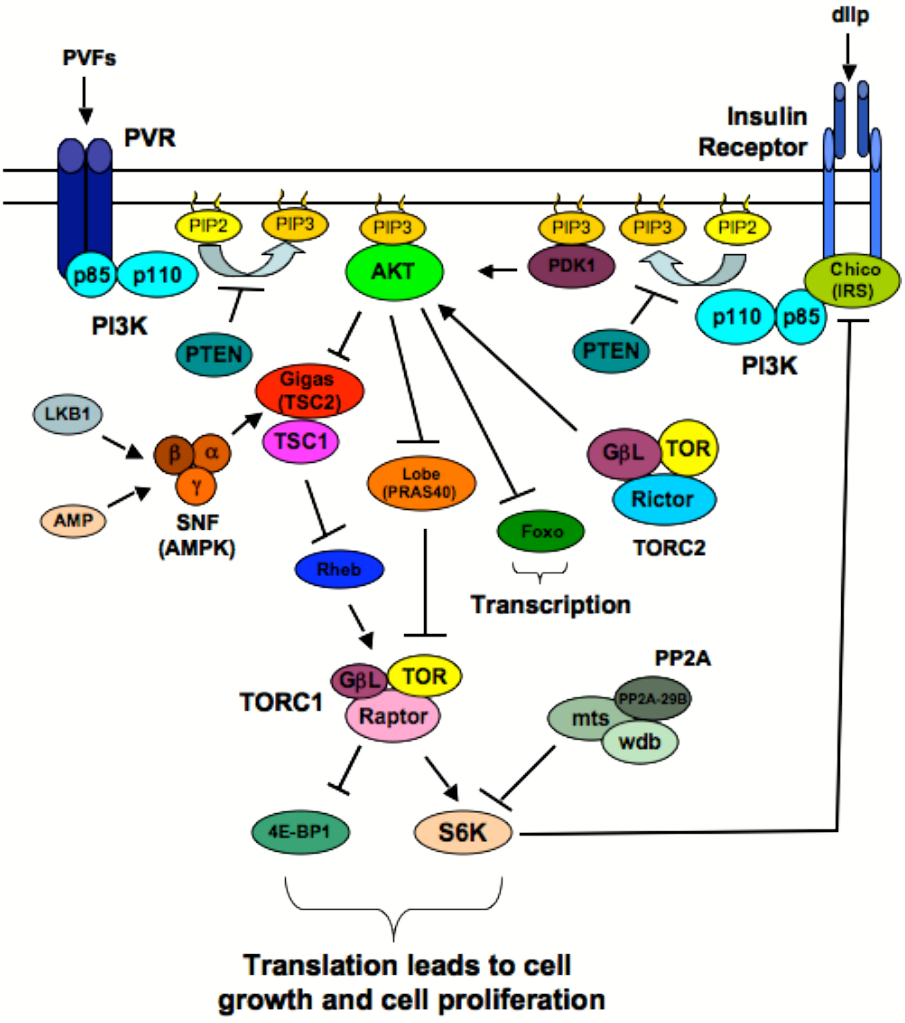
PI3K/Akt Pathway.

The PI3K/Akt signaling cascade regulates both cell proliferation and cell survival. The major components of this pathway are PI3K, Akt, TOR complex 1 (TORC1) and S6K (Figure 1.1).

Class 1 PI3Ks are heterodimers comprising of a catalytic p110 subunit and adaptor

proteins (p85α, p85β and p55γ). PI3K is activated through direct binding with specific phosphotyrosine consensus motifs in RTK's TK domains or to Chico, in the case of insulin signaling. PI3K phosphorylates membrane lipid PtdIns(3,4,)P_2 (PIP2) to generate PtdIns(3,4,5)P_3 (PIP3) (Vanhaesebroeck et al., 1997), whereas PTEN, a tumor suppressor with lipid phosphatase activity, works antagonistically (Furnari et al., 1998;Maehama and Dixon, 1998;Myers et al., 1998) to PI3K. Both PDK1 and TOR complex 2 (TORC2) activate Akt by phosphorylation (Alessi et al., 1997;Stokoe et al., 1997), which will phosphorylate three substrates- TSC2, PRAS40 and Foxo.

Figure 1.1 PI3K/Akt signaling cascade. See text for description



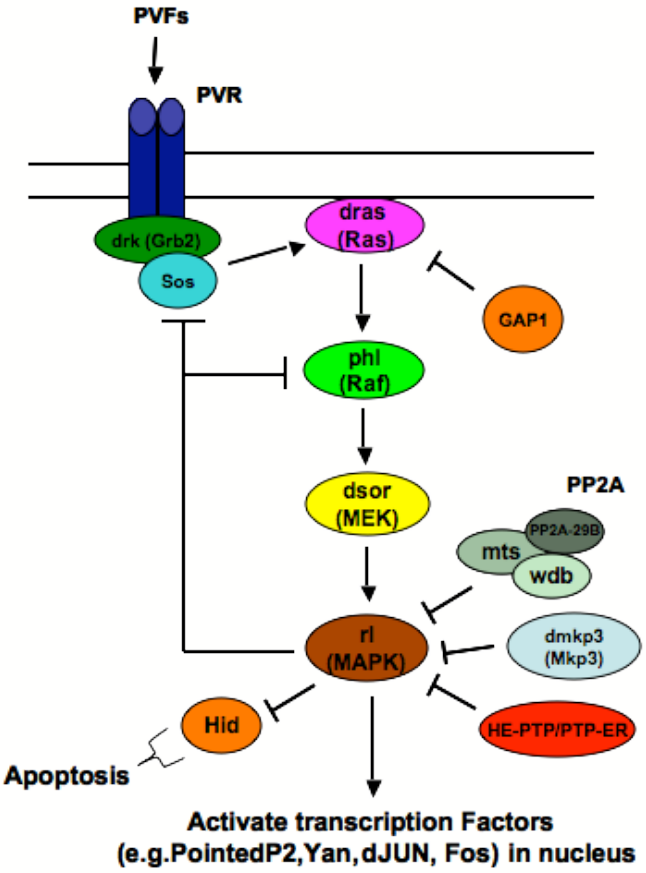
Rheb is an essential regulator of S6K who is inhibited by the GTPase activity of TSC1/2 complex (Saucedo et al., 2003;Stocker et al., 2003). Phosphorylation of TSC2 by Akt (Potter et al., 2002) inhibits the formation of TSC1/2 complex, thus permitting activated Rheb-GTP to activate TORC1 (Manning and Cantley, 2003), which phosphorylates the downstream S6K and 4E-BP (Brunn et al., 1997;Burnett et al., 1998) thus driving cell growth and cell proliferation (Brennan et al., 1999;Dufner and Thomas, 1999;Feng et al., 2000). PRAS40 is an inhibitor of TORC1 (Sancak et al., 2007). Akt inhibits PRAS40 by phosphorylating it, thus allowing activation of TORC1 (Kovacina et al., 2003;Sancak et al., 2007). Akt phosphorylation of Foxo transcription factor promotes cell survival (Tran et al., 2003).

Tumor suppressor LKB1 activates TSC2 indirectly by phosphorylating and activating a heterotrimeric complex, AMPK (Shaw, 2003; Woods, 2003). Subsequently, the activated AMPK phosphorylates and activates the TSC protein (Inoki et al., 2003). AMPK is also allosterically activated by the accumulation of AMP molecules (i.e. high AMP:ATP ratio) when the cell is subjected to metabolic stress (Hardie, 2007). Protein phosphatase PP2A inhibits S6K (Bielinski and Mumby, 2007).

Ras/MAPK Pathway.

The major components of this signaling cascade are Ras, Raf, MEK and MAPK (Figure 1.2).

Figure 1.2. Ras/MAPK signaling cascade. See text for description.



The activation of Ras, a guanine nucleotide-binding signal transducer, is an essential early step in signaling cascade initiated by RTKs. For many RTKs, Ras activation is dependent on the binding of adaptor protein Grb2 (via SH2 domian) to tyrosine-phosphorylated RTK. Grb2 recruits Sos, a guanine nucleotide exchange factor, via its two SH3 domains and this brings Sos close to Ras. Sos activates Ras by catalyzing the exchange of GTP for GDP in Ras. The active GTP-bound Ras then stimulates a conserved protein kinase cascade consisting of Raf, MEK and MAPK (McKay and Morrison, 2007). Activated MAPK will translocate into the nucleus to phosphorylate and activate transcription factors that control cellular responses to extracellular signals

(Rubinfeld and Seger, 2005;Seger and Krebs, 1995). MAPK also phosphorylate death gene, Hid, to suppress cell death (Bilak and Su, 2009). A few of the negative regulators of Ras/MAPK pathway are GAP1 (Gaul et al., 1992) which increases the intrinsic GTPase activity of Ras, hence inactivating Ras; and Mkp3 (Kim et al., 2002;Rintelen et al., 2003), PTP-ER (Karim and Rubin, 1999) and PP2A (Alessi et al., 1995) which catalyze the dephosphorylation of MAPK.

Regulatory loops and feedback mechanisms in RTK signaling

RTK signaling can be attenuated by negative feedback mechanisms through (1) expression of inhibitors (e.g. Argos, Kekkon 1 and Sprouty), or (2) modulation of the activity of signaling mediators within the pathway (e.g. MAPK and S6K modulates activity of Sos and Raf, and IRS upstream mediators).

(1) *Expression of feedback inhibitors*.

Argos and Kekkon 1 expression is induced by EGFR activation (Ghiglione et al., 1999;Musacchio and Perrimon, 1996;Schweitzer et al., 1995) and both were shown to negatively regulate *Drosophila* EGFR signaling by different mechanisms- Argos sequesters EGFR activating ligand (Klein et al., 2004) and Kekkon1 inhibits ligand binding and autophosphorylation of EGFR (Ghiglione et al., 1999;Ghiglione et al., 2003), thus both proteins form a negative feedback loop to downregulate *Drosophila* EGFR signaling. Sprouty was identified as an inhibitor of Ras/MAPK signaling (Casci et al., 1999;Mason et al., 2006). Growth factor stimulation activates the Ras/MAPK pathway which leads to the transcription of target genes, one of which is Sprouty. Sprouty, in a

negative feedback loop is able to inhibit Ras/MAPK pathway by inhibiting Ras and Raf activation (Gross et al., 2001;Mason et al., 2006).

(2) Modulation of the activity of signaling mediators within signaling pathway.

In Ras/MAPK signaling pathway, MAPK is able to phosphorylate the upstream regulators, Sos and Raf, to dampen the Ras/MAPK signaling pathway (Buday et al., 1995;Ueki et al., 1994) (Figure 1.2). In PI3K/Akt/mTOR signaling an important negative regulatory feedback loop is mediated by S6K. Studies show that high levels of constitutive mTOR activity strongly downregulate insulin/IGF-1 signaling because of the inactivation of IRS-1 (insulin receptor substrate-1) exerted through S6K-dependent suppression of gene transcription and by direct phosphorylation by S6K in a region critical for IRS-1 adaptor function (Harrington et al., 2004), as well as a downregulation of IRS-1/2 levels (Shah et al., 2004) (Figure 1.1). Shah et al also found that expressing a kinase-dead version of S6K1 can block this negative feedback loop and activate Akt (Shah et al., 2004). Recent findings by Kockel et al, show that negative feedback signaling to Akt is S6K-independent in wild-type fly but instead mediated by TSC1/TSC2 and TORC1, and S6K-dependent in conditions with high TORC1 activity (Kockel et al., 2010).

RTK signaling has the capability to amplify signals through positive feedback loops in the signaling pathway. First, RTK does this by inactivating negative regulators, such as protein tyrosine phophastases (PTP), of the signaling pathway. For example, EGFR signaling results in localized production of reactive oxygen species (ROS), which inhibit

PTP activity by oxidizing the active site of the phosphatase (Tonks, 2006). Secondly, a positive feedback loop can recruit more signal activators to enhance RTK downstream signaling (e.g. Gab1 recruitment for sustained EGFR signaling (Rodrigues et al., 2000). Gab1 is recruited to activated-EGFR via adaptor protein Grb2, whereupon it is tyrosyl phosphorylated to recruit PI3K p85 subunit. This facilitates the activation of PI3K that leads to the generation of PIP3 lipids, which promotes the translocation of more PH domain-containing Gab1 (Gu, 2003), thus increasing PI3K activation. Thirdly, autocrine signaling provides another positive feedback loop. For example, EGFR activation of Ras-MAPK pathway stimulates the production of ligands EGF or ErbB that bind to EGFR (Shilo, 2005).

1.3 *Drosophila melanogaster* as a model organism

Advantages of *Drosophila* as a model organism

Using *Drosophila* as a model organism traces back to more than a century ago to the studies by Thomas Hunt Morgan. Today, labs all over the world capitalize on this powerful model system, which has numerous advantages. (1) *Drosophila* has a very short generation time (roughly 10 to 12 days from embryo to adult), is easy to maintain in large populations, and has a low cost of upkeep. (2) *Drosophila* has low genetic redundancy, as vertebrates often have 2 or more homologous genes that mask the phenotype of a single mutant (Bier, 2005). This allows the analysis of complex signaling networks and translates the findings to identify novel drug targets for human diseases. (3) Despite its simplicity, *Drosophila* shows a high degree of evolutionary conservation with vertebrates regarding biological and cellular processes and genes that control basic developmental programs and that are also related to human disease (Bier, 2005). A survey of 287 human disease genes showed that 62% have homologues in *Drosophila*, also for cancer genes, 47 out of 65 (72%) have homologues in *Drosophila* (Fortini et al., 2000). (4) *Drosophila* has outstanding publicly shared resources and genetic tools allowing classical genetic and transgenic manipulations (Adams and Sekelsky, 2002;Parks et al., 2004;Ryder et al., 2004;Ryder et al., 2007;Venken and Bellen, 2005), and large public stock collections (e.g. Bloomington Stock Center, VDRC, etc) of strains with defined genotypes (e.g. deletions, GAL4/UAS transgenic for overexpression, P-element inserted, etc) for in vivo research. *Drosophila* cDNA clones are also available publicly (e.g. BDGP). (5) For *Drosophila* RNAi, long dsRNAs are used for gene silencing in vivo (Perrimon et al.,

2010) and in cell culture (Bjorklund et al., 2006;Boutros and Ahringer, 2008;Caplen et al., 2000;Clemens et al., 2000;Friedman and Perrimon, 2006;Hammond et al., 2000;Perrimon and Mathey-Prevot, 2007a). Recently, shorter miRNAs (i.e. shRNAs), is used for in vivo silencing as it is more efficient in somatic cells and female germline than long hairpin dsRNAs (Haley et al., 2008;Ni et al., 2011). Use of RNAi in *Drosophila* will be discussed in detail in Chapter 1 introduction.

Drosophila **development**

Drosophila has a generation time of about 10 days at 25^0C. *Drosophila* life cycle consist of a number of stages- embryogenesis, three larva stages, a pupal stage and the adult stage (Ashburner et al., 2005;Campos-Ortega and Hartenstein, 1997;Hartenstein, 1995).

Following fertilization, mitosis occurs in the embryo to form a multinucleated syncytium. This is then followed by cellularization to form the blastoderm after thirteen rounds of cell division. Following that, a complex series of gastrulation and morphogenetic movement events occur to shape the body plan of the embryo to three segments- head, thorax and abdomen. The body plan of the embryo is established by segmentation genes (i.e. gap genes and pair-rule genes). And these segmentation genes regulate the expression of two groups of homeotic selector genes (i.e. Antennapedia and bithorax) which specify the properties of the three segments. At approximately 22 hours after fertilization, the embryo hatch to the 1^{st} instar larva. The larval stage has three instars, or molts. The first 2 instars last roughly 24 hours each, the third requires 3 days to complete. The 3^{rd} instar larva molts to form the pupa, where metamorphosis occurs. During

metamorphosis, histolysis of larval tissues, cellular proliferation, and differentiation of adult structures from imaginal cells take place. Metamorphosis takes about 5 days and at the end, the adult ecloses from the pupal case and is fertile about 12 hours after hatching.

Hematopoiesis in *Drosophila*

Hematopoiesis in *Drosophila* occurs in at least three waves- embryonic hematopoiesis, larval hematopoiesis, and lymph gland hematopoiesis.

The first wave occurs during embryogenesis when the hemocytes originate from the procephalic mesoderm (Holz et al., 2003a;Tepass et al., 1994b). These hemocytes differentiate into two populations of hemocytes, plasmatocytes (approximately 700 cells, comprising 95% of all embryonic hemocytes) and crystal cells. During and after differentiation, plasmatocytes migrate from the point of origin and follow an invariant pathway (Siekhaus et al., 2010;Wood et al., 2006) to be eventually scattered throughout the embryo (Tepass et al., 1994b), whereas crystal cells (around 30 cells) remain localized around the proventriculus (Lebestky et al., 2000).

Recent work has characterized the second wave of hematopoiesis (Makhijani et al., In Press). The larval hematopoietic system is founded by differentiated hemocytes of the embryo that colonize resident hematopoietic sites in the larva and expand to form the larval hematopoietic system (Makhijani et al., In Press). Indications for such a 'carry-over' of embryonic hemocytes had also been reported by others previously (Holz et al., 2003b;Honti et al., 2010).

The third wave of hematopoiesis takes place in the lymph gland (LG) (Jung et al., 2005b;Lanot et al., 2001b;Shrestha and Gateff, 1982). The thoracic mesoderm in the early embryo (Holz et al., 2003b) gives rise to hemangioblast-like cells that subsequently differentiate into LG and dorsal vessel precursors (Mandal et al., 2004). Lymph gland cells form two clusters which coalesce to form the paired primary lobes of the lymph gland in late embryogenesis; the organ then grows by cell proliferation until 2nd larval instar stages (Crozatier and Meister, 2007;Jung et al., 2005a). In early 3rd instar, the secondary lobes develop and hemocyte differentiation takes place (Jung et al., 2005a;Lebestky et al., 2000). The hemocytes remain in the lymph gland until the onset of metamorphosis, where they are released into circulation (Lanot et al., 2001a).

