blmp-1 is a putative Ras-Cooperating Tumor Suppressor Gene in Caenorhabditis elegans

By

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Abstract

While hypermorphic Ras mutations are found in approximately 35% of colorectal, 45% of lung and 90% of pancreatic cancers, activated Ras alone is not sufficient to transform normal cells into cancerous tumors [1-4]. Additional driver mutations or epigenetic alterations are required to cooperate with the activating Ras mutations in tumorigenesis [5]. In *C. elegans*, worms homozygous for activated Ras mutations have 60 - 80% penetrant visible multivulval (Muv) phenotype. Several suppressors and enhancers of this phenotype have been shown to be orthologous to Ras-cooperating proto-oncogenes and tumor-suppressor genes, respectively [6-9]. Because several epigenetic regulators are now targets of cancer treatments and many cancer genomes exhibit changes in epigenetic regulation [10,11], I sought to systematically identify chromatin regulators that cooperate with activated Ras using RNAi screening in *C. elegans*.

After screening 215 *C. elegans* chromatin remodeling factors and 193 *C. elegans* orthologs of known driver genes from Tumor Sequencing Project [12], I identified several attenuators of Ras signaling. By generating double mutants, I was able to confirm that loss of PRDM1/Blimp1 homolog *blmp-1* served as a strong enhancer of the *let-60(n1046)* Muv phenotype. In addition, *blmp-1(tm548)* mutant worms are not Muv. I chose to investigate one of these genes, called *blmp-1*, in great detail because of known roles of its mammalian ortholog PRDM1/Blimp1 in cancer.
PRDM1/Blimp1 is a known tumor suppressor gene in activated-B cell diffuse large B-cell lymphoma (ABC-DLBCL) [13,14]. Whether PRDM1/Blimp1 cooperates with activated Ras is unknown. Therefore, I further sought to understand how BLMP-1 attenuates Ras signaling in C. elegans. blmp-1 mutants have mildly elevated Ras signaling in the vulval cells. In addition, blmp-1(RNAi) and null mutant blmp-1(tm548) are strong enhancers of let-60(n1046) Muv phenotype, resulting in nearly 100% penetrance Muv. blmp-1 expression is downregulated in response to vulval induction, and this downregulation is important for correct vulval induction. However, bypassing this downregulation reduced, but did not abolish vulval induction, suggesting that blmp-1 functions late in vulval development. In addition, I also identified distinct key regions within the blmp-1 promoter which are necessary and sufficient for the regulation of blmp-1 expression in the gut, seam cells, hypodermal cells and the vulva cells.

In conclusion, this is the first characterization of blmp-1 as a putative Ras-cooperating tumor suppressor gene as blmp-1 enhances the activated Ras phenotype through a novel mechanism. These results may shed light into our understanding of ABC-DCLBL as a Ras pathway-driven cancer, and PRDM1/Blimp1 as a Ras-cooperating tumor suppressor genes.
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List of Abbreviations

ABC-DLBCL – Activated B Cell Diffuse Large B Cell Lymphoma

AC – Anchor Cell

ACK – Activated Cdc42-associated Kinase

AUC – Area Under the Curve

BCR – B Cell Receptor

BM – Basement membrane

bZIP – Basic Leucine Zipper Motif

CDC – Cell Division Cycle

cDNA – Complementary DNA

ChIP-Seq – Chromatin Immunoprecipitation - Sequencing

CKI – Cyclin Dependent Kinase Inhibitor

CNS – Central Nervous System

CRISPR – Clustered Regularly-Interspaced Short Palindromic Repeats

Dpy – Dumpy Phenotype

DTC – Distal Tip Cell

EGF – Epidermal Growth Factors

ETS – E26 transformation-specific family of transcription factors

Evl – Everted Vulva Phenotype
GDP – Guanosine Diphosphate

GFP – Green Fluorescent Protein

GTP – Guanosine Triphosphate

HDAC – Histone Deacetylase

Hox – Homeobox

IPTG – Isopropyl β-D-1-thiogalactopyranoside

MAPK – Mitogen Activated Protein Kinase

MAPKK – Mitogen Activated Protein Kinase Kinase

miRNA – microRNA

modENCODE – Model Organism Comprehensive Encyclopedia of Functional Elements

Muv – Multivulva

NGM – Nematode Growth Media

PAX – Paired Box

PDK – Pyruvate Dehydrogenase Kinase

PGC – Premordial Germ Cell

PI3K – Phosphoinositide-3-kinase

Ppa – Pristionchus pacificus

PRDM1 – PR Domain Containing 1

Pvl – Protruding Vulva

Rb - Retinoblastoma
RFP – Red Fluorescent Protein

RISC – RNA-Induced Silencing Complex

RNAi – RNA interference

ROC – Receiver Operating Characteristic

RTK-Ras-MAPK – Receptor Tyrosine Kinase – Ras – Mitogen-activated Protein Kinase

SEM – Standard Error of the Mean

SET – Su(var)3-9, Enhancer of Zeste, Trithorax

SUMO – Small Ubiquitin-like Modifier

TLE – Transducin-Like Enhancer

TRIM-NHL – Tripartite-containing motif; RING, B-Box, coiled coil and NHL (NCL-1, HT2A and LIN-41)

TSG – Tumor Suppressor Gene

TSP – Tumor Sequencing Project

UCSC – University of California Santa Cruz

UTR – Untranslated Region

VPC – Vulvul Precursor Cells

Vul - Vulvaless
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Chapter 1
Introduction

1.1 Overview

Cancer is a disease of genetic interactions in which the cooperations between driver mutations allow the cells to acquire the hallmarks of cancer [15]. Typically, a tumor genome carries many mutations, but only a subset of these mutations drive tumorigenesis, while other mutations do not [16]. Keys to successful diagnosis and treatments of cancers include identifying driver genes and driver mutations, as well as understanding how the genetic interactions occur among them. While cancer genome sequencing and profiling provide a massive amount of data, functional studies are necessary to prioritize the list of cancer-relevant candidates and to understand how these driver mutations contribute to tumorigenesis.