In *Drosophila*, there are at least three types of terminally differentiated hemocyte lineage: plasmatocytes, crystal cells and lamellocytes. Plasmatocytes, which are professional phagocytes, are the predominant hemocyte population. Their main role is phagocytosis which is carried out during (1) development by engulfing apoptotic corpses and unwanted tissues (Franc et al., 1996;Franc et al., 1999;Lanot et al., 2001a;Rizki and Rizki, 1980) and (2) cell-mediated immunity by carrying out phagocytosis of invading pathogens (Hoffmann and Reichhart, 2002). In response to infection, plasmatocytes mediate innate immunity by releasing antimicrobial peptides (Ramet et al., 2002) and cytokines such as upd3 and spz to signal to the larval fat body (Agaisse et al., 2003;Irving et al., 2005) to secrete antimicrobial peptides. Plasmatocytes have important roles in wound healing. They migrate to wounds by chemotactic attraction (Moreira et al., 2010;Stramer et al.,

2005;Wood et al., 2006) and a subpopulation of plasmatocytes (and crystal cells) participates in wound healing through the initiation of clotting by their expression of hemolectin (Goto et al., 2003;Scherfer et al., 2004) and other proteins (Lemaitre and Hoffmann, 2007). Plasmatocytes also produce and secrete pericellular extracellular matrix (ECM) proteins that are essential for cells in contact with the hemolymph to receive signal input and for the maintenance of the cells (Fessler and Fessler, 1989;Fessler et al., 1994).

Crystal cells make up 5% of the total hemocyte population and exist only in the embryo and larvae (Crozatier and Meister, 2007). They contain enzymes (e.g. phenoloxidease) that catalyze the melanization cascade leading to the melanization of invading pathogens.

Lamellocytes are rarely found in a healthy fly, but may appear in low numbers at the time of metamorphosis (Gupta, 1986). However, their number is significantly increased in response to the parasitization of the larvae (Crozatier et al., 2004;Rizki and Rizki, 1992;Sorrentino et al., 2002). Lamellocyte main role is in the encapsulation and neutralization of foreign objects (e.g. wasp egg) that are too large to be engulfed by the plasmotocytes. Subsequently, the capsule is melanized by crystal cells and the parasite is killed possibly by cytotoxic reactive oxygen species (Nappi et al., 1995).

Several transcription factors and signaling pathways are implicated in Drosophila hematopoiesis. Serpent (srp), a GATA transcription factor, specifies the hemocyte primordium in the embryo and drives hematopoietic development (Rehorn et al., 1996).

Additional transcription factors determine the differentiation of the hemocyte precursors into plasmatocytes or crystal cells. Zinc-finger transactivators, Glial cells missing (gcm) and gcm2, specify plasmotocytes (Alfonso and Jones, 2002;Bernardoni et al., 1997;Lebestky et al., 2000) but antagonize the differentiation of crystal cells. When their expression is downregulated in some prohemocyte, RUNX family transcription factor lozenge (lz) is initiated (Bataille et al., 2005) to drive crystal cell differentiation. Transcription factor U-shaped (ush) also inhibits crystal cell development (Fossett and Schulz, 2001) and it promotes differentiation of plasmatocytes. These transcription factors act in combination to regulate crystal cell lineage commitment (Fossett et al., 2003;Waltzer et al., 2003), i.e. coexpression of lz and an srp isoform (srpNC) inhibits ush expression to drive crystal cell specification, and conversely coexpression of srpNC and ush blocks crystal cell production. Transcription factors, srp, lz and ush that regulate *Drosophila* hematopoiesis (Crozatier and Meister, 2007;Wood and Jacinto, 2007) are conserved in mammals as well. Mammalian orthologue of srp- GATA1, GATA2 and GATA3, are important for erythropoiesis (Pevny et al., 1991), primitive hematopoiesis and definitive hematopoiesis (Tsai et al., 1994) and T-cell lymphopoiesis (Ting et al., 1996). The mammalian transcription factors cooperatively regulate hematopoiesis, thus mirroring that seen in *Drosophila*. For example, RUNX1 (mammalian orthologue of lz) has shown functional and physical interactions with GATA1 to drive megakaryocyte differentiation (Elagib et al., 2003), which is synonymous with lz and srp during crystal cell differentiation. Meanwhile, FOG (the vertebrae orthologue of ush) cooperates with GATA1 to synergistically regulate erythroid and megakaryocytic differentiation (Tsang et al., 1997); which parallels the interaction between srpNC and ush in hemocyte

specification.

PVR, a *Drosophila* homologue of the vertebrate PDGF and VEGF receptor (Duchek et al., 2001 ;Heino et al., 2001), regulates embryonic hemocyte survival (Brückner et al., 2004) and hemocyte proliferation (Munier et al., 2002). PVR mutants show high embryonic hemocyte cell death and this death can be rescued by the specific expression of baculovirus pan-caspase inhibitor p35 in the hemocytes (Brückner et al., 2004) By overexpressing Pvf2, one of PVR putative ligands, this leads to a dramatic increase (of up to 300-fold) in hemocyte numbers (Munier et al., 2002). PVR signaling also regulate embryonic hemocytes migration (Brückner et al., 2004 ; Cho et al., 2002 ; Heino et al., 2001; Wood et al., 2006). Evidences suggest Pvfs may act as chemoattractants to hemocytes (Cho et al., 2002 ;Wood et al., 2006). Wood et al, showed that Pvf2 and Pvf3 ligands expression during embryogenesis coincide spatially and temporally with the migration of hemocytes. In Pvf RNAi silenced conditions, the hemoyctes failed to migrate to the ventral midline and dorsal vessel (Wood et al., 2006). Using p35-rescued Pvr null hemocyte, Brückner et al showed that PVR supports hemocyte migration to reach targets in the ventral-posterior area (Brückner et al., 2004). Lastly, PVR plays a specific role in plasmatocyte differentiation during lymph gland hematopoiesis (Jung et al., 2005a) since loss of PVR function abolishes the expression of plasmatocyte markers in hemocytes (however the hemocytes are still able to differentiate into crystal cells).

A number of other key signaling pathways are known to regulate hematopoiesis in *Drosophila.* Notch signaling controls the haemangioblast cell-fate choice by inducing

lymph gland cell specification during lymph gland development (Mandal et al., 2004) and also it drives differentiation of crystal cells (Duvic et al., 2002). In the lymph gland, cells of the posterior signaling center express Hedgehog signaling molecule, which instructs hematopoietic precursors within the neighboring medullary zone to maintain an undifferentiated state while preventing hemocyte differentiation (Mandal et al., 2007). Similarly, Wingless (Wg) signaling pathway occurs in the hematopoietic precursors to maintain their undifferentiated state (Sinenko et al., 2009).

With the above-listed strengths of using Drosophila as the model system, this thesis intends to capitalize on these advantages to study blood cell number control.

1.4 Overview

The aim of this thesis project is to characterize novel genes that previously were identified in a genome-wide RNAi screen for modifiers of cell number control. Studies focused on the role of these genes within the complex signaling network downstream of or in parallel to PDGFR and VEGFR signaling. In future, the result from this study can be recapitulated in the mammalian system to test its relevance. Further understanding of these biological networks potentially increases the repertoire of targets for countering the diseases attributed to the dysregulation of PDGFR and VEGFR signaling.

In Chapter 2 of this dissertation, I describe the sensitized genome-wide RNAi screen in *Drosophila* cell culture that was used to identify novel modifiers of PVR signaling that are regulators of cell number.

In Chapter 3, I summarize my studies on a PVR modifier gene identified in the screen, using cell culture and in vivo approaches. The methodology can be recapitulated for other candidate genes from the screen.

In Chapter 4, I extended my study to determine if the candidate gene from Chapter 2 may have a role in determining *Drosophila* embryonic blood cell lifespan.

Chapter 2: Genome-wide RNAi screen reveals known and novel modifiers of *Drosophila* PDGF/VEGF receptor signaling

2.1 Introduction

The *Drosophia* PDGF/VEGF Receptor (PVR) is the only ortholog of the PDGFR and VEGFR families in vertebrates (Duchek et al., 2001; Heino et al., 2001; Cho et al., 2002). PVR signaling is found to have a variety of biological effects and developmental roles in *Drosophila*. As mentioned in 'General Introduction' chapter, PVR signaling was shown to have a role in hemocyte development in terms of cell surival (Brückner et al., 2004), migration (Heino et al., 2001; Cho et al., 2002; Brückner et al., 2004; Wood et al., 2006), proliferation (Munier et al., 2002) and differentiation (Jung et al., 2005). PVR ligand, PVF1, is suggested to be a guidance cue for border cells migration (Duchek et al., 2001;McDonald et al., 2003). In addition, PVR signaling is involved in epidermal cell migration during wound healing (Wu et al., 2009), thorax closure during metamorphosis (Ishimaru et al., 2004), differentiation of intestinal stem cell (Park et al., 2009) and immunity, by repressing the immune deficiency (IMD) signaling (Ragab et al., 2011).

In vertebrates, extensive studies have shown that PDGFR and VEGFR to trigger cell survival and proliferation during normal development and in myeloid malignancies and other human cancers. Examples include acute myeloid leukemias (AMLs), that in more than one third of the cases carry activating mutations of flt3 and c-kit (Ashman, 1999), and chronic myeloid leukemias (CMLs) that in some cases arise from oncogenic chromosomal translocations TEL/PDGFRβR or HIP1/PDGFβR (Kelly and Gilliland,

2002). Both PDGFR and VEGFR are known to transmit signals to downstream cell survival and proliferation pathways, notably the Ras/Erk and Akt/TOR (Ferrara et al., 2003;Hoch and Soriano, 2003) pathways. Similarly, in *Drosophila*, PVR was suggested to transmit signals to both the Ras/MAPK and Akt/TOR pathways (Brückner et al., 2004;Cho et al., 2002;Duchek et al., 2001;Ishimaru et al., 2004). While PDGFR and VEGFR pathways have been studied extensively, our knowledge of the signaling networks that interact with these pathways, that control or execute their activities, or that play redundant roles, are incomplete. Thus, the identification of novel regulators or effectors of these pathways may expand the repertoire of molecular markers and targets for cancer diagnosis and therapy.

RNA interference (RNAi) is a process within living cells that regulates gene expression at a post-transcriptional level (Fire et al., 1998;Hammond et al., 2001b;Meister and Tuschl, 2004;Timmons and Fire, 1998). Ever since RNAi was first discovered to be effective in *Drosophila* (Kennerdell and Carthew, 1998), several groups were able to demonstrate RNAi in *Drosophila* cell lines (Caplen et al., 2000;Clemens et al., 2000;Hammond et al., 2000). The RNAi pathway in *Drosophila* can as in *C.elegans*, utilize long double-*stranded* RNAs (dsRNAs) without inducing an adverse interferon response as seen in mammalian cells (Echeverri and Perrimon, 2006;Sledz et al., 2003). In many cell lines, extracellulary supplied long dsRNAs are incorporated spontaneously by phagocytosis (Saleh et al., 2006;Ulvila et al., 2006). This RNAi uptake pathway is suggested to deviate from standard endocytic uptake at some point in order to deliver dsRNA to the cytoplasm (Saleh et al., 2006). In the cytoplasm, long dsRNA are being

processed into short double-stranded RNA (siRNA) of 20-25 nucleotides long by the RNase III type endonuclease enzyme Dicer2 (called the initiation step) (Meister and Tuschl, 2004;Tomari and Zamore, 2005). siRNA is unwound into two single-stranded RNA (ssRNA). The anti-sense strand ssRNA (guide strand) will be assembled into Argonaute-containing complexes known as RNA-induced silencing complexes (RISCs) (Meister and Tuschl, 2004;Tomari and Zamore, 2005). The guide strand subsequently guides the RISCs to complementary messenger RNA (mRNA) (Martinez et al., 2002), where they cleave and destroy the cognate RNA (effecter step) (Schwarz et al., 2002). Argonaute, the catalytic component of the RISC complex, induces the cleavage of the mRNA (Hammond et al., 2001a). The cleaved mRNA is subsequently degraded, thus gene expression is silenced.

The success of RNAi in *Drosophila* cell line provides an efficient approach for systematic loss of function phenotype analyses (Kiger et al., 2003;Ramet et al., 2002). With the knowledge of *Drosophila* genome sequence (Adams et al., 2000), several academic initiatives (Boutros et al., 2004;Foley and O'Farrell, 2004;Lum et al., 2003) and commercial entities (OpenBiosystem and Ambion/Cenix) began to generate genome-wide dsRNA libraries targeting up to nearly all the predicted genes in the *Drosophila* genome. The initial used of these large-scale and genome-wide libraries proved to be a great success in studying and identifying novel genes regulating Hedgehog signaling pathway (Lum et al., 2003), cell growth and viability (Boutros et al., 2004) and immune deficiency pathway activity (Foley and O'Farrell, 2004). Subsequently, many genome-wide RNAi screen were carried out to identify novel candidates in cell cycle (Bjorklund

et al., 2006), major key signaling pathway (DasGupta et al., 2005;Friedman and Perrimon, 2006;Muller et al., 2005;Nybakken et al., 2005), cell death (Chew et al., 2009;Chittaranjan et al., 2009), cell size (Bjorklund et al., 2006;Sims et al., 2009) and infection (Agaisse et al., 2005;Hao et al., 2008). Despite the excitement of this new technology platform, the problem of false discovery in RNAi screens was discussed (Kulkarni et al., 2006;Ma et al., 2006) and revealed in poor reproducibility between or among related screens (Bushman et al., 2009;Muller et al., 2008). Many causes of false discovery are inherent to high-throughput studies but these problems can be resolved or minimized, e.g. conducting replicates can minimize statistical noise from the screen data thus allowing increased in confidence of positives and to avoid false negatives (Boutros and Ahringer, 2008). However, a greater concern is presented by the issue of off-target effects (OTEs), i.e. sequence-specific recognition of transcripts other than the intended target by the RNAi reagent (Perrimon and Mathey-Prevot, 2007b). One of the ways to limit OTEs is by the successful application of '19 based-pair rule' i.e. avoiding regions in the target sequence that have 19 or more base pairs of contiguous nucleotide identity to another gene, as a 19-mer is sufficient to induce RNAi knockdown (Booker et al., 2011;Horn et al., 2011;Kulkarni et al., 2006). In addition, a general approach is also to verify a primary screen by testing two or more nonoverlapping RNAi reagents per gene (Echeverri et al., 2006).

In this chapter, I summarize work by others using a *Drosophila* cell culture system in a genome-wide RNAi screen under sensitized conditions to identify novel signaling pathways that mediate blood cell numbers. In brief, RNAi-mediated silencing of PVR

was used to induce apoptotic cell death (Brückner et al., 2004) providing a sensitized background that allows the identification and characterization of genes that drive cell survival or compensatory cell proliferation. Further, I show that PVR signaling triggers several redundant downstream survival pathways, such as the Erk (Cho et al., 2002;Duchek et al., 2001) and Akt (Brückner et al., 2004;Ishimaru et al., 2004), which may play a role in the system. Under PVR RNAi knockdown conditions, re-activation of one of these pathways, e.g. by inactivation of a negative regulator, is sufficient to suppress the cell death phenotype and rescue cell numbers. Using this system, a genome-wide RNAi screen was completed to identify novel candidate suppressors of cell numbers. Double-knockdown screens were used to identify genes that show differential effects under sensitized condition (Bakal et al., 2008;Horn et al., 2011). This combinatorial RNAi can reveal results that could not have been predicted based on conventional single gene analyses (Horn et al., 2011). The screen presented here was designed to eliminate general regulators of cell number and focus those genes that show differential effects under sensitized conditions. This revealed genes, PVR suppressor, with tumor suppressor-like activities, many of which have not been identified in conventional RNAi screens for cell proliferation and surivival (Bettencourt-Dias et al., 2004;Bjorklund et al., 2006;Boutros et al., 2004;Chew et al., 2009;Yi et al., 2007).

2.2 Results

PVR signaling in the embryonic hemocyte cell line Kc

It has been demonstrated that the *Drosophila* PDGF/VEGF Receptor, PVR, is essential for anti-apoptotic survival of embryonic hemocytes, and Kc cells in culture (Brückner et

al., 2004). In Kc cells, PVR signaling is autonomously activated due to simultaneous expression of PVR and its ligands PVF2 and, to a weaker extent, PVF3 (DRSC/ModEncode, and K. Brückner not shown). This prompted us to examine the signaling networks that mediate anti-apoptotic effects in these systems. Establishing Kcp35, a stable pool of Kc cells expressing the baculovirus inhibitor of apoptosis, p35 (Hay et al., 1994), we confirmed that RNAi-mediated knockdown of PVR (PVRRNAi) induces apoptotic cell death, which is suppressed by p35 (Figure 2.1A). Suppression of apoptosis revealed that PVRRNAi also results in reduced incorporation of the thymidine nucleoside analog EdU (5-ethynyl-2'deoxyuridine, Click-iT, Invitrogen) suggesting that PVR signaling also mildly contributes toward cell proliferation in this system (Figure 2.1B), an effect that could not be distinguished previously (Brückner et al., 2004).

Using Kc and Kcp35 cells we next investigated which signaling pathways are involved in PVR-controlled cell survival and proliferation. Using antibodies to phosphorylated signaling mediators of the Akt/Tor and Mek/Erk pathways, we found that, in Kc cells, both pathways are active as judged by phosphorylation of S6K, Mek, and Erk. PVRRNAi led to a dramatic reduction in the phosphorylation level of these proteins, indicating that PVR is a major activator of these pathways in Kc cells (Figure 2.1C).

Next we asked whether silencing of either or both of these pathways is sufficient to affect cell viability and mimic loss of PVR function. Using RNAi silencing of various mediators of the Akt/Tor or Mek/Erk pathways, we found that generally even combined inhibition of both pathways decreased cell numbers mildly. To quantify cell populations,

we used CellTiter-Glo (Promega), a luminescence-based cell viability assay that quantifies ATP content as a readout of cell number, and actual cell counts (Figure 2.1D, E). Despite efficient RNAi knockdown of the genes studied (Suppl. Fig. 1), the effects were never as dramatic as upon PVRRNAi, suggesting that additional redundant cell survival and/or proliferation pathways operate downstream of PVR (Fig. 2.1F).

In summary, our data suggest that PVR triggers activation of the Akt/TOR and Mek/Erk pathways in Kc cells, thereby supporting anti-apoptotic cell survival and proliferation. Our findings predict the presence of one or more additional, redundant cell survival/proliferation pathway(s) 'X' downstream of PVR, and we anticipate further parallel, anti-apoptotic and pro-apoptotic signaling pathways 'Y' and 'Z' that contribute to the overall lifespan and proliferation of the cell (Fig. 2.1F).