Several signaling pathways that are perturbed in cancers are highly conserved, thus genetic screening and additional characterization of cancer signaling pathways in model organisms have shed tremendous light into our understanding of cancer [17-19]. *C. elegans* is an excellent model for understanding pathways that contribute to cancer with both genetic conservation and well-characterized phenotypes [19]. For example, many processes that are misregulated in cancer, including cell-cell communication requiring RTK-MAPK signaling, apoptosis, DNA damage and DNA repair have been characterized in *C. elegans* development and shown to involve pathways similar to those found in mammals [20-22].

Notably, in *C. elegans*, *let-60(n1046)* (G13E) is an activated Ras mutation that constitutively activates Ras in the same manner as do Ras mutations found that are found in human tumors. These worms have a multivulva (Muv) phenotype, with 60 – 80% penetrance at 20°C, which allows for screening of genetic suppressors and enhancers of activated Ras. Therefore, *C. elegans* possesses several qualities that make it a great model organism for identifying and characterizing genetic interactions that play important roles in tumorigenesis.

Aside from well-characterized phenotypes and several amenable genetic tools in *C. elegans*, it has been established as an excellent metazoan model for reverse genetics. RNAi allows for rapid, potent and specific *in vivo* inactivation of gene function [23]. An RNAi library covering
~90% of the *C. elegans* genome, referred to as the Ahringer library, has been made available to the research community [24,25]. This RNAi library has been used extensively to map genetic interactions, in order to identify new driver genes and characterizing new driver gene interactions [6-9].

The work described in this thesis involves identification of putative driver genes that cooperate with Ras using RNAi screening in *C. elegans*. My effort was focused on orthologs of known cancer genes as defined by The Tumor Sequencing Project [12], and chromatin remodeling factors: a group of genes that are increasingly implicated in cancer. In addition, I sought to understand how one such gene identified by the screen, *blmp-1*, the *C. elegans* homolog of Blimp1, cooperated with the mutant Ras gene.

In this chapter, I set the stage for this work by—first—providing the relevant background with respect to Ras signaling and cancer. Second, I describe relevant features of this model system for studying the Ras signaling pathway. Third, I will go into the details of employing RNAi in *C. elegans* as a reverse genetic approach to identifying driver genes. Finally, I discuss relevant previous findings with respect to using RNAi to identify putative driver genes, which provided guidelines for further characterization of genes that were identified in the screen.

### 1.2 Cancer as a disease of genetic interactions, which reflect the hallmarks of cancer

Tumorigenesis is a complex and multi-step process where mutations, epigenetic changes in and the environment allow tumor cells to acquire the hallmarks of cancer. These hallmarks, described by Hanahan and Weinberg, include self-sufficiency in growth signals, insensitivity to anti-growth signals, the ability to evade apoptosis, limitless replicative potential of DNA, the ability to sustain angiogenesis, and, finally, tissue invasion and metastasis [27]. More recently, four additional hallmarks of cancer have been proposed by Hanahan and Weinberg, including reprogrammed metabolism, the ability to evade the immune system, genomic instability, and chronic inflammation [28].

A tumor genome typically harbours many mutations, but only a subset of them contribute to the hallmarks of cancer. These genes are called “driver mutations”, and the genes in which driver
mutations are found are called “driver genes”. Other mutations that do not contribute to the tumorigenesis process are called “passenger mutations.” Identification of driver mutations within hundreds or tens of thousands of mutations present in cancer presents a challenge in cancer research.

Cancer is a disease of genetic interactions; this adds to the complexity as multiple driver mutations cooperate to drive the hallmarks of cancer [29]. Genetic interactions in cancer or “oncogene cooperation,” are certain combinations of oncogenes and tumor suppressor gene mutations that, together, would transform NIH-3T3 cells [30].

One of the most important oncogenes that cooperates with other driver genes is the activated Ras oncogene. Activated Ras is found in approximately 35% of colorectal, 45% of lung and 90% of pancreatic cancers cases [2,3]. However, introduction of activated Ras into untransformed cells is not sufficient to transform these cells into cancers, as additional driver mutations are required to cooperate with Ras to achieve cell transformation [5]. Central to this thesis is how I identified new driver genes that cooperate with activated Ras using an RNAi screen in C. elegans. In the next section, I will discuss the relevant backgrounds of Ras signaling in cancer.

1.3 Ras signaling and cancer

Ras belongs a family of small (ca 21 kDa) GTPases, which act as molecular switches that relays extracellular growth signals. The mammalian Ras gene family consists of three genes, i.e. H-Ras, K-Ras and N-Ras [31]. These different Ras genes are highly related but differ in their expression patterns, suggesting that they perform different cellular functions [32].