A genome-wide RNAi screen for modifiers of PVR

Loss of PVR function in Kc cells and embryonic hemocytes leads to enhanced apoptotic cell death and reduced proliferation, while re-activation of just one survival or proliferation pathway is sufficient to rescue cell numbers (Brückner et al., 2004). Based on this, K. Brückner developed and conducted a genome-wide screen for modifiers of cell number specific for the PVRRNAi silenced condition, using the DRSC Genome-Wide RNAi library 1.0 (*Drosophila* RNAi Screening Center, DRSC, Harvard Medical School), and analyzed the data from this screen (Fig. 2.2A). The DRSC 1.0 set targets 22,915 distinct amplicons of version 1 of the *Drosophila* genome, corresponding to 13,592 unique genes (Adams et al., 2000), and 7463 Sanger predictions (Hild et al., 2003), 1378 of which have been confirmed to be expressed (Stolc et al., 2004;Yandell et

al., 2005). Screening was performed in 384-well format, using CellTiterGlo (Promega) as an ATP-based readout of cell number. To determine an increase or decrease over the average value of ATP content, Z scores were calculated for each well. Focusing on those dsRNAs that show differential effects in PVR knockdown (PVRRNAi) versus control cells (GFPRNAi), the difference of each of the Z scores (ZDiff=Z[PVR]-Z[GFP]) was calculated, and selected amplicons with ZDiff>2 and ZDiff<-2 were selected as primary screen hits (Suppl Table 1). Cluster analysis (Eisen, 1998) of the values Z[PVR], Z[GFP], and ZDiff for each amplicon revealed three distinct classes of signatures, i.e. PVR Suppressors, PVR Enhancers, and PVR Upstream Regulators, which could not be distinguished based on the final ZDiff alone (Figure 2.2B). By these cutoff criteria, 64 amplicons are Suppressors of the PVR phenotype, that rescue CellTiter numbers specifically in the PVR kd background, and have lesser effects on controls. 65 amplicons are PVR Enhancers that exacerbate PVR RNAi-dependent effects. 290 amplicons are PVR Upstream Regulators, which reduce CellTiter numbers in control cells, but have rather minor effects in PVRRNAi cells. Among this group were amplicons targeting PVR itself as well as many ribosomal proteins, suggesting that many of the targeted genes play a role in the production or activation of PVR (Suppl. Table 1).

For subsequent analyses we focused on the classes of PVR Suppressors and PVR Enhancers. To verify the primary screen data, K. Brückner selected 47 Suppressor genes and 48 Enhancer genes (Table 1) based on a cutoff of ZDiff>2.2 and ZDiff<-2.2 (Suppl. Table 2), and synthesized non-overlapping verification amplicons that were free of 19bp or larger overlaps with other genes, in order to avoid off-target effects (Kulkarni et al.,

2006;Perrimon and Mathey-Prevot, 2007b).

As in the primary screen, amplicons were tested for their ability to modify cell number, specifically of PVRRNAi cells relative to controls, using CellTiter-Glo (Suppl. Table 2). Assays were performed in replicate and repeated in two independent duplicates. For the 22 most promising Suppressors we performed live/dead cell counting to confirm their effect on the rescue of cell numbers, ruling out other possibilities such as variations in the intracellular ATP balance or cell size (Suppl. Fig. 2). To identify promising 'high confidence candidates' for further analysis, we calculated the average of the ZDiff scores of the primary and verification screens (ZDiffFinal) (Suppl. Table 3). Based on ZDiffFinal values of ZDiffFinal>1.9 or ZDiffFinal<-1.2, and excluding genes involved in the RNAi process that underlies the assay conditions (e.g. AGO2), we report 26 high-confidence PVR Suppressors and 5 high-confidence PVR Enhancers (Table 2).

Only relatively few genes scored as PVR Enhancers by these criteria. Among those, we identified the RTK InR (Insulin-like Receptor) (Goberdhan and Wilson, 2003), the helix-loop-helix transcription factor crp (cropped) or dAP-4, which is a homolog of the mammalian transcription factor AP-4 (King-Jones et al., 1999), and tna (tonalli), a protein similar to mammalian ZMIZ1 (ZIMP10) and ZMIZ2 (ZIMP7) involved in sumoylation (Rodriguez-Magadan et al., 2008), that in *Drosophila* is known to genetically interact with the Brahma ATP-dependent chromatin remodeling complex.

The screen yielded a group of Suppressors of the PVR phenotype, i.e. genes, which,

when silenced, rescue cell numbers under the PVR kd condition. This group contains all negative regulators of the Akt/Tor pathway, many of which are known tumor suppressors in mammalian systems (Manning and Cantley, 2007;Sarbassov et al., 2005;Zoncu et al., 2011). Further, it contains negative regulators of the Erk pathway such as mts and wdb, both components of the PP2A complex (Janssens and Goris, 2001;Van Hoof and Goris, 2003), and Mkp3, known to negatively regulate Erk (Kim et al., 2004). This confirms our initial hypothesis and the basis for our screen, as re-activation of one of the redundant pro-survival pathways downstream of PVR is sufficient to rescue cell numbers in the PVR kd background. In addition, two of the strongest hits among the PVR Suppressors were members of a functional group of genes called nuclear hormone receptors- EcR (Ecdysone Receptor) and usp (ultraspiracle) (Koelle et al., 1991;Yao et al., 1992).

2.3 Discussion

A sensitized screen to identify novel regulators of cell number

Drosophila PVR mediates cell survival in the *Drosophila* embryonic hematopoietic system and in *Drosophila* Kc cells in culture (Brückner et al., 2004), and similar roles in other cell populations such as glia were reported subsequently (Learte et al., 2008). Further, by suppressing PVR-dependent cell death we reveal a new role for PVR in the proliferation of Kc cells, similar to a previous report on PVR-supported cell proliferation in larval or lymph gland hemocytes (Munier et al., 2002). These functions are conserved with mammalian PDGF/VEGF Receptors, and are highly relevant for normal development (Breen, 2007;Hoch and Soriano, 2003) as well as the pathology of leukemias and other forms of cancer (Gerber and Ferrara, 2003;Kelly and Gilliland,

2002;Scheijen and Griffin, 2002). Our findings on the activation of the Mek/Erk and Akt/Tor pathways downstream of PVR in Kc cells are consistent with PVR-dependent phosphorylation of Erk in embryonic hemocytes and in the cell line S2 (Cho et al., 2002;Duchek et al., 2001), and biochemical interaction of PVR with Grb2, Shc, and the regulatory subunit of PI3K in cell culture (Ishimaru et al., 2004). While previous reports described synergism of the Akt and Erk pathways in PVR-mediated cell size control (Sims et al., 2009), we find that PVR survival signaling in Kc cells cannot be phenocopied by a combination of Akt/Tor and Mek/Erk silencing, suggesting additional, redundant pro-survival pathways downstream of PVR.

Resulting from a genome-wide RNAi screen under sensitized conditions, we identified known and novel regulators of cell number. Since the screen was designed to eliminate general regulators of cell number and focus those genes that show differential effects under sensitized conditions, it predominantly revealed genes with tumor suppressor-like activities (PVR Suppressors), many of which have not been identified in conventional RNAi screens for cell proliferation or survival previously (Bettencourt-Dias et al., 2004;Bjorklund et al., 2006;Boutros et al., 2004;Chew et al., 2009;Yi et al., 2007). For example, with the exception of dpr6, the PVR modifier screen identified a unique set of genes not detected in a screen for general regulators of growth and viability using the same genome-wide dsRNA library (Boutros et al., 2004). As expected, some genes identified in the PVR modifier screen also scored in screens for signaling mediators of the Akt/TOR and RTK/Erk pathways, respectively (Friedman and Perrimon, 2006;Kockel et al., 2010).

Among the screen hits, we distinguish three major classes of modifiers. First, a large group of 'Upstream Regulators' of PVR that specifically affect cell number in control cells, but not in PVR kd cells, was identified, exemplified by PVR itself and a large number of ribosomal protein genes, that presumably participate in the production of PVR or PVR-activating factors. Although some of these genes might be very interesting to follow, in particular in light of the possibility of target-specific ribosomal activities that may influence the cellular signaling makeup in development and tumorigenesis (Kondrashov et al., 2011;Silvera et al., 2010;Stumpf and Ruggero, 2011) we decided, for the purpose of my project, not to focus on this large group of genes.

Second, the screen yielded relatively few confirmed Enhancers of PVR, which further reduce cell numbers under PVR kd conditions, but have less noticeable effects in control cells. Interestingly, we found InR among the PVR enhancers, consistent with the idea that other RTKs provide pro-survival signals in Kc cells, in particular InR being stimulated by low levels of insulin present in the cell culture medium. A role of InR in cell number control is further supported by our observation that insulin stimulation can compensate for PVRRNAi in the rescue of cell numbers and an increase in phosphorylation of signaling mediators (see Chapter 3). Similar situations of InR and other RTKs redundantly supporting cell survival and proliferation are known in *Drosophila* development (Friedman and Perrimon, 2006;Learte et al., 2008;Marin-Hincapie and Garofalo, 1999), and vertebrate signaling (van der Geer et al., 1994). Additional examples of PVR enhancers are helix-loop-helix transcription factor crp (cropped) or

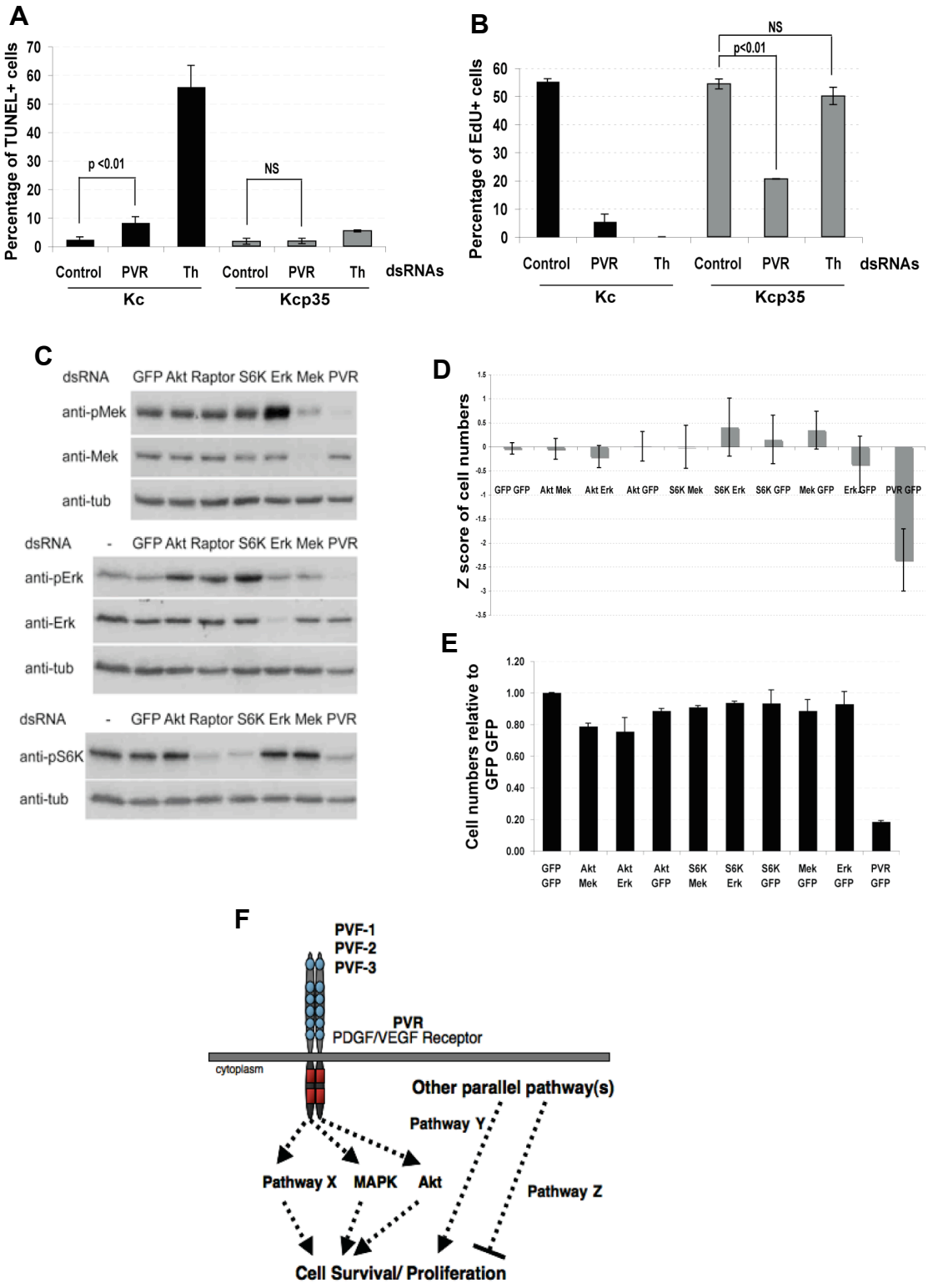
dAP-4, which is a homolog of the mammalian transcription factor AP-4 (King-Jones et al., 1999), and tna (tonalli), a protein similar to mammalian ZMIZ1 (ZIMP10) and ZMIZ2 (ZIMP7) involved in sumoylation (Rodriguez-Magadan et al., 2008), that in *Drosophila* is known to genetically interact with the Brahma ATP-dependent chromatin remodeling complex (Gutierrez et al., 2003).

Third, the screen yielded a group of Suppressors of the PVR phenotype, i.e. genes, which, when silenced, rescue cell numbers under the PVR kd condition Among the PVR Suppressors, the screen yielded all known tumor suppressors and negative regulators of the Akt/TOR pathway, i.e. PTEN (Phosphatase and Tensin Homolog), Tsc1 (Tuberous Sclerosis Protein 1), gig (gigas) or Tsc2 (Tuberous Sclerosis Protein 2), AMPK-α and –γ (AMP-Activated protein Kinase subunits α and γ), FOXO (Forkhead Box Protein), and L (Lobe), a protein with similarities to the vertebrate proline-rich Akt substrate of 40 kDa (PRAS40)) (Manning and Cantley, 2007;Sarbassov et al., 2005;Zoncu et al., 2011). We further identified negative regulators of the Ras/Erk pathway such as Mkp3 (Mitogen-activated protein kinase phosphatase 3), and mts (microtubule star) and wdb (widerborst), components of the protein phosphatase PP2A complex (Janssens and Goris, 2001;Kim et al., 2004;Van Hoof and Goris, 2003). This confirms our initial hypothesis and the basis for our screen, as re-activation of one of the redundant pro-survival pathways downstream of PVR is sufficient to rescue cell numbers in the PVR kd background. Importantly, the screen revealed novel genes, some of which only recently have been described in other systems. For example, the screen yielded CG6182, an ortholog of the mammalian TBC7 (TBC1 domain member 7), that was suggested to interact with Tsc1

(Nakashima et al., 2007), and CG5169, a counterpart of mammalian STK25 (Serine Threonine Kinase 25), also known as SOK1, that localizes to the Golgi (Matsuki et al., 2010) and induces cell death upon overexpression in mammalian cell culture (Nogueira et al., 2008). In addition, we identified genes such as CG31635, corresponding to mammalian LRRC68, a SH3-domain-, and leucine-rich-repeat- containing protein for which no function has been assigned to date (NCBI). Besides these 'CG number' genes, we identified genes that have been known in *Drosophila* previously, yet no role in cell number control has been described in the embryo. For example, we identified multiple members of the Brahma SWI2/SNF2 family ATPase chromatin-remodeling complex (Papoulas et al., 1998;Tamkun, 1995), with osa and dalao scoring as PVR Suppressors, and Bap60 (Brahma associated protein 60kD) and mor (moira) partially behaving as PVR Upstream Regulators. Consistently, the *Drosophila* Brahma complex has recently been described as negative regulator of signaling by the RTK EGFR (Epidermal Growth Factor Receptor) (Rendina et al., 2010) with a role in the growth and survival of wing disc cells (Terriente-Felix and de Celis, 2009), and mammalian SWI2/SNF2 members are well-known tumor suppressors (Reisman et al., 2009). Two of the strongest hits among the PVR Suppressors were the nuclear hormone receptors EcR (Ecdysone Receptor) and usp (ultraspiracle) (Koelle et al., 1991;Yao et al., 1992), and for further analysis we focused on the role of EcR signaling in embryonic blood cells (see Chapter 3).