When an extracellular ligand binds to an upstream receptor tyrosine kinase (RTK), the RTK dimerizes and trans-autophosphorylates the tyrosine residues. The phosphorylated tyrosine residues in the RTKs recruit adaptor proteins such as GRB2 and Sos, which subsequently activate Ras by converting GDP-bound (inactive) to GTP-bound (active) form of Ras [4,32]. The GTP-bound Ras then triggers downstream effectors, including mitogen activated protein kinase (MAPK), PI3-kinase and Ral-GDS signaling pathways [4,33]. After this signal
transduction, GTP-bound Ras can hydrolyze the bound GTP into GDP and cycle Ras-GTP to its inactive Ras-GDP form [32].

Constitutive activation of Ras signaling pathway may confer limitless replicative potential, which is a key hallmark of cancer. In addition, several activating mutations in Ras signaling or related signaling pathways have been implicated in cancers [4,27]. These include the G12V, G13E and G61E mutations in Ras that abolish its GTPase activity, resulting in constitutively active Ras-GTP [4,32].

In *C. elegans*, the let-60(n1046) (G13E) mutation constitutively activates Ras in a manner similar to activating Ras mutations that are found in human tumors. *let-60(n1046)* worms have a quantifiable multivulva (Muv phenotype) that is approximately 60 - 80% penetrant, which allows for genetic screening for suppressors and enhancers of this phenotype. In the next section, I describe *C. elegans* vulval development as it relates to Ras and other interacting pathways.

### 1.4 Ras signaling and other cancer signaling pathways in *C. elegans* vulval development

Although *C. elegans* does not develop tumors of somatic tissues in the same way mammals do, it has several relevant phenotypes that allow for studies of genetic interactions among cancer signaling pathways. In this section, I first describe the relevant features of vulval development and mutant phenotypes, and then transition into the characterized cancer signaling pathways in *C. elegans*.

#### 1.4.1 Vulval development

In *C. elegans*, vulval development is a well-orchestrated organogenesis process throughout the 4 larval stages (L1 – L4) and adulthood. During the L2 stage, six vulval precursor cells (VPCs, P3.p – P8.p) are equally potent to assume vulval or non-vulval cell fates. The anchor cell (AC) is a secretory cell located above P6.p. The AC functions by secreting the EGF ligand LIN-3, to induce the VPCs to assume the vulval cell fate. The EGF ligand then activates RTK-Ras-MAPK signaling within these cells in the vulval induction process. In wildtype worms, three of the six Vulval Precursor Cells (P5.p, P6.p and P7.p) are induced to assume the vulval cell fate.
P5.p, P6.p and P7.p cells then undergo precisely 3 rounds of cell division to become the 22-cell vulva by late L4 stage (Figure 1.1).

1.4.2 Vulval induction index as a quantitative readout of Ras signaling

In addition to the visible phenotypes under the microscope, *C. elegans* vulval induction can be quantified as the number of cell divisions, typically denoted as the vulval induction index. This allows for finer characterization of vulval phenotypes as it relates to the strength of vulval induction. To provide relevant background, this section of the introduction summarizes the vulval cell lineage as described by Sternberg and Horvitz (1986) [34].

In wildtype vulval development, the VPCs P5.p, P6.p and P7.p divide three times to become the 22-cell organ called the vulva. Secondary cells, P5.p and P7.p undergo 2 completes rounds of cell division, and in the third cell division, one of the granddaughter cells does not undergo cell division. The cell divisions of each of P5.p and P7.p therefore result in 7 descendants. Whereas, the primary cell P6.p undergo three complete rounds of cell division, resulting in 8 descendants. The vulva induction index denotes the number of VPCs that assume the primary or secondary vulval cell fate, as quantified by the number of VPC granddaughters that undergo terminal cell divisions following the wildtype patterns.

In addition to the number of cell division, the primary and secondary cell divisions are also distinguished by the planes of cell division. VPC granddaughters can divide longitudinally or anteroposteriorly (L), transversely (T), or not divide at all (N). For the primary cell, P6.p, all 4 of its granddaughter cells divide transversely, producing 8 descendants, which is typically denoted in shorthand as TTTT. For secondary cells, two of the granddaughters divide laterally, one of them divides transversely, and another one does not divide. Secondary cell divisions are typically denoted in shorthand as LLTN for P5.p and NTLL for P7.p, for division of VPC granddaughters. In wildtype worms, the vulval cell division pattern can be denoted as LLTN TTTT NTLL, which correspond to 3 VPC cells undergoing vulval induction, and the vulval induction index is 3.0.
For simplicity, in this thesis, I follow the alphabetical nomenclature system [35,36]. Descendants of secondary cells are named vulA, vulB1, vulB2, vulC and vulD from the outside of the vulva towards the center. Whereas, descendants of primary cells are named vulE and vulF (Figure 1.1, VPC great-granddaughters).

In wildtype worms, this pattern of cell division is consistent from animal to animal and can be observed under Nomarski optics. While comparison of penetrance levels can provide crude comparison within the same mutant strain background, scoring the vulval induction index provides a more quantitative readout for Ras signaling output in the vulval cells. This allows for categorization of genes that modify vulval phenotype into strong or weak modifiers of vulval induction which may not be visible through superficial phenotype, and hence may shed light into their molecular function.

1.4.3 *C. elegans* vulval phenotypes

Generally, disruption of Ras signaling pathway results in *vul*valess worms (Vul), whereas gain of function result in *multi*vulva worms (Muv) [37]. Worms with only one but abnormally protruded *vul*va have protruding vulva phenotype (Pvl), which may result from reduced vulval cell division, abnormal anchor cell invasion or abnormal attachments to the gonads [36,38]. When the anchor cell fails to invade the divided vulval cells, the worm may have a *squashed* vulva phenotype (Sqv) [36]. Mutants whose vulva evert incorrectly during the morphogenesis process have *everted* vulva (Evl), which include several mutations in the Notch signaling pathway [39].

While the vulval induction index for wildtype worms is consistently 3.0, various degrees of vulval induction in mutants correspond to a continuum of vulval induction indices. In Vul worms where vulval induction is abolished, the vulval induction index is 0.0. In partially Vul mutants, vulval cell division is decreased but not completely abolished; these worms may have vulval induction of over 0.0 but less than 3.0. In Muv worms, P3.p, P4.p and P8.p may also assume either the primary or secondary vulval cell fate and divide, so their total vulval induction is over 3.0.
1.4.4 Cancer signaling pathway in *C. elegans*

Forward genetics has identified genes that function in Receptor Tyrosine Kinase-Ras-MAPK (RTK-Ras-MAPK) signaling, Wnt Signaling and Notch signaling as important in wildtype vulval development [20]. In wild type worms, the anchor cell secretes LIN-3 (an Epidermal Growth Factor or EGF ligand) that binds to LET-23 (the EGF receptor) on the surface of three vulval precursor cells that are closest to the anchor cells: P5.p, P6.p, and P7.p. LET-23, which is an RTK, then dimerizes and undergoes autophosphorylation, which allows it to relay signals to the downstream signaling steps involving Ras (LET-60) and downstream MAP kinase cascades (MAP3K LIN-45, KSR KSR-1 and KSR-2, MAPK2K MEK-2, and ERK MPK-1) [20,40]. Among targets of MPK-1 are the transcription factors Winged Helix LIN-31 and ETS LIN-1, which heterodimerize and regulate downstream gene expression to determine vulval cell fate [41,42]. The combination of graded LIN-3 signals and Notch ligands from P6.p (1° cell), downregulates Ras-MAPK activation in P5.p and P7.p, which then allows them to assume the secondary vulval cell fate [34,43,44].

Towards the end of cell division, these vulval cells undergo vulval invagination process prior to cell fusion (reviewed in [36]). The potency of VPCs to assume the vulval cell fate and divide accordingly is tightly regulated via homeobox genes, cell cycle regulators and heterochronic pathways [37].

Beyond vulval development, RTK-Ras-MAPK signaling also has other functions in the worms. Loss-of-function mutants are sterile because Ras signaling is required for progression through pachytene in meiosis [45]. During larval development, Ras signaling is required to specify the excretory duct cell fate, which is important for osmoregulation [46]. As a result, hypomorphic *let-60* and certain other mutants in the signaling pathway die as rod-like larvae with a fluid-filled appearance due to improper osmoregulation [20]. Ras signaling promotes the correct formation of male tail spicules, so male mutants in this pathway are mating defective [47]. Lastly, Ras signaling is also responsible for correctly specifying the uv1 cell fate, which is involved in the establishment of a ventral-uterine connection, so many Ras signaling mutants are egg-laying defective [48].
1.4.5 *C. elegans let-60(n1046)* Muv phenotype

Ras in *C. elegans* is encoded by the *let-60* locus on LG IV. The gain of function allele of Ras *let-60(n1046)* has a point mutation (G13E) that constitutively activates Ras in a similar manner as do G12V or G13E mutations, which are found in human cancers. At 20°C, in *C. elegans*, approximately 60 - 80% of *let-60(n1046)* worms are Muv, whereas the remainder of worms develop wildtype vulva. This forms a basis for genetic screening for enhancers and suppressors of this activated Ras phenotype.

In the next section, I will discuss examples of known genetic suppressors and enhancers of the *let-60(n1046)* Muv phenotype. These genes were included as positive controls in my RNAi screen. In addition, results from studies that characterize these genes will provide a framework for characterizing genes that are newly identified.

1.5 Genetic suppressors of the *let-60(n1046)* Muv phenotype

Forward genetic screens have revealed several genes that function in the RTK-Ras-MAPK signaling pathway as suppressors of the Muv phenotypes of *let-60(n1046)* or other *let-60* mutants [49]. Several of these suppressors are orthologous to known oncogenes. Other suppressors are genes that are required to maintain potency of the VPC to assume vulval cell fates such as Wnt and Homeobox genes including *lin-39*. Mutations in these genes may prevent vulval induction, resulting in the Vul phenotype.

1.5.1 Suppressors of *let-60(n1046)* which function in the RTK-Ras-MAPK signaling pathway

Many genes that function downstream of Ras in the MAPK cascade have been identified as suppressors of *let-60(n1046)* Muv phenotype. These genes were originally named suppressor of ras, or *sur* genes. For example, the *C. elegans* MAP kinase (*mpk-1*) was one such gene and was originally named *sur-1*, and a KSR (*ksr-1*) was originally named *sur-3* [50,51]. *sur-2* encodes a *C. elegans* ortholog of the MED23 mediator subunit which functions downstream of Ras [52]. Epistatic analyses placed *mpk-1*, *ksr-1* and *sur-2* downstream of Ras, but *mpk-1* and *ksr-1* act upstream of the transcription factors *lin-1* and *lin-31* [50-52].
Because RTK-Ras-MAPK signaling pathway has pleiotropic function and is involved in embryonic and larval development, most genetic suppressors of activated Ras that are in the Ras signaling pathway are also required for viability. In addition, strong mutant alleles typically result in vulvaless phenotype and penetrant rod-like larval lethality.