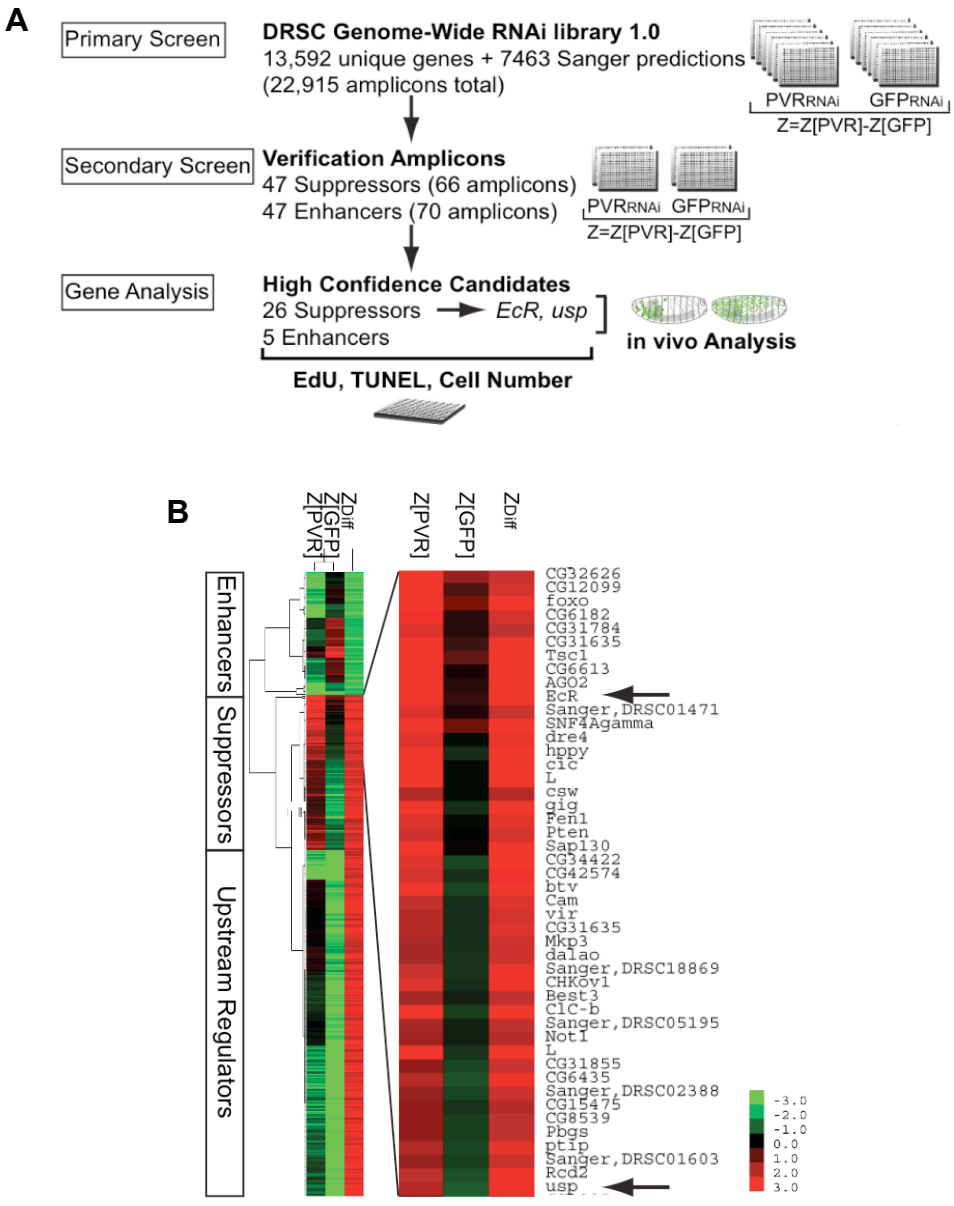
2.4 Figures

Figure 2.1. Signaling pathways in PVR survival signaling



(A, B) Comparison of Kc and p35-expressing Kcp35 cells to distinguish between effects on cell death and cell proliferation. (A) dsRNA treatment targeting PVR or Th (Diap1) causes apoptosis, which is suppressed in Kcp35. (B) EdU proliferation assay in Kc and Kcp35 cells shows almost normal proliferation of th kd cells, while impairment of proliferation in PVR knockdown is revealed. (A, B) n = 3, Mean ± s.d. Statistical analysis performed using two-tailed T test. (C) Western blots showing phospho-mediators of the Mek/Erk and Akt/Tor pathways. Silencing of genes as indicated on top. PVR kd leads to reduced activity of both pathways. (D, E) Combined knockdown of Akt/Tor and Mek/Erk pathway components does not match the effect of PVR silencing, indicating that additional, redundant survival pathways downstream of PVR. (D) CellTiter-Glo assay measuring ATP concentration as readout of cell number. Z score relative to all experimental samples is shown. n = 4, Average Z score ± s.d. (E) Cell counts demonstrate close correlation with CellTiter-Glo results in (D). (F) Model: Hypothesis of PVR-mediated survival and proliferation signaling. In addition to signaling pathways downstream of PVR, additional parallel pro-and anti-death pathways are hypothesized. Selective re-activation of an anti-apoptotic pathway by silencing of a suppressor, or inhibition of a pro-apoptotic regulator, suffices to restore cell numbers in this system.

Figure 2.2. PVR modifier screen



(A) Screen scheme of Primary and Verification Screens, and subsequent single gene analysis. (B) Cluster analysis of primary screen hits, highlighting a fraction of PVR suppressors including EcR and usp (arrows).

Table 1. Table of Primary Screen Hits

Suppressor genes	Enhancers genes
AGO2	Aly
btv	CG11255
Cam	CG12454
CG11006	CG12977
CG12099	CG13530
CG14220	CG13737
CG14656	CG14163
CG31635	CG14459
CG31855	CG17124
CG32133	CG17723
CG32626	CG17742
CG40188	CG31145
CG4786	CG32432
CG5169	CG33110
CG6182	CG4880
CG6435	CG5079
CG6613	CG8745
CG7097	CG9132
CG7274	CkIalpha
CG8594	crp
cic	CycE
dalao	Egfr
dpr6	GNBP1
dre4	HDC03105
EcR	HDC07158
Fen1	HDC16392
foxo	HDC19527
gig	HDC20097
Grip84	His2A:CG31618
HDC02668	His2B:CG17949
HDC02990	His-Psi:CR31615
HSC03502	InR
HDC05451	Irbp
L	I(3)82Fd
Mkp3	mio
mtm	mod(mdg4)
mts	Or67d
osa	par-1
Pp4-19C	pcm
Pten	Proct
raw	Ptp99A
SNF1A	Rgk1
SNF4gamma	sns
Tsc1	svp
usp	th
vir	tkv
wdb	tna
	wts

Selected 47 Suppressor genes and 48 Enhancer genes from primary screen.

Table 2. Table of PVR Suppressors and Enhancers

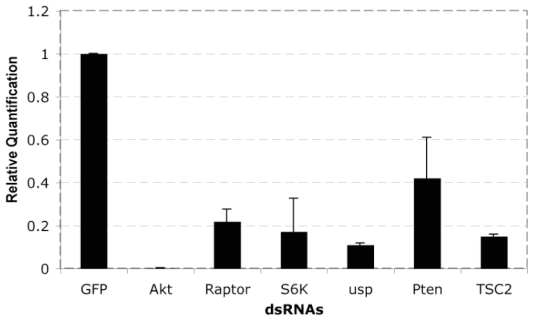
Gene ID	Gene name	Known mammalian orthologue	Gene ontology	Functional Class	Averaged Z-score
CG11006	Sin3A-associated protein 130	N.A.	N.A.	mitotic spindle organization	2.2
CG14656	N.A.	N.A.	N.A.	N.A.	3.1
CG31635	N.A.	LRRC68	N.A.	N.A.	2.2
CG32626	N.A.	AMPD2	AMP deaminase	Metabolism	2.8
CG5169	Germinal centre kinase III	serine/threonine kinase 25	protein serine/threonine kinase activity	actin filament organization	1.9
CG6182	N.A.	TBC1 domain family, member 7	Rab GTPase activator	Rab GTPase activity	3.1
CG43122	capicua	CIC	Transcription factor	Polarity determination/Cell growth	2.3
CG7055	dalao	SMARCE1	Component of chromatin remodeling complex	Chromatin remodeling	1.9
CG14162	dpr6	N.A.	N.A.	N.A.	1.9
CG1765	Ecdysone receptor	NR1H3	Nuclear hormone receptor	Morphogenesis	2.6
CG8648	Fen1	FEN1	Flap endonuclease	Flap endonuclease activity	2.4
CG3143	forkhead box, sub-group O	FOXO1/3	Transcription factor	Cell growth/ Autophagy	4.5
CG6975	gigas	TSC2	Kinase binding	Cell growth	11.3
CG10109	Lobe	PRAS40	N.A.	Cell death/ Signaling	3.9
CG14080	Mitogen-activated protein kinase phosphatase 3	DUSP7	MAP kinase tyrosine/serine/threonine phosphatase activity	Signaling	2.0
CG9115	myotubularin	MTMR2	protein tyrosine/serine/threonine phosphatase activity	Cell Cycle	2.3
CG7109	mts	PPP2CB	protein phosphatase type 2A	Signaling	4.5
CG7467	osa	ARID1A	Component of chromatin remodeling complex	Chromatin remodeling	4.8
CG32505	Pp4-19C	PPP4C	protein serine/threonine phosphatase activity	Cell cycle	2.1
CG5671	Pten	Pten	Phosphoprotein phosphatase	Signaling	9.0
CG3051	SNF1A/AMP-activated protein kinase	SNF1A	AMP-activated protein kinase activity	Cell growth	2.0
CG17299	SNF4/AMP-activated protein kinase gamma subunit	SNF4Aγ	AMP-activated protein kinase activity	Cell growth	8.6

CG6147	Tsc1	Tsc1	Kinase binding	Cell growth	1.9
CG4380	Ultraspiracle	RXRB	Nuclear hormone receptor	Morphogenesis	2.7
CG3496	vir	KIAA1429	Nucleic acid binding	RNA splicing	2.0
CG5643	widerborst	PPP2R5E	protein phosphatase type 2A	Signaling	3.8
CG14459	N.A.	N.A.	N.A.	N.A.	-1.3
CG7664	Cropped	N.A.	RNA Polymerase II transcription factor	Transcription	-3.1
CG18402	Insulin-like receptor	insulin-like growth factor 1 receptor	insulin receptor	Signaling	-1.2
CG7074	Missing oocyte	MIOS	Unfolded protein binding	meiosis	-1.6
CG7958	tonalli	zinc finger, MIZ-type containing 1	N.A.	Transcription	-2.2

Summary of high confidence PVR Suppressors and Enhancers (grey) showing final Z scores, vertebrate orthologs and functional domains.

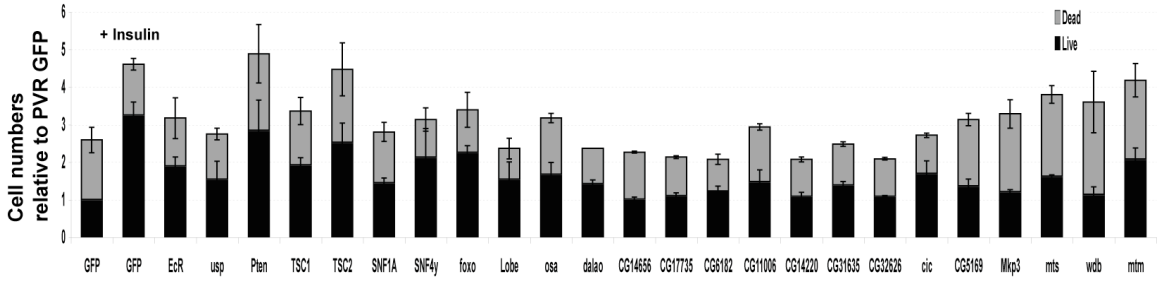
Supplemental Figure 1. Knockdown efficiency of amplicons

Gene	Knockdown Efficiency	Verified by
Pvr	>99%	Western blot
Akt	>99%	qRT-PCR
Raptor	~80%	qRT-PCR
S6K	~80%	qRT-PCR
Erk	>99%	Western blot
Mek	>99%	Western blot
EcR	>99%	Western blot
usp	~90%	qRT-PCR
Pten	~60%	qRT-PCR
TSC2	~85%	qRT-PCR



(A) Summary of dsRNA-mediated kd efficiencies. (B) kd efficiencies by qRT-PCR.

Supplemental Figure 2. Verification screen cell counts of 22 suppressors



Silencing of 22 PVR suppressors in the PVRRNAi background. Insulin stimulation and GFPRNAi as controls. Live/dead cell counts.

Figure legends for Supplemental Tables

Supplemental Table 1. Primary screen scores

Amplicons of primary PVR modifier screen with resulting ZDiff >2.0 or <-2.0. PVR Enhancers, Suppressors, and Upstream Regulators are indicated.

Supplemental Table 2. Verification screen scores

Verification screen amplicons, Z scores of all replicates with resulting ZDiff values.

Supplemental Table 3. Averaged final scores

Final scores ZDiffFinal resulting from primary and verification screen averages.

Chapter 3: Candidate genes ecdysone receptor complex regulates *Drosophila* blood cell survival

3.1 Introduction

Nuclear Receptors (NRs) are ligand-activated transcription factors. Ligands of NRs are commonly endogenous, often steroid-derived, hormones. The NR family in humans consists of 48 members divided into six subfamilies. The *Drosophila* genome contains 18 NRs, with receptors representing all six of NR subfamilies (King-Jones and Thummel, 2005). NRs typically have three main domains, a activation function-1 (AF-1) domain, a DNA-binding domain (DBD) and a ligand-binding domain (LBD) with an activation function-2 (AF-2) domain located at its C-terminus. Both AFs recruit coactivators and work synergistically to ensure full transcriptional activity of the NR (Warnmark et al., 2003). The DBD binds to target DNA hormone response element and also promotes dimerization of NRs. The LBD allows ligand binding and contributes to the dimerization of NRs (Kumar and Thompson, 1999). NRs can function as monomers, homo- or heterodimers, and their signaling occur by various mechanisms. (1) The NR steroidogenic factor (SF-1) functions as a monomer bound to the target gene response element and upon being phosphorylated, SF-1 recruits cofactors to enhance its transcriptional activity (Hammer et al., 1999). (2) Steroid hormone receptors (e.g. glucocorticoid receptor) reside in the cytoplasm and upon ligand binding translocate into the nucleus and bind to the response element as a homodimer to drive transcription (Kumar and Thompson, 2005). (3) In contrast, some homodimeric NRs (e.g. estrogen-related receptor-α), are constitutively active receptors that can function as transcriptional

activators in the absence of any added ligand (Vanacker et al., 1999), with their activity being downregulated by inverse agonists (Busch, 2004). (4) NRs such as thyroid hormone receptor can associate with rexinoid receptor (RXR) to form heterodimers. The unliganded heterodimer binds to the response element and recruits corepressor to repress transcription of target genes. Upon ligand binding, the corepressor dissociates and coactivators are recruited, thus enabling gene transcription (Zhang and Lazar, 2000).

NR signaling is involved in development as highlighted by two classes of retinoic acid (RA)-binding NRs- the retinoic acid receptors (RARs) and RXRs. Both RARs and RXRs families consist of three isotypes each- RARα, RARβ and RARγ, and RXRα, RXRβ and RXRγ; where each isotype has multiple isoforms (Mark et al., 2006). RA signaling through RARs and RXRs are indispensable during organogenesis and loss of these receptors leads to a range of developmental defects (Mark et al., 2006;Mark et al., 2009). Studies from gene knockout mice showed the important role of RARβ and RXRα during heart development. RXRα signaling within the epicardium is critical for proper cardiac morphogenesis and coronary arteriogenesis (Merki et al., 2005). RARβ and RXRα mutants exhibit defects in outflow tract septation due to excessive cell death (Ghyselinck et al., 1998). RARα and RARγ were shown to be necessary for embryonic inner ear development as RARα/RARγ null mutants displayed anomalies due to impaired differentiation of the otic placode derivatives (Romand et al., 2002). Both RARα and RARβ are expressed throughout the developing kidney and are important for renal morphogenesis (Mendelsohn et al., 1999). RARα/RARβ mutants have greatly impaired collecting ducts and ureters.

Mutation in NRs are known to lead to various diseases, notably cancer and metabolic diseases. RARβ is a tumor suppressor that regulates proliferation differentiation and apoptosis (Fan et al., 2010;Liu et al., 1996;Sun, 2004), and downregulation of its activity (due to hypermethylation of its promoter) is associated with many malignant tumors (Ivanova et al., 2002;Kuroki et al., 2003;Mehrotra et al., 2004;Widschwendter et al., 2000). Conversely, by reactivating RARβ transcription activity through histone reacetylation, there is significant inhibition in tumor growth (Sirchia et al., 2002). Acute promyelocytic leukaemia (APL) arises by chromosomal translocation involving RARα and the promyelocytic leukaemia (PML) gene; thus forming a PML-RARα fusion protein. It is suggested that PML-RARα block differentiation of promyelocytes by transcriptional repression (Puccetti and Ruthardt, 2004), which initiates and perpetuates APL. Thus, applying a differentiating agent such as RA can be potentially used as a treatment for APL (Degos et al., 1995;Huang et al., 1988). RA also promotes APL remission by inducing PML-RARα catabolism or apoptosis in PML-RARα cells (Zhu et al., 2001). However, mutation in PML-RARα RA ligand-biding domain leads to relapse of APL (Schachter-Tokarz et al., 2010). RXR is a common binding partner for a subgroup of other NRs, thus forming a resulting heterodimer (Shulman et al., 2004). RXR heterodimers are involved in many metabolic activities such as fat and glucose metabolism, cholesterol transport and bile metabolism (Francis et al., 2003). PPARγ/RXR heterodimer is expressed in adipocytes, macrophages and muscle, where it regulates lipid homeostasis and glucose metabolism (Gurnell, 2003;Rangwala and Lazar, 2004). As the result, dominant negative mutations in PPARγ leads to corresponding

metabolic disease such as severe insulin resistance, type 2 diabetes mellitus and hypertension (Barroso et al., 1999;Gurnell et al., 2003).

Much remains to be learned about NRs. *Drosophila* ecdysteroid signaling is an important model for understanding NR function and signaling. Ecdysteroids are insect moulting and sex hormones, and comprise of a steroid hormone called ecdysone (also know as 20-hydroxy ecdysone (20-E)). The ecdysone signaling pathway is an essential regulator of cell death, cell proliferation and differentiation. Ecdysone signaling is induced by the binding of ecdysone onto the heterodimeric ecdysone receptor, which is made up of ecdysone receptor (EcR) (Koelle et al., 1991) and ultraspiracle (usp) (Oro et al., 1990;Thomas et al., 1993;Yao et al., 1992). EcR and usp are the *Drosophila* homologs of the vertebrate gene NR1H3 (also known as liver X receptors: LXRA and LXRB) and RXR (retinoid X receptor) respectively.

Each phase in the *Drosophila* life cycle is characterized by a pulse of ecdysone. These ecdysone pulses trigger developmental changes as the ecdysone target tissue differentiates and/or undergo morphological changes. In larval stages, pulses of ecdysone occur during each of the larval instar stages. Ecdysone pulses during 1st and 2nd larval instar triggered molting of the larval cuticle (Schubiger et al., 1998). The high-titer ecdysone pulse at the end of 3rd instar initiates puparium formation as *Drosophila* undergoes metamorphosis (Thummel, 2001). During metamorphosis, two peaks of ecdysone pulses occur- one at 10-12 hours after puparium formation and another high titer pulse at late pre-pupal. In metamorphosis, ecdysone signaling induces histolysis of

the larval tissues such as salivary glands (Jiang et al., 1997a) and neurons (Schubiger et al., 1998;Truman et al., 1994), and the proliferation and differentiation of the imaginal tissues into their adult structures. The role of ecdysone signaling in embryonic development and in specific organ systems such as hematopoietic system is only partially understood. An ecdysone pulse occurs at the mid-embryonic stage and this is suggested to be involved in morphogenetic movements such as germ band retraction (Kozlova and Thummel, 2003). With respect to hematopoiesis, it is suggested that induction of ecdysone signaling at metamorphosis switches lymph gland hemocyte precursors from cell division to terminal differentiation (Sorrentino et al., 2002), and ecdysone arrests the embryonic hemocyte cell line Kc in G2 phase of cell cycle (Rosset, 1978;Stevens et al., 1980).

During *Drosophila* metamorphosis, ecdysone pulses induce gene transcription of ecdysone primary (early)- and secondary-response genes. It is suggested that one of the functions of this ecdysone-induced transcriptional hierarchy is to control programmed cell death (PCD) of the larval salivary gland during Drosophila metamorphosis. Three of the primary genes control salivary gland cell death by regulating death activators- head involution defective (hid) and reaper (rpr). The genes are Broad-Complex (BR-C) (DiBello et al., 1991), E74 (Burtis et al., 1990) and E75 (Segraves and Hogness, 1990). BR-C is required for both rpr and hid induction, and E74A is required for maximal hid induction (Jiang et al., 2000). Diap2, an inhibitor of rpr and hid, transcription is repressed by, thus abrogating the inhibition of PCD by Diap2. Although BR-C, E74 and E75 were shown to direct salivary gland PCD, their induction upon an earlier ecdysone pulse (in

late third instar larvae) during puparium formation did not lead to PCD. Instead it is E93 (Baehrecke and Thummel, 1995) gene that directs the stage specific salivary gland PCD (Lee et al., 2000). E93 plays an essential role in regulating steroid induced PCD by inducing E74 and E75 directly and BR-C indirectly. E93 (as well as BR-C) is also a critical regulator of larval midgut PCD (Lee et al., 2002) during the onset of *Drosophila* metamorphosis. In larval salivary gland and midgut, ecdysone triggered PCD ultimately leads to the transcription of core apoptotic genes such as dark, dronc, rpr, hid and crq. However, interestingly, larval salivary gland cells and midgut cells were suggested to die by autophagic cell death (Lee et al., 2000;Lee et al., 2002).

In the previous chapter, I describe the identification of EcR and usp as suppressors of cell number reduction in the PVR-silenced background. Since the role of ecdysone signaling in hematopoiesis and in embryonic development in general remains incompletely understood, this chapter focuses on the goal to understand how ecdysone signaling regulates hemocyte cell numbers, and to draw parallels with vertebrate orthologs, thereby providing proof-of-principle of the significance of genes identified in the screen. To characterize ecdysone signaling and determine the relationship between PVR signaling and ecdysone signaling, I utilized the *Drosophila* hemocyte cell line Kc, and in vivo genetics.

3.2 Results

The nuclear hormone receptors EcR and usp have pro-apoptotic effects in Kc cells and embryonic hemocytes

EcR and usp form a heterodimer and are induced by binding of the steroid hormone 20-hydroxy ecdysone (20E) (Oro et al., 1990;Yao et al., 1992). Signaling by the EcR complex plays a major role during molting and metamorphosis (Kozlova and Thummel, 2000), yet a role in embryonic cell death control has not been established to date (Chavez et al., 2000). First, we quantified the effects of EcR and usp on suppressing PVRRNAi-induced apoptosis using TUNEL assays, and proliferation using EdU incorporation in Kcp35 cells (Figure 3.1A, B). Tsc2 (gig), a negative regulator of the Akt/Tor pathway, served as control. All genes were targeted utilizing optimized dsRNA amplicons with kd efficiencies that generally exceeded 80% (Chapter 2 Suppl. Figure 1). Interestingly, we found that EcRRNAi and uspRNAi rescued only apoptosis, but not proliferation defects under PVR kd conditions, while Tsc2RNAi suppressed both defects in this background (Figure 3.1A, B). Further, stimulation with insulin rescued both survival and proliferation of PVRRNAi cells (Figure 3.1A, B), consistent with the classification of InR as PVR Enhancer and indicating that activation of by another upstream RTK can compensate for the lack of PVR signaling.

Based on our cell culture results, we tested whether inhibition of EcR signaling would rescue the PVR phenotype of embryonic hemocyte apoptosis in vivo (Brückner et al., 2004). Compared to control embryos that typically carry ~600 hemocytes post stage 11, Pvr1 null mutant embryos show a dramatic decline in hemocyte numbers over time,

leading to less than 200 hemocyte by stage 16 (Figure 3.1C)(Brückner et al., 2004). Since *Drosophila* EcR is expressed in three isoforms, EcR-A, -B1, -B2 (Yao et al., 1993), we tested the effect of dominant-negatives of EcR-A-W650A and EcR-B1-W650A (Brown et al., 2006;Cherbas et al., 2003) by overexpression in hemocytes using the driver srpHemo-GAL4 (Brückner et al., 2004). Indeed, suppression of EcR signaling partially rescued the hemocyte number decline in the Pvr1 mutant background, similar albeit less complete than the baculovirus inhibitor of apoptosis, p35 (Figure 3.1C, D). This suggests that under normal conditions, EcR signaling accounts for some level of pro-death signaling in embryonic hemocytes, while absence of the signal shifts the overall balance of the cell toward elevated survival levels, which become obvious under sensitized conditions such as Pvr loss of function.

Next we examined the effects of ecdysone stimulation. An anti-proliferative effect of ecdysone has been described in Kc cells previously (Andres et al., 1993;Cherbas et al., 2003;Stevens et al., 1980), however, it has not been distinguished whether ecdysone also has direct pro-apoptotic effects. To test this, we used 20-Hydroxyecdysone (20E) at concentrations close to physiological levels (0.01ug/ml) and beyond (Rosset, 1978;Stevens et al., 1980) to stimulate Kc and Kcp35 cells (Figure 3.2). Overall, 20E induced a marked reduction in cell number at stimulation times of >48h (Figure 3.2A, B). When using EdU incorporation as a measure of cell proliferation, we found as expected a decline in proliferative activity upon 20E treatment, which was, to a lesser extent, also measurable in apoptosis-resistant Kcp35 cells (Figure 3.2C). Importantly, TUNEL analysis showed a large increase in the fraction of apoptotic cells upon 20E

stimulation from 72h of stimulation onwards (Figure 3.2D). As expected, this effect was largely suppressed in Kcp35 cells, leading to a drop in TUNEL rates from >15% in Kc to 5% in Kcp35 cells (Figure 3.2D).

During metamorphosis-associated programmed cell death (PCD), several genes have been described as ecdysone-induced pro-death targets, in particular E93, BR-C (broad), E74A, and rpr (reaper) (Jiang et al., 1997a;Lee et al., 2000;Lee et al., 2002). We therefore examined the expression levels of these genes during ecdysone stimulation of Kc cells using q-RT-PCR. Indeed, we found an induction of rpr and E93 that increased over the first day of 20E stimulation (Figure 3.2E), consistent with the induction of apoptotic cell death in our assay.

Based on our cell culture results from Figure 3.2A- 3.2E, we examined the effects of ecdysone stimulation on in vivo embryonic hemocytes. We permeabilized embryos by heptane treatment to allow 20E to enter. Despite having a majority of embryos permeabilized, the viability of the permeabilized control and 20E treated embryos is very low (data not shown). Instead, we tested whether over-expression of wildtype EcR would further exacerbate PVR dominant-negative (PVRΔC) (Brückner et al., 2004) induced apoptosis of embryonic hemocyte. We tested the effect of recombinants of wildtype EcR-A and EcR-B2 (Lee et al., 2000), with PVRΔC by overexpression in hemocytes using the driver srpHemo-GAL4 (Brückner et al., 2004). Indeed, gain-of-function of EcR signaling further exacerbate the hemocyte number decline in PVRΔC background (Figure 3.2F). This suggests that under sensitized conditions such as Pvr loss of function, the effect of

EcR contribution to pro-death signaling in embryonic hemocytes becomes obvious.

In summary, we conclude that the EcR complex has pro-apoptotic functions in the cell line Kc, and in embryonic hemocytes in vivo, which become apparent under sensitized conditions such as PVR loss of function. Further, stimulation with 20E is potent in inducing programmed cell death in Kc cells, which may be caused by the upregulation of the pro-apoptotic ecdysone target genes E93 and Reaper.

PVR signaling supports ecdysone-induced EcR signaling

To characterize the relationship between PVR and EcR signaling, we first asked whether signaling by the EcR complex acts epistatic or in parallel with PVR-triggered signaling pathways. Comparing the effects of silencing of the EcR and Akt/TOR pathways either separate or in combination, we found that simultaneous knockdown of genes from both pathways resulted in increased rescue numbers (e.g. EcR+Pten), while combination of two genes from the same pathway did not show such effects (e.g. EcR+usp) (Figure 3.3A), indicating a parallel, rather than epistatic, relationship.

Next we asked whether silencing of EcR or usp would affect the activation or expression level of any of the PVR downstream signaling mediators in the PVRRNAi background (Figure 3.3B). In this assay, the controls PtenRNAi and Tsc2RNAi rescued Akt and S6K phosphorylation as expected (Figure 3.3B), control mtsRNAi rescued both S6K and Erk, and insulin stimulation rescued and superstimulated Akt, S6K, Mef and Erk phosphorylation, indicating that, also at the molecular level, activation of other RTKs can

compensate for a lack of PVR signaling (Figure 3.3B). However, EcRRNAi or uspRNAi did not lead to any changes in the phosphorylation or expression levels of Akt, S6K, Mek, or Erk, once more suggesting that EcR signaling does not epistatically link to these pathways (Figure 3.3B). The same result was obtained in control cells with normal PVR levels (data not shown).

The activation of pro-apoptotic proteins known as IBM (IAP binding domain) is the major mechanism for apoptosis where different signaling pathways that trigger apoptosis converge at (Steller, 2008). One of the IBM proteins is reaper. We asked whether apoptosis induced by PVRRNAi and 20E induction occur via the similar cell death machinery. PVRRNAi induces negligible expression of reaper (Figure 3.3C) in comparison to 20E stimulation. However, 20E stimulation on cells pretreated with PVRRNAi shows a further upregulation of reaper expression (Figure 3.3C). This suggests apoptosis induced by both ways (PVRRNAi and 20E stimulation) converged in a same cell death pathway.

Interestingly, we found an effect of PVR signaling on EcR under 20E stimulated conditions. Previous studies demonstrated that 20E can induce expression of its own receptor, EcR, in a feed-forward loop (Varghese and Cohen, 2007), and indeed, when stimulating Kc cells with 20E, we saw a strong induction of EcR protein levels at 24 hours of stimulation (Figure 3.3D, lane 5). Surprisingly, silencing of PVR strongly attenuates 20E-dependent EcR expression (Figure 3.3D, lane 5 vs 6), an effect that cannot be reproduced by silencing single signaling mediators of the Akt/Tor and/or Mek/Erk

pathways (data not shown) and combinatorial silencing of Akt/Mek and Akt/Erk (Figure 3.3D, lane 9 and 10). To determine if this effect is PVR specific, we silence insulin receptor (InR) but did not see an attenuation of 20E-dependent EcR expression (Figure 3.3D, lane 7). However, combinatorial silencing of PVR and insulin receptor (InR) further attenuates 20E-dependent EcR expression (Figure 3.3D, lane 8). Insulin stimulation leads to attenuation of 20-E dependent EcR expression (Figure 3.3D, lane 5 vs 11).

Based on these results, we conclude that EcR and PVR signaling function in parallel, but ultimately may converge at the downstream cell death machinery. Interestingly, PVR signaling supports the sensitivity of Kc cells towards ecdysone-induced EcR upregulation. This reverse relationship of pro-survival PVR signaling and pro-death EcR signaling may prevent an excess of death signals, acting as a buffer to balance overall cell lifespan.

3.3 Discussion

The EcR complex in embryonic cell death

EcR and usp have been extensively studied in their roles in cell death, proliferation and differentiation at major transitions of *Drosophila* development, such as larval molting and metamorphosis (Kozlova and Thummel, 2000;Thummel, 1995). With respect to hematopoiesis, ecdysone plays a role in the terminal differentiation of lymph gland hemocytes (Sorrentino et al., 2002). Similarly, in Kc cell in culture, ecdysone stimulation arrests cells in the G2 phase of the cell cycle and triggers a cell differentiation program

(Andres et al., 1993;Cherbas et al., 1989;Stevens et al., 1980). In the embryo, ecdysone signaling was reported to play roles in morphogenetic movements such as germ band retraction (Kozlova and Thummel, 2003), yet it has not been associated with cell death previously (Chavez et al., 2000).

Here, we describe for the first time a role of ecdysone signaling in embryonic hemocyte death, both in vivo and in Kc cells in culture. This effect is revealed only under sensitized conditions of PVR silencing or loss-of-function that lead to a reduced viability of cells, or upon 20E stimulation in cell culture that overactivates the EcR pathway. When stimulating Kc cells with 20E, we find that the EcR targets E93 and rpr are transcriptionally upregulated, consistent with EcR complex binding to the E93 promoter that was reported previously (Gauhar et al., 2009). E93 controls pro-apoptotic genes such as the capase dronc (Baehrecke and Thummel, 1995;Lee et al., 2000), and rpr inactivates the caspase inhibitor Diap-1, thereby triggering apoptotic cell death through activation of Dronc and other caspases (Goyal et al., 2000;Meier et al., 2000). E93 and rpr are required in ecdysone-induced death of larval salivary gland and midgut during metamorphosis (Choi et al., 2006;Jiang et al., 1997a;Lee et al., 2000;Lee et al., 2002), and in the larval cell line l(2)mbn (Chittaranjan et al., 2009;Kilpatrick et al., 2005). Our findings show that ecdysone-induced cell death in embryonic Kc cells follows a similar mechanism. Consistent with (Gauhar et al., 2009;Hu et al., 2003;Roesijadi et al., 2007), we find that, in Kc cells, ecdysone treatment leads to a strong transcriptional upregulation of EcR, thereby positively amplifying the ecdysone response, an effect also known for many *Drosophila* tissues (Karim and Thummel, 1992;Koelle et al., 1991). This effect is

mediated through direct transcriptional regulation by the EcR complex (Gauhar et al., 2009), and indirect modulation through the micro-RNA mi-R14 (Varghese and Cohen, 2007). The mechanism of cell number rescue by EcR lof in the PVR kd background is less obvious. Since Kc cells express only very low levels of the known Halloween genes disembodied (dib), shadow (sad), shade (shd), spook (spo), phantom (phm) (DRSC, FLIGHT, ModEncode) required for the biosynthetic maturation of functional 20E ligand (Rewitz et al., 2006), we speculate that the EcR complex may have residual pro-death functions even in the absence of ligand. Previous publications have suggested that the unligated EcR complex has an active role and can bind to ecdysone response elements but frequently represses basal transcription (Cherbas et al., 1991;Schubiger and Truman, 2000). However, there are reports suggesting that mammalian estrogen-related receptor-α are constitutively active receptors that can function as a transcriptional activator in the absence of any added ligand (Vanacker et al., 1999). Thus, EcR complex may be involved in a residual transcription of pro-death genes even in the absence of ligand.

In addition, our data suggest PVR signaling and EcR signaling function in a parallel fashion. Our studies show that, surprisingly, PVR is permissive for efficient ecdysone-induced upregulation of EcR. In the presence of functional PVR signaling in wt control cells we find ecdysone-induced upregulation of EcR, while upregulation of EcR is attenuated in the PVRRNAi background. This effect is not seen when silencing mediators of the Akt/Tor or MAPK pathways, indicating that combined loss of PVR downstream signals, and/or a so far unidentified PVR-specific downstream pathway, is required for this effect. This positive epistasis of PVR signaling and EcR upregulation stands in

contrast to the previously described antagonistic relationship of InR and EcR in larval fat body and *Drosophila* S2 cells, where insulin stimulation leads to downregulation of the EcR coregulator dDor, and decreased EcR signaling (Francis et al., 2010). We interpret the inverse regulatory relationship of PVR and ecdysone signaling as a mechanism for the cell to buffer its pro-and anti-death signals in an attempt to maintain a defined lifespan. However, more studies will be required to dissect this mechanistic connection in more detail.