1.5.2 Genes that maintain VPC competence for vulval induction

The competence of the VPC cells to be induced by LIN-3 ligand is partly mediated by the Wnt signaling pathway. VPC cells express the Hox gene lin-39, and loss of lin-39 functions result in all VPC cells all fuse with the hypodermal cells at one-cell stage, rendering the VPCs incompetent to further inductions and causing the worms to be Vul [53]. Gleason et al (2002) showed that overactivation of Wnt signaling pathway not only bypasses the requirement of the Ras signaling pathway for vulval induction, but also results in Muv worms [54]. This Ras-independent Muv phenotype is regulated by Wnt signaling, as β-catenin bar-1 mutants phenocopy the lin-39 vulval phenotype [54,55]

1.6 Attenuators of Ras signaling may be genetic enhancers of the let-60(n1046) Muv phenotype

Genetic screens for attenuators of Ras signaling have shown that vulval development is subject to redundant negative regulation. A single mutation in one of these genes is typically not sufficient to induce ectopic pseudovulva in laboratory conditions. However, mutations of these attenuators enhance the let-60(n1046) Muv phenotype, and some combinations of these mutations result in ectopic pseudovulval induction. These categories include genes encoding products that function in the cytoplasm to attenuate EGFR-RTK-Ras signaling, transcriptional regulators including synMuv genes, and components of the heterochronic pathways that regulate let-60 function. Importantly, several of these genes are orthologous to known tumor suppressor genes that cooperate with Ras.

1.6.1 Cytoplasmic attenuators of RTK-Ras-MAPK signaling

In the cytoplasm, RTK-Ras-MAPK signaling is subject to control at many levels. For example, genes that encode proteins that negatively regulate LET-23/EGFR functions include the AP-1 receptor UNC-101, Adaptin DPY-23, Cbl Ubiquitin ligase SLI-1 and nonreceptor tyrosine
kinase ACK ARK-1 [43,56]. MAPK phosphatase LIP-1 functions to antagonize MAPK signaling downstream of LET-60 [43,44]. Many of these genes are also transcriptional regulation targets of Notch signaling, that are activated in 2° cells downstream of vulval induction [43].

All cytoplasmic attenuators of vulval induction characterized to date have several properties in common. First, single mutants of these genes do not result in the Muv phenotype in laboratory conditions, but mutations of these genes do typically increases the penetrance of the Muv phenotype in the let-60(n1046) background. Second, while the increased vulval induction in a single mutant is not visible as an ectopic pseudovulval induction, there is a detectable increase in expression of genes whose expression is activated downstream of RTK-Ras-MAPK, such as egl-17 in P5.p or P7.p [43,57]. Third, a combination of mutations among these group of genes, or with RasGAP gap-1, typically result in ectopic pseudovulval induction [43].

1.6.2 The synMuv genes

synMuv genes function redundantly to inhibit ectopic pseudovulval induction. Single mutations of these genes do not result in Muv phenotypes, but a combination of mutations from any two groups of these genes will result in the Muv phenotype. synMuv genes are defined as three classes of genes: A, B, and C. All known synMuv genes encode nuclear proteins. Many synMuv genes encode transcriptional regulators, while others encode proteins with unknown biochemical functions. Notably, the synMuv class B genes include the C. elegans Retinoblastoma (RB1) ortholog lin-35 and several other C. elegans orthologs of genes that function with RB1, such as RBBP4 lin-53, E2F efl-1 and TFDP1 dpl-1 [58]. Class C genes encode chromatin remodeling factors, such as the components of the Tip60 chromatin remodeling complex [59]. Class A genes encode SUMO and enzymes that function in the sumoylation pathway [6], but the molecular of several other class A genes remain unknown [58].

The first characterized synMuv genes (lin-15A and lin-15B) were shown genetically to regulate EGFR LET-23 function [60]. Cui et al demonstrated that synMuv genes redundantly inhibit LIN-3/EGF expression in hyp7 hypodermal cells to prevent inappropriate vulval induction [61]. However, certain synMuv gene pairs have Muv phenotypes independent of LIN-3 [6,59]. Other
synMuv genes were shown via mosaic analysis to function cell-autonomously in the VPCs [62]. To date, the precise mechanism by which these synMuv genes redundantly prevent ectopic vulval induction remain to be elucidated.

Since synMuv genes function redundantly to prevent ectopic pseudovulval induction, they are considered to be negative regulators of vulval induction [58]. Several of these genes, such as egl-27 and egr-1, are known genetic enhancers of let-60(n1046) Muv phenotype [63].

1.6.3 Heterochronic genes, miRNA and cell cycle regulators

Heterochronic regulation is important for correct timing or rate of developmental changes in a multicellular organism. In C. elegans vulval development, miRNA is employed to regulate heterochrony by coordinating vulval cell cycles and development with the rest of the animal. let-60 is a gene with pleiotropic functions that is subject to post-transcriptional control, including by miRNAs. Not surprisingly, some genes that regulate cell cycle, i.e. C. elegans CKI cki-2 and Cdc42 cdc-42 are genetic enhancers of the let-60(n1046) Muv phenotype [64].

let-60 is a target of let-7 family miRNAs (including miR-84, miR-48 and miR-241) [65]. In addition, the TRIM-NHL protein NHL-2 is part of the miRNA-induced silencing complex that regulates LET-60 levels. Hammell and colleagues found that nhl-2 is a genetic enhancer of the let-60(n1046) and let-60(ga89) Muv phenotype [66]. Overexpression of miR-84 partially suppressed the let-60(ga89) and let-60(n1046) Muv phenotype [65,66]. This regulation of Ras expression by let-7 family miRNAs is conserved between worms and mammals, as some let-7 family miRNAs have been characterized as Ras-cooperating tumor-suppressor genes [65].

While expression patterns of let-7 family miRNAs appear to correlate with vulval induction [65], it remains unclear whether Ras signaling also functions to regulate transcription of these miRNAs.

In vulval development, the role of miRNAs is not limited to the Ras signaling pathway alone. Other opposing pathways, such as Notch signaling, also involve miRNA regulation. Yoo and Greenwald (2005) showed that miR-61 is a transcriptional target of Notch signaling [67]. For example, one target gene for miR-61 is vav-1, the C. elegans ortholog of Vav proto-oncogene [67].
1.7 *C. elegans* as model organism for identifying and characterizing genetic interactions related to cancer biology

1.7.1 *C. elegans* biology

*C. elegans* is a free-living, soil-dwelling nematode, which has been established as one of the best multicellular genetic models to study animal development. It has simple anatomy and short life cycle of 3.5 days but also has many sophisticated developmental processes seen in other animals. Adult hermaphrodite worms have 959 cells whose lineages have been rigorously followed and described [68]. Genome sequencing and bioinformatic studies estimate that approximately 60 – 80% of genes are conserved between *C. elegans* and humans [69]. Therefore, *C. elegans* is an excellent system for genetic screening and *in vivo* characterization of several processes relevant to human [69].

1.7.2 RNAi in *C. elegans*

Central to this thesis is the use of RNAi interference (RNAi), which is a highly potent and sequence-specific mechanism to inhibit gene function. In *C. elegans*, systemic RNAi can be induced by introducing double-stranded RNA either by microinjection or by feeding worms with *E. coli* expressing dsRNA [70].

RNAi by feeding with bacteria is by far the least labor-intensive method of inducing RNAi as this bypasses *in vitro* RNA synthesis and purification, and because the bacteria cultures can easily be replenished. When worms ingest *E. coli* cells containing dsRNA, the intestinal cells uptake the dsRNA, which is then transported throughout the animal. Inside the cells, Dicer processes dsRNAs into small pieces of double-stranded RNA before amplification by RNA-dependent RNA polymerases [71]. These small double-stranded RNAs are then used for guiding the RNA-induced silencing complex to cleave endogenous mRNA molecules with identical or complementary sequences. These knock-down effects are heritable as these siRNAs may diffuse into the germline and subsequently be packaged into the embryos [23]. RNAi transiently inhibits gene function in nearly every tissue of the animal, with the exception of neurons and sperms. This allows for rapid assay of reduction of function phenotypes of genes of interest within 1 – 2 generations (3 – 7 days).
dsRNA-expressing bacteria libraries have been made for the purpose of RNAi screening in *C. elegans*, with ~90% coverage of the *C. elegans* genome. These include the Ahringer library, which includes 16,757 genes, and the Vidal ORFeome RNAi library, which includes 11,511 genes [72,73]. In addition, the Ahringer group established an optimized assay for *C. elegans* phenotypes, and estimated that 92% of RNAi knockdown phenotypes resemble that of known mutants [24]. This has enabled reverse genetic approaches, by targeting known genes with RNAi and looking for phenotypes or synthetic phenotypes caused by genetic interactions.

In conclusion, *C. elegans* has been established as an excellent system for in vivo RNAi screening. In the next section, I will discuss how reverse genetics screening by RNAi in *C. elegans* has been a powerful approach which has been effectively employed to identify genes involved in diseases like cancers [6-9].

### 1.7.3 Timeline, throughput and scaling of this RNAi screening to identify modifiers of the *let-60(n1046)* Muv phenotype in *C. elegans*

Presently, RNAi screening for vulval phenotypes in *C. elegans* relies on manual setup of the screen apparatus and scoring of phenotypes. The throughput of the screen depends on the screen designs and whether the end phenotypes can be rapidly scored. Specifically, the RNAi screens for enhancers and suppressors of *let-60(n1046)* Muv phenotypes involve manually counting the number of Muv and non-Muv worms. While it is possible to set up RNAi by feeding experiments for a few hundred genes in each round of the screen over the course of a week, the manual scoring is limited to ~100 genes a day because it takes 5 minute to score 1 gene (or 12 genes per hour). In addition, at least three independent experimental replicates would be required to determine the repeatability of the results and achieve statistical power. Screening the whole RNAi library of 16,757 genes was therefore not feasible, so I carried out a screen through 408 candidate genes instead.

In the next section, I will discuss a few case studies to demonstrate how this approach complements other driver gene identification strategies. In addition, I discuss how data from these case studies provided a framework for characterization of genes identified in the screen.
1.8 Case studies using a reverse genetic approach to identify genes that cooperate with Ras in C. elegans

Reverse genetics involves the evaluation of gene function by starting from the targeted perturbation of known genetic sequence, in contrast to forward genetics which starts from a phenotype. With the advent of tools like RNAi libraries, it has become possible to comprehensively query a library of genes of interest for possible roles in a biological process. Reverse genetics may identify genes that may be missed in forward genetics screens, or genes involved in a process for which mutants have yet to be identified [74]. Therefore, this approach complements previous findings from forward genetic screens. Various screen designs also make it possible to identify genes with certain epistatic relationships to biological processes. In addition, because the gene identity is known, this approach bypasses the mapping steps required in forward genetic screens and allows for rapid identification of novel positives.