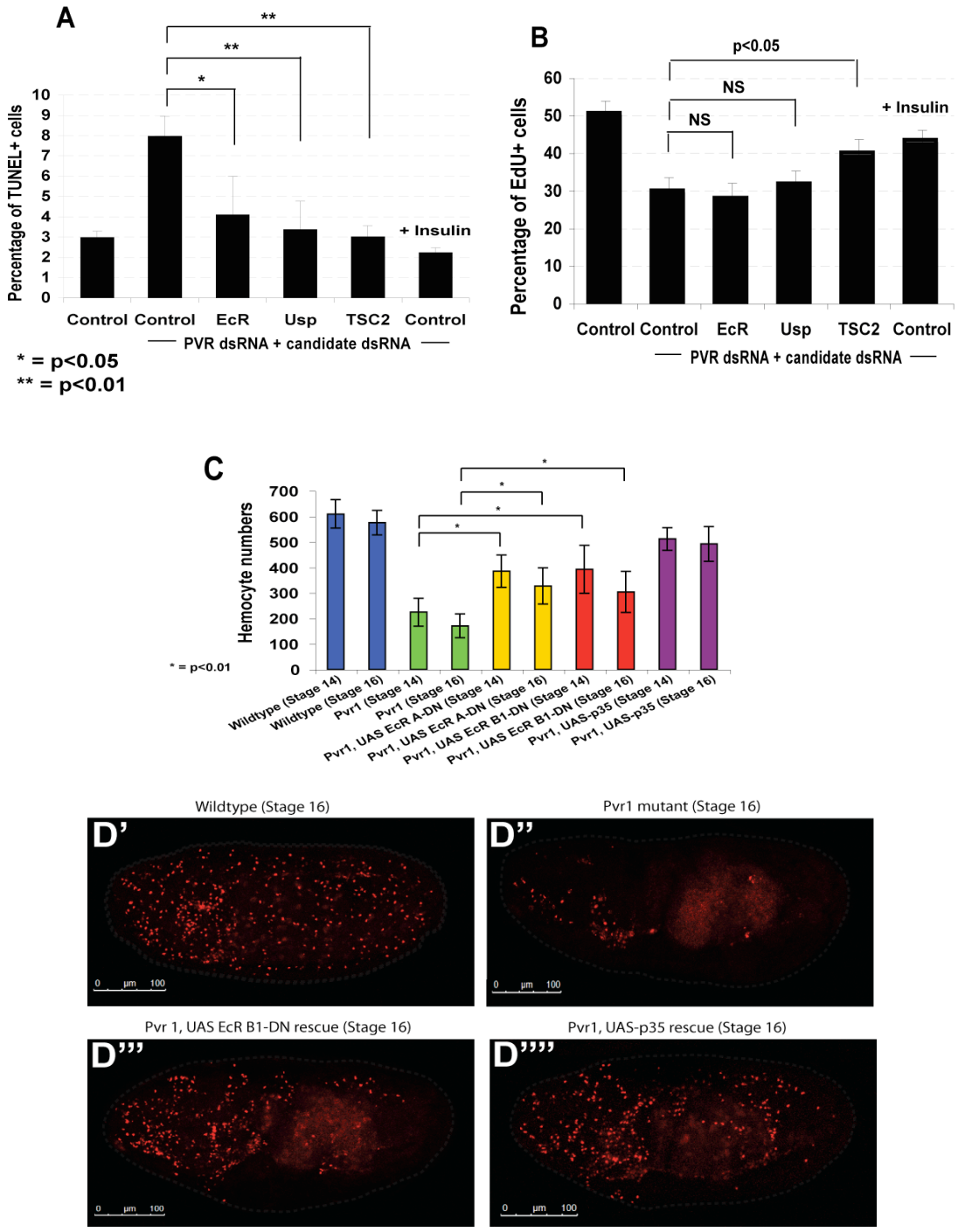
Mammalian nuclear hormone receptors in apoptosis and cancer

The vertebrate counterparts of EcR and usp are the LXRs (liver X receptors), and RXR (retinoid X receptor), respectively (King-Jones and Thummel, 2005;Owen and Zelent, 2000). LXRa and b are oxysterol sensors that control transcriptional programs in the regulation of macrophage metabolism and function (Janowski et al., 1996;Peet et al., 1998;Repa and Mangelsdorf, 2000), and their misregulation has been associated with metabolic disorders, artherosclerosis, and lack of immune functions (Korf et al., 2009;Repa and Mangelsdorf, 2002;Tontonoz and Mangelsdorf, 2003). RXR acts as a heterodimer with RAR (Retinoid Acid Receptor) that is stimulated by vitamin A-derived retinoids and rexinoids (Bastien and Rochette-Egly, 2004;Bushue and Wan, 2010). From our findings, we find there are similarities with what has been reported in the mammalian nuclear receptors, in particular RXR/RAR. We find EcR signaling regulates *Drosophila* blood cell survival. Similar to EcR, RXR plays central roles in cell proliferation, apoptosis, and differentiation (Altucci and Gronemeyer, 2001;Bastien and Rochette-Egly, 2004;Ross et al., 2000) during development and in pathologies such as cancer and

metabolic disease (Altucci et al., 2007;Means and Gudas, 1995). Lack of activation of the RXR/RAR pathways causes cancer due a lack of cell differentiation and increased cell survival (Bushue and Wan, 2010) and treatment with synthetic retinoids or rexinoids promotes cancer remission by inducing catabolism or apoptosis in cancer cells (Zhu et al., 2001). Here, we report ecdysone induction also leads to apoptosis and reduction in cell proliferation in Kc cells. Additionally, we found an interesting relationship between PVR and ecdysone signaling, where functional PVR signaling permits ecdysone-induced upregulation of EcR, while loss of PVR signaling attenuates the upregulation of EcR. By analogy with the *Drosophila* system, it will be interesting to investigate whether signaling by PDGF/VEGF Receptors or other RTKs may provide a regulatory link with RXR signaling, with potential implications for cancers in which either or both signaling pathways would be deregulated.

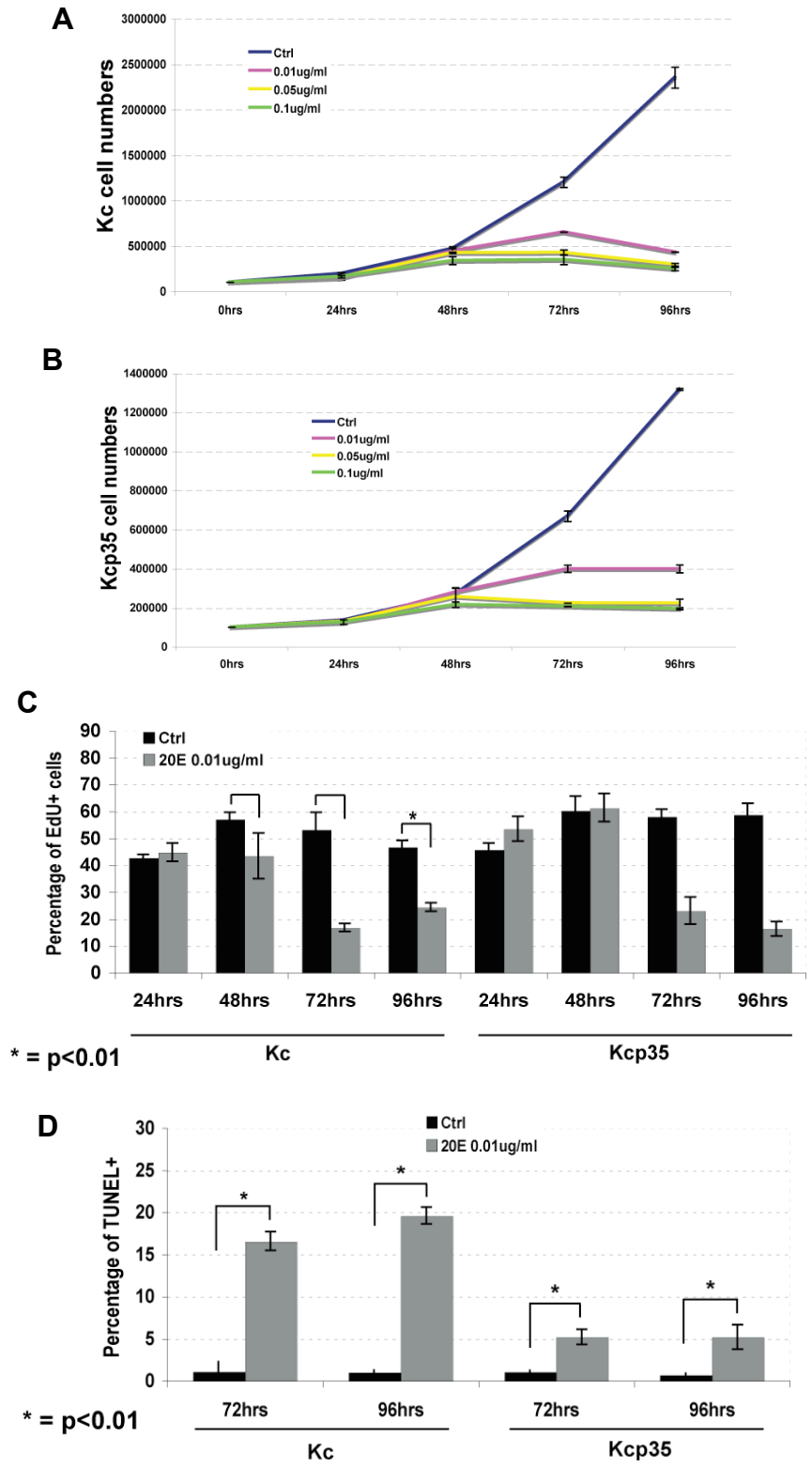
3.4 Figures

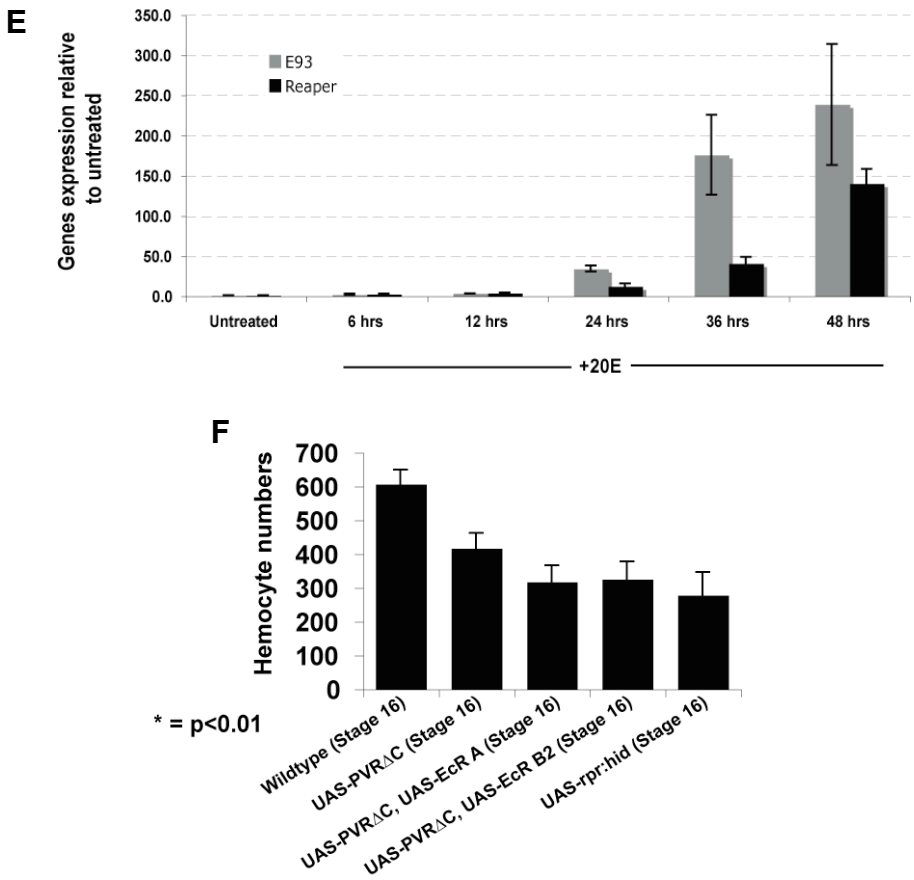
Figure 3.1. EcR/usp loss-of-function rescues cell death



(A) Silencing of the PVR Suppressors EcR, usp, and Tsc2 rescues PVR-induced cell death. TUNEL assay in Kc cells. Quantification of % TUNEL positive cells from total cells counted. (B) EdU incorporation assay in Kcp35 cells to quantify proliferation rescue by PVR Suppressors. While re-activation of the Akt/Tor pathway by Tsc2 kd rescues proliferation in PVR kd cells, EcR and usp kd do not. Quantification of % EdU positive cells from total cells counted. (A, B) n = 3 independent experiments. Statistical analysis performed with two-tailed t-test. (C, D) Inhibition of EcR signaling by expression of dominant-negative EcR rescues hemocyte numbers in Pvr1 mutant embryos. (C) Comparison of embryonic hemocyte numbers in wildtype, PVR loss-of-function and rescue embryos by expressing EcR dominant negative. Hemocytes were marked by nuclear β-Gal driven by srpHemoGAL4 and counted. Average hemocyte numbers (in brackets) were as follows: wildtype stage 14 (610), stage 16 (575); *Pvr1*/*Pvr1* stage 14 (224), stage 16 (171); Pvr^{1},UAS-EcR A W650A / Pvr^{1} stage 14 (385), stage 16 (327); Pvr^{1},UAS-EcR B1 W650A / Pvr^{1} stage 14 (393), stage 16 (304); p35 rescue of *Pvr1*/*Pvr1* stage 14 (512), stage 16 (492). 10 independent embryos per genotype and stage were assessed. Statistical analysis performed with two-tailed t-test. (D) Confocal images of lateral view (anterior left, dorsal top) of stage 16 embryos. Hemocytes were marked by nuclear β-Gal driven by srpHemoGAL4 for confocal images showing the distribution of the hemocytes.

Figure 3.2. Ecdysone stimulation induces cell death

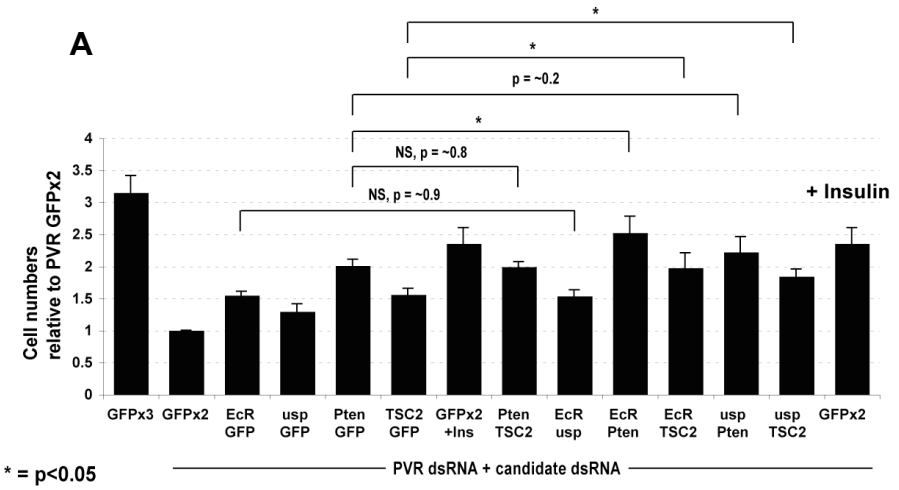


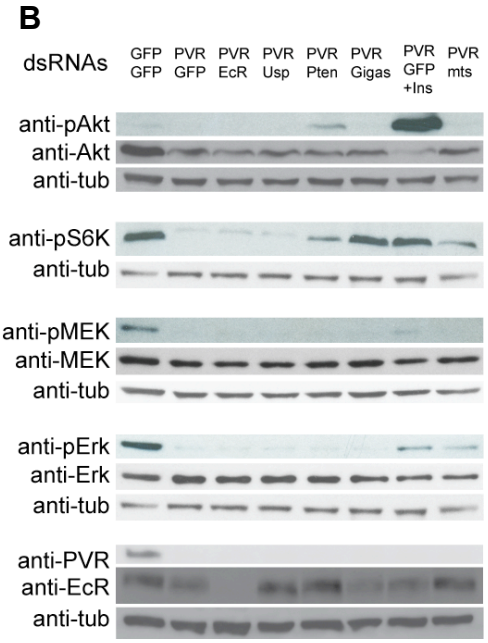


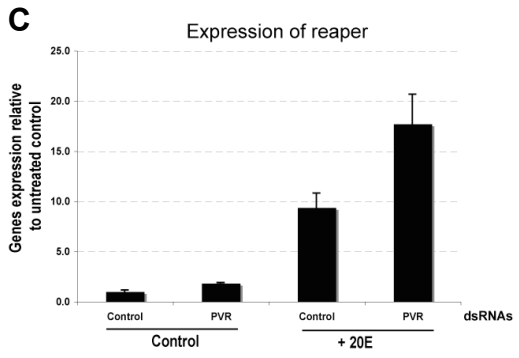
(A,B) Increase in cell numbers over time; Kc (A) and Kcp35 cells (B), treated with various concentrations of 20E. Data presented are an average of 2 independent duplicate experiments. (C) EdU incorporation in Kc and Kcp35 cells, treated with 20E and vehicle control. (D) Percentage of TUNEL positive cells in Kc and Kcp35 cells, treated with 20E and vehicle control. (C, D) n = 3 independent experiments. Statistical analysis performed with two-tailed t-test. (E) Stimulation of Kc cells with 20E leads to upregulation of rpr and E93. Gene expression measured by q-RT-PCR. 2 independent triplicate experiments were performed. Representative data shown. (F) Comparison of embryonic hemocyte numbers in wildtype, PVR dominant negative, PVR dominant negative with wildtype EcR recombinants and rpr:hid control. Hemocytes were marked by nuclear β-Gal driven

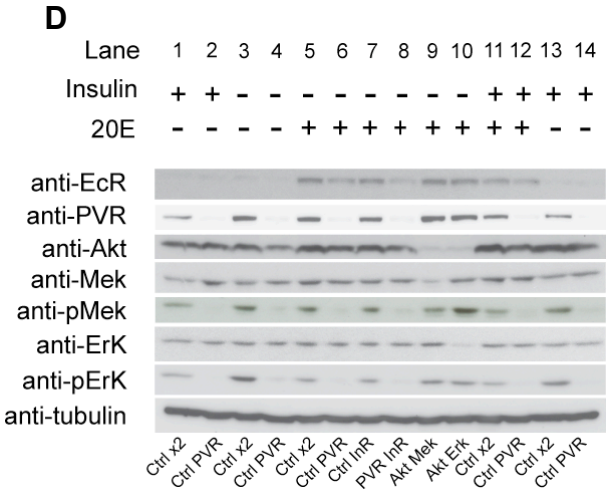
by srpHemoGAL4 and counted. Average hemocyte numbers (in brackets) were as followed: wildtype stage 16 (608); UAS-PVRΔC stage 16 (417); UAS-PVRΔC, UAS-EcR A stage 16 (317); UAS-PVRΔC, UAS-EcR B1 stage 16 (325); UAS-rpr:hid stage 16 (279). 10 independent embryos per genotype and stage were assessed. Statistical analysis performed with two-tailed t-test.

Figure 3.3. Relationship between PVR and EcR signaling









(A) Effects of combinatorial RNAi on cell numbers. Note additive effects of combinations of genes from the Akt and EcR pathway on PVR rescue. n = 3 independent experiments. Statistical analysis performed with two-tailed t-test (B) Western blot for phosphorylated signaling mediators, using various RNAi conditions. Note that PtenRNAi rescues Akt and S6K phosphorylation, Tsc2RNAi rescues S6K phosphorylation and mtsRNAi rescues both S6K and Erk phosphorylation in the PVR RNAi background, while EcR RNAi and usp RNAi have no effect. Super stimulation by Insulin treatment phosphorylates all signaling mediators in the PVR RNAi background. (C) PVRRNAi and 20E induction synergizes reaper expression. q-RT-PCR of rpr in Kc cells pretreated by PVRRNAi and control kd (GFPRNAi) for 2 days. Cells were stimulated with 20E or vehicle control for 2 more days. Note further upregulation of rpr expression in 20E-stimulated PVRRNAi cells. 2 independent triplicate experiments were performed. Representative data shown. (D) WB of EcR. 20E stimulation induces EcR upregulation, which is attenuated in the PVRRNAi background. Cells pretreated with dsRNAs for knockdown 2 days before 20E is added and left for additional 24 hours, before cell lysis for protein analysis.