1.8.1 An RNAi screen for new synMuv genes identified novel synMuv group members and implicated the sumoylation pathway as an attenuator of vulval induction

Two genome-wide RNAi screens in worms have been carried out to identify class A and class B synMuv genes that were not identified by forward genetic screens [6]. The authors were able to identify 9 new synMuv genes, including chromatin factors and genes that function in sumoylation. The identification of *ubc-9*, *uba-2* and *smo-1* in these genetic screens suggested that SUMOylation might attenuate vulval induction. This study found that 7% of *smo-1* worms are Muv. Interestingly *ubc-9*, *ubc-2* and *smo-1* are found to be synthetic Muv with all three groups of synMuv genes. Also, targeting these genes in the reduction-of-function *let-60(n2021)* background actually resulted in Muv phenotype through a mechanism that is suppressed by a *lin-12* loss of function mutation. Therefore, *lin-12* may be a sumoylation target [6].

In a follow-up RNAi screen for sumoylation targets, Gee *et al* screened for chromatin factors that, when targeted with RNAi, resulted in the Muv phenotype in a *smo-1* background [75]. In a subsequent egl-17 reporter assay, the authors were able to confirm some of these positives as novel attenuators of vulval induction, including the bromodomain protein BET-1 [75].
1.8.2 RNAi screens for attenuators of RTK-Ras-MAPK signaling

Previously, a large scale RNAi screen to identify genes that are synthetic Muv with gap-1 identified 39 candidates [76]. Five of these genes were components of the C. elegans MLL-like complex. The MLL complex functions as histone methyltransferases and demethylases. These five MLL components identified in the screen include set-16, rbbp-5, wdr-5, ash-2 and utx-1. Additional analyses demonstrated that, similar to genes that function in the sumoylation pathway, set-16 was a synthetic Muv with all three classes of synMuv genes. They also found that these MLL-like complexes function together to regulate expression of ajm-1, an epithelial cell junction marker, which is also an attenuator of Ras signaling. Interestingly, MLL does genetically interact with activated Ras in human cancers [77-79]. Therefore, such RNAi screens that identify genes that inhibit pseudovulval induction may shed light into our understanding of cancer.

1.8.3 SynMuv suppressors

An RNAi screen to identify synMuv suppressors has identified several transcriptional regulators which suppress the Muv phenotype without abolishing endogenous vulval induction [8]. Several of these genes also suppress the let-60(n1046) Muv phenotype, including isw-1 and zfp-1 [8]. Therefore, RNAi screening for suppressors of the Muv phenotype may possibly identify suppressors that suppress pseudovulval induction in genetic backgrounds other than synMuv.

1.8.4 Kinases that attenuate vulval induction

Previously, Wilson and colleagues carried out an RNAi screen for suppressors and enhancers of the let-60(n1046) Muv phenotype among the C. elegans kinases [9]. They also identified the NRBP1 pseudokinase homolog H37N21.1, Diacyl glycerol kinase dgk-2, Nck-interacting kinase mig-15, and homeodomain interacting protein kinase hpk-1 as genetic enhancers of the let-60(n1046) Muv phenotype. Like synMuv genes and cytoplasmic attenuators of RTK-Ras-MAPK signaling, mutations in these genes do not yield any visible superficial vulval phenotypes, yet mutating or targeting these genes by RNAi in a let-60(n1046) background significantly increases the penetrance of the let-60(n1046) Muv phenotype.
The authors were able to show that \textit{N37N21.1} is an attenuator of vulval induction signal, as targeting \textit{H37N21.1} resulted in increased ectopic expression of \textit{egl-17} reporters in daughters of secondary VPC cells [9]. \textit{NRBP1} is known to function as a modulator of Wnt signaling. Reduction of \textit{Nrbp1} expression is found in a wide variety of human tumors, and is correlated with poor prognoses [80,81]. In addition, using a cell transformation assay, the authors were able to confirm that that the cooperation between activated Ras and \textit{NRBP1} is conserved between worms and mammals [9]. These examples demonstrate that \textit{C. elegans} is tractable system for identifying putative driver genes that cooperate with Ras, which may lead to novel insight about cancer biology.

1.9 Project overview and rationale

Cancer is a complex and heterogeneous disease driven by the cooperation of multiple driver mutations. For example, while activated Ras is frequently found in cancers, activated Ras alone is not sufficient to drive tumorigenesis [5]. Key questions in cancer research therefore include the identification of driver genes, and the understanding of how driver mutations cooperate to drive tumorigenesis \textit{in vivo}. For this thesis, I focus on the identification of driver genes that may cooperate with activated Ras using RNAi screening in \textit{C. elegans}.

\textit{C. elegans} vulval phenotype is a robust \textit{in vivo} readout of the Ras signaling pathway, which allows for identification of potential Ras-cooperating cancer genes. Several genetic screens and RNAi screens in \textit{C. elegans} have identified novel Ras-cooperating genes, many of which were confirmed to be Ras-cooperating driver mutations in human cancers [9,19].

The role of epigenetic regulation is increasingly implicated in cancer pathogenesis and treatments. For example, HDAC inhibitors are emerging as treatments for colorectal cancers and DNA methyltransferase inhibitors are being used in ovarian and endometrial cancers [82]. However, epigenetic regulators have not been systematically queried in this activated Ras background. Therefore, in Chapter 2 of this thesis, I describe an RNAi screen in which I systematically screened the chromatin remodeling factors for genetic modifiers of the \textit{let-60(n1046)} Ras phenotype.
One such chromatin factor identified in my RNAi screen, BLMP-1, is an ortholog of a known tumor-suppressor gene PRDM1/Blimp1. While \textit{blmp-1} has been implicated in B-cell lymphoma and several lymphoid malignancies [14,83], it has never been associated with activated Ras. Therefore, in Chapter 3, I characterized \textit{blmp-1} in \textit{C. elegans} and sought to understand how this \textit{blmp-1} functions as an attenuator of Ras signaling.