Chapter 4: Studies on blood cell lifespan in *Drosophila* embryo and larva

4.1 Introduction

In a multi-cellular organism, the lifespan of cells range from a few days to even the whole lifetime of the organism (Alberts et al., 2007;Nowakowski, 2006;Parihar et al., 2010;Pillay et al., 2010;Sprent and Surh, 2011;Tough and Sprent, 1995), as determined by the survival signals the cells received from growth factors, cell surface interactions, etc. The regulation of blood cell lifespan by the inputs of pro-apoptotic and anti-apoptotic signals has been extensively studied in view of understanding hematopoiesis and hematologic diseases. Blood cell lifespan ranges from a few days (e.g. neutrophils and monocytes) to a few months (e.g. memory T cell) (Parihar et al., 2010;Pillay et al., 2010;Sprent and Surh, 2011;Tough and Sprent, 1995). Most naïve T cells lives as long as the periods of infection, as the cells lose their survival signals (Berard, 2002). However, a small of percentage effector T cells survives to be long-lived memory T cells as they received survival signals (Akbar et al., 1993;Grayson et al., 2000;Pilling et al., 1999).

The dysregulation of blood cell lifespan can generally lead to human diseases including immunodeficiency, autoimmunity and cancer. Neutrophils are short-lived cells which are cleared as inflammation resolves. They undergo apoptosis and eventually phagocytosed by macrophages (Haslett et al., 1994). If the neutrophil lifespan is shortened (i.e. die prematurely), this leads to immunodeficiency called neutropenia with incomplete clearance of infection or higher chances of infection (Aleman et al., 2004;Ramirez et al.,

2004). Conversely, if the neutrophil survives and persists at the sites of inflammation this can potentially lead to chronic inflammatory diseases such as rheumatoid arthritis (Edwards and Hallett, 1997).

The understanding of the mechanisms of cell lifespan allows the design of therapies that alter the abnormal longevity of the cells in a pathological setting such as cancer. To combat cancer, there is an array of FDA-approved small-molecule inhibitor and monoclonal antibodies against receptor tyrosine kinases for cancer therapy. The molecular multikinase inhibitors (e.g. Sorafenib, Gleevec, Dasatinib, Nilotinib) induce apoptosis in human leukemia cells and other cancer cells (Belloc et al., 2007;Belloc et al., 2009;Okabe et al., 2011;Rahmani et al., 2007a;Rahmani et al., 2007b;Steinberg, 2007) and, therapeutic monoclonal antibodies like Herceptin induces breast cancer cell apoptosis (Mohsin et al., 2005).

The control of cell lifespan is also being displayed in *Drosophila* development. Just as programmed cell death (PCD) is involved in neurogenesis in vertebrates, this happens so for the *Drosophila* central nervous system (CNS) (Buss et al., 2006) as well. For example, *Drosophila* neuroblasts (NB) in the developing ventral nerve cord (VNC) undergo apoptosis in late embryoenesis (Truman and Bate, 1988;White et al., 1994). Of the 30 NBs originally present in the VNC, only 3 survived at the end of embryogenesis (Truman and Bate, 1988). These 3 NBs persist through the larval life and proliferate to give rise to neuronal lineages, until mid-third instar where the NBs are eliminated by apoptosis (Bello et al., 2003). By blocking the expression of cell death genes (e.g. reaper,

grim and sickle), the death of neuroblasts is prevented and the VNC becomes hypertrophic (Peterson et al., 2002;Tan et al., 2011).

The regulation of the lifespan of *Drosophila* hemocyte has not been characterized previously, although there is a suggestion that embryonic hemocyte persist through the whole lifetime of the fly (Holz et al., 2003a), this report does not distinguish whether it is the same cells or their descendents that persist into later stages. Instead, reports suggest that hemocyte numbers in 1st instar larva is significantly lower than in late embryos (Lanot et al., 2001a;Makhijani et al., In Press). The hemoycte precursors are specified in the procephalic mesoderm (Rehorn et al., 1996). These undifferentiated prohemocytes undergo four divisions during embryonic stages 8-11, and after at the final division stop proliferating. The hemocyte numbers then remains constant throughout embryogenesis (Tepass et al., 1994a). Therefore, hemocyte proliferation is separated in time from other later developmental events such as survival (Brückner et al., 2004). Taking all these evidences, it suggests the observed phenomenon from Lanot, et al and Makhijani, et al is independent of proliferation and could be due to hemocyte cell death.

The goal of this chapter is to characterize the lifespan of *Drosophila* hemocyte during embryonic and postembryonic development. First, I characterize hemocyte number development at the embryo-larva transition, followed by experiments that aim to understand putative underlying mechanisms for the apparent hemocyte changes.

4.2 Results

Hemocyte development at the embryo-larva transition

After mid-embryogenesis, the hemoycte numbers remain constant throughout

embryogenesis (Tepass et al., 1994a). Based on previous reports that suggest hemocyte number in 1st instar larva is much lower than in late embryos (Lanot et al., 2001a;Makhijani et al., In Press), we investigated the temporal changes in hemocyte numbers during the embryo-larva transition. First, we quantified hemocyte numbers in vivo from late embryogenesis to late larva 1st instar (Figure 4.1). In vivo quantification of embryonic hemocytes by expression of the nuclear GFP (i.e. stinger) under control of srpHemo-GAL4 (Brückner et al., 2004) driver, showed that during late embryogenesis hemocyte numbers remains the same until 16 hours after egg laying (AEL). Interestingly, we found that towards the end of embryogenesis, the number of GFP-positive hemocytes drops by about one-third (16 hours AEL 313±34 hemocytes vs 20 hours AEL ~207±23 hemocytes) (Figure 4.1), suggesting termination of cell lifespan. The hemocyte numbers then remained constant during the transition from embryo to 1st larva instar, and postembryonically, the number of GFP-positive hemocytes increases gradually.

Putative mechanism of hemocyte changes at the embryo-larva transition

Since we observed a drop in hemocyte counts towards the end of embryogenesis, we are investigated the putative underlying mechanisms. Hypothesizing that hemocytes undergo apoptosis towards the end of embryogenesis, we used the GAL-UAS system (Brand and Perrimon, 1993) to overexpress proteins that block the apoptotic pathway and determined the effects on hemocyte number at the embryo-larva transition. It has been demonstrated that the *Drosophila* PDGF/VEGF Receptor, PVR, is essential for anti-apoptotic survival of embryonic hemocytes (Brückner et al., 2004). In addition, we have previously shown

that suppression of pro-apoptotic EcR signaling by the expression of dominant-negative forms of EcR partially rescues the hemocyte number decline in the Pvr1 mutant background (see Figure 3.1). Based on these results, we investigated whether by increasing a pro-survival signal through the expression of activated-PVR, λPVR, (Brückner et al., 2004) or blocking pro-death signal through the expression of dominant-negatives of EcR is able to rescue the drop in hemocyte numbers during late embryogenesis.

We tested the effect of λPVR and dominant-negatives of EcR (i.e. EcR A-W650A and EcR B1-W650A) by overexpression in hemocytes using the driver srpHemo-GAL4 (Brückner et al., 2004). Hemocytes were quantitated, by co-expression of the fluorescent protein stinger, at two time points (i.e. 16 hours and 18 hours AEL) (Figure 4.2). Expression of λPVR and dominant-negative forms of EcR did not rescue the drop in hemocyte numbers during late embryogenesis (Figure 4.2), thus suggesting the decline in hemocyte numbers may not be attributed to a death pathway shared by PVR signaling and EcR signaling. Likewise, expression of baculovirus caspase inhibitor- p35 (Hay et al., 1994) did not rescue hemocyte numbers at the embryo-larva transitions (Figure 4.3). As expected concerted expression of the two pro-apoptotic genes, rpr and hid induced a further reduction in hemocyte numbers (Figure 4.3), suggesting induction of apoptosis can lead to reduction in hemocyte numbers. In conclusion, I conclude that hemocyte loss at the embryo-larva transition may not occur through apoptosis, but another cell death mechanism, or may occur through a p35-insensitive apoptotic mechanism.

4.3 Discussion

While it has been reported that the total number of hemocytes in the first larval instar is significantly lower than the hemocyte number observed at the end of embryonic development (Lanot et al., 2001a;Makhijani et al., In Press), this phenomenon has not been well characterized. Here, we report the embryonic hemocyte population declines about one-third at the end of embryogenesis (Figure 4.1). We hypothesized that a subpopulation of the embryonic hemocytes has a limited lifespan and termination of lifespan is regulated by a specific balance of pro-survival and pro-death signals within the cell, similar to vertebrate examples of defined cell lifespan (Opferman, 2007;Parihar et al., 2010;Wright and Deshmukh, 2006). Postembryonically, hemocyte numbers increase gradually in the first instar. This corresponds well with recent findings by Makhijani et al. In the paper, the authors did lineage tracing experiments showing embryonic differentiated hemocytes persist into the larval stages to be the founders of the larval hematopoietic system. These differentiated hemocytes proliferate to constitute the population of larval hemocytes (Makhijani et al., In Press).

Earlier, in Chapter 3, PVR and EcR signaling were both shown to regulate the survival of embryonic hemocytes as an anti-apoptotic (Brückner et al., 2004) and pro-apoptotic mediator respectively. Our results suggesting the decline in hemocyte numbers may be attributed to a death pathway not shared by PVR signaling and EcR signaling (Figure 4.2) or p35-related apoptotic pathway (Figure 4.3).

Further investigation will be needed to understand the mechanism behind the decline in

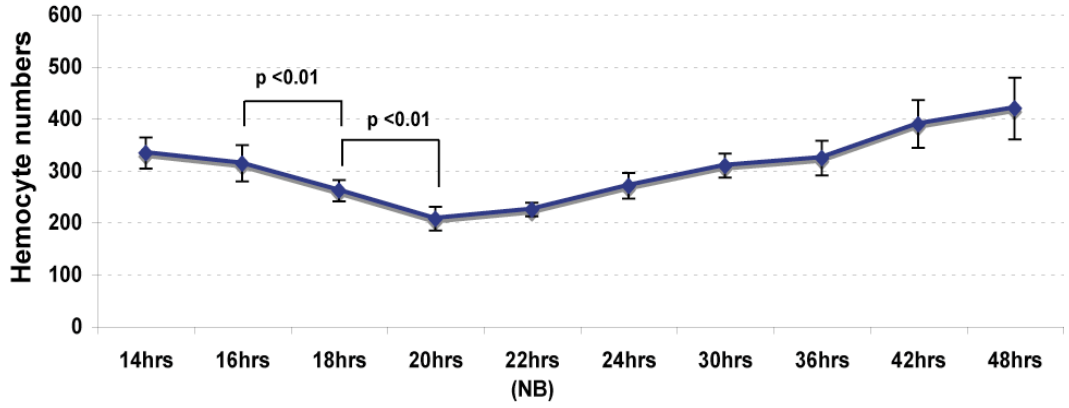
hemocyte numbers during late embryogenesis. The possibility that the embryonic hemocytes de-differentiate, thereby losing their nuclear GFP expression is very unlikely, based on an experiment by Makhijani et al. The authors of the paper did a EOS-FP lineage tracing experiment of embryonic hemocytes from stage 16 embryos to the 1st instar. EOS-FP is a color-switch fluorescent protein that fluoresces green in its native state, and by exposure to UV is photoconverted to emit a stable red fluorescence (Wiedenmann et al., 2004). By photoconverting stage 16 embryos and observing 1st instar larvae the next day, the authors found that the vast majority of EOS-green hemocytes was positive for the red label, indicating that the majority of larval hemocytes derive directly from the differentiated population of embryonic hemocytes, and the hemocytes stay in their differentiated state from stage 16 to 1st instar (Makhijani et al., In Press). If the hemocyes de-differentiated and stay in this state, they will not express EOS-FP, and most hemocytes will only be positive red label.

Therefore, I propose that an alternative form of PCD, e.g. autophagic cell death (ACD), could be responsible for the decline in hemocyte numbers at the embryo-larva transition. It has been reported that *Drosophila* larval salivary gland and midgut undergoes ACD during metamorphosis (Berry and Baehrecke, 2007;Lee and Baehrecke, 2001;Lee et al., 2002). Caspase activity has been widely reported to function during ACD (Berry and Baehrecke, 2007;Lee and Baehrecke, 2001;Lee et al., 2002), however, it has also been shown that ACD and caspases function in parallel pathways during cell death and that both independently contribute to cell death (Berry and Baehrecke, 2007;Yu et al., 2004). Gain-of-function and loss-of-function of autophagy dependent genes (atg) experiments

had been done to ascertain the role of ACD in cell death during *Drosophila* development (Berry and Baehrecke, 2007;Scott et al., 2007), therefore, similar experiments can probably reveal the role of ACD in the control of hemocyte lifespan.

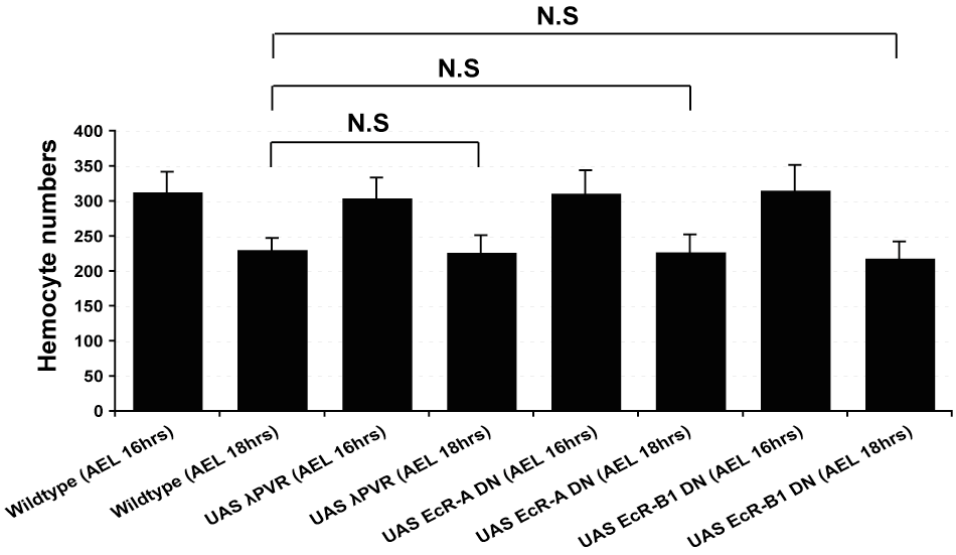
4.4 Figures

Figure 4.1. Decline of blood cell numbers at the embryo-larva transition



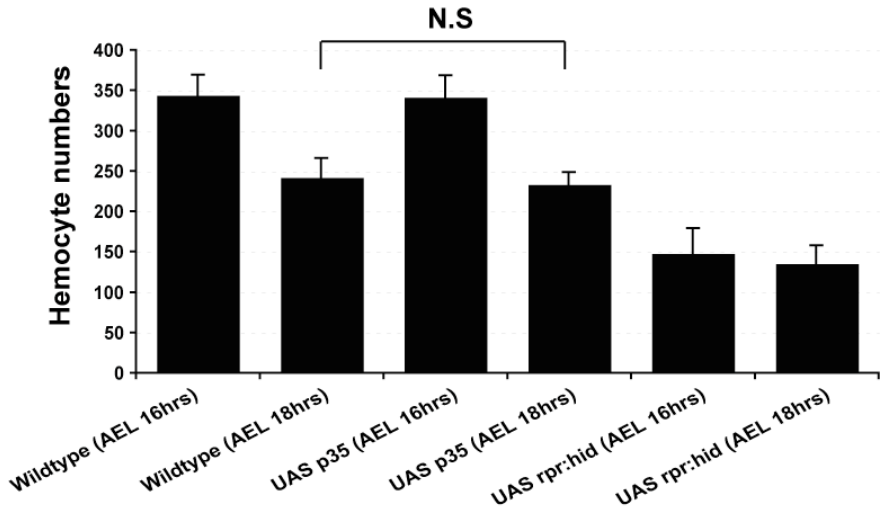
In vivo quantification of hemocytes from late stage embryos to first instar larvae. Hemocytes were marked by srpHemoGAL4 (Brückner et al., 2004) driven expression of nuclear GFP stinger and then counted. Average hemocyte numbers (in brackets) were as followed: AEL 14 hours (333±30); AEL 16 hours (313±34); AEL 18 hours (261±21); AEL 20 hours (207±23); AEL 22 hours (224±13); AEL 24 hours (270±25); AEL 30 hours (309±23); AEL 36 hours (324±33); AEL 42 hours (389±46); AEL 48 hours (419±59). 10 independent embryos per genotype and stage were assessed. Statistical analysis performed with two-tailed t-test.

Figure 4.2. Activated PVR and EcR dominant negative failed to rescue drop in hemocyte numbers



Comparison of embryonic hemocyte numbers in wildtype, expressing activated PVR and expressing EcR dominant negative. Hemocytes were marked by srpHemoGAL4 driven expression of nuclear GFP stinger and then counted. Average hemocyte numbers (in brackets) were as followed: wildtype AEL 16 hours (312±29), AEL 18 hours (231±16); UAS-λPVR AEL 16 hours (305±28), AEL 18 hours (227±24); UAS-EcR A W650A AEL 16 hours (311±32), AEL 18 hours (227±25); UAS-EcR B1 W650A AEL 16 hours (316±35), AEL 18 hours (219±24). 10 independent embryos per genotype and stage were assessed. Statistical analysis performed with two-tailed t-test. Results based on a single experiment.

Figure 4.3. p35, baculovirus inhibitor of apoptosis does not rescue drop in hemocyte numbers



Comparison of embryonic hemocyte numbers in wildtype, expressing p35 and expressing rpr:hid as control. Hemocytes were marked by srpHemoGAL4 driven expression of nuclear GFP stinger and then counted. Average hemocyte numbers (in brackets) were as followed: wildtype AEL 16 hours (344±25), AEL 18 hours (242±24); UAS-p35 AEL 16 hours (342±27), AEL 18 hours (234±15); UAS-rpr:hid AEL 16 hours (148±31), AEL 18 hours (135±22). 5 independent embryos per genotype and stage were assessed. Statistical analysis performed with two-tailed t-test. Results based on a single experiment.

Chapter 5: Concluding remarks

5.1 Summary of findings

To our knowledge, the double-knockdown screen, on which this thesis is based, has been the only screen that investigates cell number regulators in relation to *Drosophila* PVR. This allowed the detection of many genes that have not been identified in conventional RNAi screens for cell proliferation and survival (Bettencourt-Dias et al., 2004;Bjorklund et al., 2006;Boutros et al., 2004;Chew et al., 2009;Yi et al., 2007). Many of the identified genes are tumor suppressor genes from the PI3K/Akt/Tor and Ras/MAPK pathway, thus validating the approach and the candidate genes. The screen identified heterodimeric nuclear hormone receptor (i.e. EcR and usp) as two of the strongest hits among PVR suppressors and we describe the characterization of both genes as a proof of principle study. Our studies show a novel role of ecdysone signaling in blood cell survival and by manipulating EcR signaling in PVR silencing and loss-of-function will have an effect on the overall survival of hemocytes. Also, we reported an inverse regulatory relationship of PVR and ecdysone signaling which may be a mechanism for the cell to buffer its pro-and anti-death signals in an attempt to maintain a defined lifespan. However, more studies will be required to confirm this hypothesis. Lastly, we attempted to characterize the lifespan of *Drosophila* hemocyte during embryonic and postembryonic development, so as to use it as a model system for studying blood cell lifespan. However, our results in this study have yet to be conclusive.

5.2 Multiple signaling networks involved in cancer

Cellular signaling pathways are not independent from each other but are interconnected to form complex signaling networks (Martin, 2003). To understand how these complex signaling networks lead to cancer progression is a major challenge due to our limited understanding of the pathways (Martin, 2003). In recent years, researchers have embarked on systematic sequencing studies of many human tumors from different cancer types to detect new cancer genes (Cowin et al., 2010;Greenman et al., 2007). Cancer cells have many mutations, and most mutations do not contribute to cancer (i.e. passenger mutations) (Greenman et al., 2007). Thus, finding a carcinogenic mutation (i.e. driver mutation) is like finding the right needle from a plethora of needles in a haystack. While systematic sequencing studies has made contributions in identifying numerous novel candidates, one of the difficulties posed by the studies is separating passenger from driver mutations, and the need to develop high-throughput functional assays for this purpose (Cowin et al., 2010). Here, a functional approach, i.e. change in cell numbers, in a genome-wide *Drosophila* cell culture screen was used, which identified many known tumor suppressors, and uncharacterized candidates, who display the same phenotype as the tumor suppressor, and may potentially be novel tumor suppressors. This highlights the success of the system in carrying out a genome-wide functional assay to identify novel genes and the system can probably complement the studies done in mammals. *Drosophila* model system has been the pioneer in the discovery of many cancer genes, signaling pathways and in understanding how multiple signaling pathways interact in driving cancer (Brumby and Richardson, 2003;Brumby and Richardson, 2005;Simon et

al., 1991;Simon et al., 1993;Wu et al., 2010). With this reliable system, we can only look forward in uncovering more novel cancer-causing genes and understand how different signaling pathways synergistically drive cancer.

5.3 Future directions

Certainly, there are still numerous uncharacterized PVR suppressors that we have yet to study in depth. 24 out of 26 PVR suppressors has a mammalian orthologue. To bring our studies closer to clinical relevance, it might be worthwhile to evaluate the functional conservation of these mammalian orthologues in their regulation of cell numbers.

Lastly, we hope that the basic research efforts in this thesis contribute to further the knowledge of clinical problem arose due to dysregulation of cell numbers, especially in the field of cancer. The studies presented in this thesis could be the first step on the road to potential novel therapies against tumor diseases.

Chapter 6: Materials and Methods

Fly stocks and crosses

Fly lines used were: *Pvr^{1}/CyO* (Brückner et al., 2004), *Pvr^{4}/CyO* (Brückner et al., 2004), *srpHemoGAL4* (Brückner et al., 2004), *UAS-PVRΔC* (Brückner et al., 2004), *UAS-λPVR* (Brückner et al., 2004), *UAS-p35* (Hay et al., 1994), *UAS-srcEGFP* (E. Spana), *UAS-lacZnls* (E. Spana), *UAS-mCD8:GFP* (Lee and Luo, 1999), *UAS-Stinger* (Barolo et al., 2000), *UAS-EcR A*, *UAS-EcR B1*, *UAS-EcR B2* (Lee et al., 2000), *UAS-EcR B1 W650A* (Cherbas et al., 2003), *UAS-EcR A W650A* (Brown et al., 2006) and UAS rpr;; UAS hid (Zhou et al., 1997).

Genotypes of Pvr rescue experiments were: *Pvr^{1},UAS-srcEGFP/ Pvr^{1},srpHemoGAL4; UAS-p35/UAS-lacZnls* and *Pvr^{1},UAS-EcR (A/B1) W650A / Pvr^{1},srpHemoGAL4; UAS-mCD8::GFP/UAS-lacZnls*. For overexpression studies in PVR sensitized background genotypes generated are *srpHemoGAL4,UAS-srcEGFP/+; UAS-PVRΔC, UAS-EcR (A/B2)/UAS-lacZnls*. *srpHemoGAL4, UAS-Stinger* (K. Makhijani) was generated for live assessment of hemocytes in late embryos and 1st instar larvae. Genotypes of the live assessment experiments are *srpHemoGAL4, UAS-Stinger/+; UAS-λPVR/+, srpHemoGAL4, UAS-Stinger/+; UAS-EcR A W650A /+, srpHemoGAL4, UAS-Stinger/+; UAS-EcR B1 W650A /+, srpHemoGAL4, UAS-Stinger/+; UAS-p35 /+ and srpHemoGAL4, UAS-Stinger/+; UAS-rpr:hid /+*.

Embryo stainings, stimulation, and microsopy

Fly crosses were kept in collection baskets with apple juice flavored *Drosophila* agar plates as caps. Embryos laid on the agar plates were dechorionated by freshly made 50% bleach, washed, fixed in 8% formaldehyde and then devitellized by methanol/heptane, before stored in fresh methanol at -20^{C}. Collections for each specific cross is consolidated after few days of collections and stained. Primary antibodies used were goat anti-GFP (1:1500) (Molecular Probes) and mouse anti-β-Gal (1:750) (Promega), and corresponding Alexa Fluor® secondary antibodies (Invitrogen) were used for fluorescent microscopy (Leica DMI 4000B) . Hemocyte counts were conducted under fluorescent microscopy at 40X, assessing 10 independent embryos per genotype and stage.

Live assessment of hemocytes. Embryos and 1st instar larvae were harvested from 1 hour timed collection that had been aged for a specific time after egg laying (AEL), transferred to a drop of halocarbon oil (Sigma-Aldrich) on microscope slide and anesthetize by incubating for 5-7 minutes in a chamber with vaporized heptane (Sigma-Aldrich). Hemocyte counts, of the anesthetized embryos and larvae, were conducted under fluorescent microscopy (Leica DMI 4000B) 40X, assessing 5 or 10 independent larvae per genotype.

Cell maintenance and stimulation

Kc 167 cells {Echalier, 1969 #745}, here simply called Kc, were cultured in Schneider's *Drosophila* Medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) and

1000 units/ml Penicillin and 1000mcg/ml Streptomycin.

20-E experiments: 20-Hydroxyecdysone (Sigma-Aldrich), 20-E, was dissolved in ethanol to make a 5mg/ml stock. A subsequent stock of 1μg/ml stock was made by diluting in ddH_2O. 0.1x10^6 cells was seeded into each well of a 24 well plate and required amounts of 20-E stock was added immediately to achieved the desired concentration. 100X (0.5mg/ml) insulin stock is prepared and required amounts of insulin stock were added to experiments to achieved 1X insulin.

p35 stable cell line

Effectene Transfection Reagent (Qiagen) is utilized for the transfection. Kc 167 cells were co-transfected with driver Actin-GAL4, UAS-puromycin, UAS-GFP and UAS-p35 plasmid constructs. 3 days after transfection, puromycin was added to medium for a final concentration of 10ug/ml. Cells were left for further 2 weeks for stable cells with puromycin resistance to expand. Surviving cells were harvested and FACs sorted to isolate the highest 20 percentile of GFP-expressing cells. To further select cells that are resistant to apoptosis, DIAP knockdown is induced to eliminate cells with weak resistance to caspase-dependent apoptosis. The surviving cells are expanded for experimental use. Presence of p35 transgene in p35 stable cell line was confirmed by PCR verification.

Cell-based RNAi

RNAi kd was performed as described previously (Clemens et al., 2000). Briefly, Kc 167 cells were re-suspended and diluted in serum free medium before seeding. dsRNA targeting each specific genes knockdown was added and incubated for 45 minutes before supplementing with complete medium with FBS.

Genome-wide RNAi screening

K. Brückner screened a set of 62 384-well plates that were pre-arrayed with dsRNAs, corresponding to 22,915 distinct amplicons of version 1 of the *Drosophila* genome corresponding to 13,592 unique genes (Adams et al., 2000), and 7463 Sanger predictions (Hild et al., 2003) (DRSC). To determine differential effects between PVR silenced and control cells each plate was screened under two conditions, kd of PVR or a control (GFP). All experiments were performed in replicate. Each well contained 0.25ug of pre-arrayed dsRNA. In addition, before seeding, Kc cell suspensions were pre-mixed with PVR or control (GFP) dsRNAs in batch, corresponding to a final concentration of 0.3ug per well. Cells were seeded at a density of 7,000 cells/well and incubated for 4 days. CellTiter-Glo assay (Promega) was performed according to the instructions of the manufacturer, and luminescence was read using Analyst GT or SpectraMax plate readers (Molecular Devices). All liquid handling was performed using WellMate (Matrix), MicroFill (BioTek), or MultiDrop (Thermo), high-throughput dispensers. Z scores [$z=(\chi-\mu)/\sigma$] were calculated as follows: μ = Mean of readings from controls wells (i.e. wells without pre-arrayed candidate dsRNAs), σ = Standard deviation from readings of the control wells. χ = Reading of candidate gene well. Z score for PVR knockdown condition

(Z[PVR]) and for control knockdown condition (Z[GFP]) were generated and the differential effects in PVR knockdown condition and control knockdown were calculated by the difference of each Z scores (i.e. Z[PVR]- Z[GFP]).

Cell counting, EdU and TUNEL assays

To obtain cell counts, $0.1x10^{\wedge 6}$ cells was seeded into each well of a 24 well plate follow by treatment with dsRNAs or 20E. 3.3ug of dsRNA targeting each specific gene knockdown was added. After culturing for the indicated period of time, cells are re-suspended and diluted 1:1 with 0.4% Trypan Blue. Numbers of viable/dead cells were assess by hemocytometer counting based on Trypan Blue exclusion/staining.

For EdU and TUNEL assays, 20,000 Kc cells were seeded into each well of 96-well black clear bottom plate (Corning Costar) and immediately treated with dsRNAs or 20-E. 0.825ug of dsRNA was used to target each specific gene knockdown. For assessing cell proliferation, cells were incubated for 4 hours with 10uM of Click-iT® EdU (Invitrogen) one or several days after the dsRNAs or 20-E treatment. The Click-iT® EdU cell proliferation assay was conducted according to manufacturer's instructions. For assessing cell death, post-incubated cells were processed by TUNEL assay according to manufacturer's instructions (Invitrogen).

The stained cells were counted visually and with ImageJ from still images. Briefly, for ImageJ protocol, still images were converted to 8 bit images and cells were selected by

setting a threshold against the background. Highlighted cells were then counted by 'Analyze Particles'. At least 3 still images for each sample were taken at random sites using a 40X objective. Percentage of EdU or TUNEL positive cells were calculated as follow- (No. of EdU or TUNEL positive cells/ Total number of cells) * 100.

dsRNAs design and generation

In most cases, dsRNA amplicon sequences were selected by the *Drosophila* RNAi Screening Center (DRSC), as indicated by DRSC amplicon numbers (See below). Primers used for generating the amplicon template contain a 5' T7 RNA polymerase binding site (TAATACGACTCACTATAGG) following by the amplicon sequences. dsRNAs were generated by in vitro transcription using Megascript T7 transcription kit (Ambion). Generated dsRNAs were purified by RNeasy Mini Kit (Qiagen) and dsRNAs product size is confirmed by 1% agarose gel electrophoresis. The dsRNAs amounts were quantitated by Nanodrop 2000C spectrophotometer (Thermo Scientific). See below for dsRNA amplicon template primers.

Real time PCR

Total RNA was extracted by using RNeasy mini kit (Qiagen), done according to manufacturer's instruction. Total purified RNA amount extracted were quantitated by Nanodrop 2000C spectrophotometer (Thermo Scientific). 1ug – 0.1ug of purified RNA was reversed transcribed into cDNA using iScrpit cDNA synthesis kit (Biorad). Real time PCR reactions were carried out using iQ SYBR Green Supermix (Bio-Rad) on Bio-Rad

CFX96™ Real Time System and gene expression levels were analyzed with CFX Manager™ Software (Bio-Rad). Primers for real time PCR assays were designed using web-based software ProbeFinder (Roche Applied Science Universal ProbeLibrary Assay Design Center) or by the author. Primer sequences for real-time PCR assessment will be made available upon request.

PCR primers and program. Primers used for PCR are as follow.

qRT-PCR

For Akt: Forward primer 5' AGCGGCGTTAAGAAAGTGAC 3'; Reverse primer 5' TTTTGATCGCGTACAGCTTG 3'

For Raptor: Forward primer 5' AAGATTCCAGGCAAGGTCAA 3'; Reverse primer 5' GAAAGAGCTCGCGAGGAAGT 3'

For S6K: Forward primer 5' GAGCTGGACGATGTTGACCT 3'; Reverse primer 5' CCTCAGTGTCTTGGTGCAGA 3'

For usp: Forward primer 5' CCACGATGGCTCCTTTGA 3'; Reverse primer 5' GGCTTTGATCGCACTGTTG 3'

For TSC2: Forward primer 5' GGTATGTGGAGTTCCTGCGTA 3'; Reverse primer 5' TGGAACGTAACCTGCAGTATGTC 3'

For Pten: Forward primer 5' CGAAAGTAAGCCTTAACGTATGTG 3'; Reverse primer 5' TTGCATTTTCTGTGGCTGAG 3'

For E93: Forward primer 5' TGGCTTTTGGGCAGAGATAA 3'; Reverse primer 5'

AGCAACGCGTTCTAGGGATA 3’

For Reaper: Forward primer 5’ TCGATTTCTACTGCAGTCAAGG 3’; Reverse primer 5’ GAGTAAACTAAAATTGGGTGGGTGT 3’

For Rp49 control: Forward primer 5’ AGCACTTCATCCGCCACC 3’; Reverse primer 5’ ATCTCGCCGCAGTAAACG 3’

For Actin 5C control: Forward primer 5’ GTCGCGATTTGACCGACTA 3’; Reverse primer 5’ AGGGCAACATAGCACAGCTT 3’

qRT-PCR program is as follow: (1) 94^0C – 4 minutes, (2) 94^0C – 30 seconds, (3) 60^0C – 30 seconds, (4) 72^0C – 45 seconds, (5) 78^0C – 10 seconds, Repeat steps 2 to 5 for 39 additional cycles, (6) 72^0C – 7 minutes.

Genotyping

UAS F - 5’ CGGAGTACTGTCCTCCGAG 3’

EcR R - 5’ CTGCTCGAAGGCGAGAGAT 3’

PVR R - 5’ ACTTAAGCGTGGTGCTCGTC 3’

Akt F - 5’ GCTCTGGGCTATCTGCATTC 3’

Akt R - 5’ GAGGCCATGTCTCCTTGGTA 3’

PCR program is as follow: (1) 94^0C – 5 minutes, (2) 94^0C – 45 seconds, (3) 55^0C – 30 seconds, (4) 72^0C – 3 minutes, Repeat steps 2 to 4 for 34 additional cycles, (6) 72^0C – 10 minutes.

dsRNA amplicon template primers

All primers contain a 5' T7 RNA polymerase binding site (TAATACGACTCACTATAGG) followed by the primer sequences.

EGFP Sense- 5' CAAGGGCGAGGAGCTGTT 3'

EGFP Antisense - 5' GTCGTCCTTGAAGAAGATGGTG 3'

SID-1 Sense - 5' GCCTATCACGCATTTGGTTT 3'

SID-1 Antisense - 5' CAGGAAATGACAGCAGCAAA 3'

PVR 3' Sense - 5' GACGTCCCGGAGCCATTAG 3'

PVR 3' Antisense - 5' CATGGAGTGAGTGTGTGGTCC 3'

PVR int Sense - 5' GCACAACCCTCGGACACTGGTCTATACAAG 3'

PVR int Antisense - 5' GAAGAAGGTCACGATAGCCGGCGGATAG 3'

Other primer sequences are retrieved from DRSC- Akt (DRSC14108), phl (DRSC31072), rl (DRSC31396), th (DRSC11404), EcR (DRSC04910), mts (DRSC30716), S6K (DRSC11276), dsor1 (31052), raptor (DRSC18359), Pten (DRSC30687), TSC2 (30904) and usp (DRSC36079).

Protein lysates and Western Blotting

Kc cells were lysed using Triton lysis buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% Trition X-100, 30mM NaF) freshly supplemented with 1mM Na_3VO_4 and protease inhibitors (Roche) {Brückner, 2004 #280}. Lysates were precleared by centrifugation and protein concentrations were measured by colorimetric Protein DC Assay (Bio-Rad) with

bovine β-globulin (Bio-Rad) as standard. Equal amounts of proteins were separated by SDS polyacrylamide gel and semi-dry transferred to nitrocellulose membrane. Membrane blots were blocked with 5% non-fat milk in TBS-T (20mM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.4) follow by incubation with TBS-T diluted primary antibodies for overnight at 4^{o}C. Anti-phospho primary antibodies were diluted in 1% non-fat milk in TBS-T whereas all other primary antibodies were diluted in 5% non-fat milk in TBS-T. Primary antibodies were obtained from Cell Signaling Technology except monoclonal anti-β-tubulin (Sigma T5168), anti-PVR {Brückner, 2004 #280} and anti-EcR (Developmental Studies Hybridoma Bank, DSHB). HRP conjugated secondary antibodies (Amersham NA934V/NXA931 and Jackson ImmunoResearch 706-035-148) were diluted in 5% non-fat milk in TBS-T.

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