**Figure 1.1: Wildtype vulval cell division (dorsal view with the head of the worm towards the left hand side), cell lineages and the vulval induction index**

Adapted from references [37,68]

Cell nomenclature: AC = Anchor cell. BM = basement membrane. The first two cell divisions of the VPCs happen longitudinally or anteroposteriorly, so the daughter cells are annotated with an “a” for anterior and “p” for posterior appended to the names of the mother cells. For cell divisions that happen transversely, daughter cells are annotated with “l” for left and “r” for right. VPC great-granddaughters are also named vulA1 (P5.paaa), vulA2 (P5.papa), vulB1 (P5.papa), vulB2 (P5.papp), vulC (P5.ppaa and P5.ppar), vulD (P5.ppp), vulE (P6.paal and P6.paar) and vulF (P6.pppl and P6.pppr). For the posterior half of the vulva, vulF (P6.pppl and P6.pppr), vulE (P6.paal and P6.ppar), vulD (P7.paa), vulC (P7.papl and P7.papr), vulB2 (P7.ppaa), vulB1 (p7.ppap), vulA2 (P7.pppa) and vulA1 (P7.pppp). vulA1 and vulA2 eventually fuse to become a single vulA toroid.

During vulval induction, P5.p – P7.p receives LIN-3 EGF ligand from the AC which is located above P6.p cell, separated by a BM (omitted after the first cell division for simplicity). P5.p – P7.p then divide three times cell to form the 22-cell vulva. Vulval induction index involves \textit{in vivo} scoring for the total number of Pn.p granddaughters that assume the vulval cell fate and undergo the final cell division. Wildtype worms have 1.0 from each of P5.p, P6.p and P7.p, with a total of 3.0 vulval induction index. If one of the Pn.p granddaughters do not undergo the final cell division, then the vulval induction index is reduced by 0.25 for each of these undivided mother cells. Ectopic vulval induction in P3.p, P4.p and P8.p can also be scored using the same method. In ~50% of WT worms, P3.p assume the F cell fate and fuse with the hyp7 cell before the first VPC division, so only 1 P3.p descendant is observed at later stages.
Chapter 2

RNAi Screening in *Caenorhabditis elegans* Identifies putative Ras-cooperating Cancer Genes

This work is unpublished. I performed all work described in this chapter, except the cloning of 10 RNAi constructs that were missing from the Ahringer library, which was done in collaboration with Alan Jiao. Preparation of the Tumor Sequencing Project Ortholog RNAi library was done in collaboration with Tungalag Chuluunbaatar.
Chapter 2
RNAi screening in *Caenorhabditis elegans* identifies putative ras-cooperating tumor suppressor genes

2.1 Summary

While constitutively activated Ras is found in many types of cancers, activated Ras alone is typically not sufficient to drive tumorigenesis. Additional mutations are required to drive cell transformation into malignant tumors (Figure 2.1A). Identifying these key mutations and understanding how they interact with each other are important for prognosis determination and treatment of cancers. In *C. elegans*, worms homozygous for activated Ras mutation have 60 - 80% penetrant multivulva (Muv) phenotype, which can be seen as multiple ventral protrusions. Suppressors and enhancers of this phenotype were shown to be proto-oncogenes or tumor-suppressor genes, respectively [9,19,66] (Figure 2.1B). Therefore, by targeting genes with RNAi and assaying for changes in number of worms that have the Muv phenotype, we can identify new Ras-cooperating cancer genes. This formed the basis for an RNAi screen to identify such genes.

The RNAi screen requires manual phenotyping, so a midi-throughput screen using a candidate approach was more feasible than a whole-genome screen. The screen covered 215 chromatin factors and 194 orthologs of cancer associated genes [12]. I uncovered a total of 24 RNAi enhancers and 23 RNAi suppressors of *let-60(n1046)* Muv phenotype.

By generating homozygous double mutants with *let-60(n1046)*, I was able to genetically validate PRDM1 *blmp-1*, Smad *sma-4*, CKI *cki-2* and PDK *pdkh-2* as enhancers, and Akt *akt-1*, Rb *lin-35* and *pha-1* as suppressors of *let-60(n1046)* Muv phenotype. Because *let-60(n1046);blmp-1(tm548)* worms are nearly 100% Muv, and its mammalian orthologs have been implicated as a tumor suppressor gene (TSG), I decided to further characterize *blmp-1*, which is described in detail in Chapter 3.
2.2 Introduction

Tumorigenesis represents a process where the interactions between driver mutations emerge in vivo: cancer cells accumulate mutations as they progress from benign to malignant (Figure 2.1). For example, while activated Ras oncogene is found in many types of cancer, activated Ras alone is not sufficient to drive tumorigenesis. Additional mutations are required to drive cell transformation into malignant tumors. Identification of these driver mutations and understanding how they cooperate to drive tumorigenesis are critical to prognosis determination and treatment of cancers.

In *C. elegans, let-60(n1046)* mutants harbor the G13E mutation homologous to the HRAS and KRAS mutations that are frequently found in human cancers [84]. Worms homozygous for this mutation have a median of 60 – 80% penetrant multivulva phenotype at 20°C (Figure 2.2, white bar). Forward genetic screens in this mutant background have identified genes that function in the same signaling pathway as *let-60*. Loss of function of these genes suppresses the Muv phenotype by either reducing the percentage of worms with ectopic pseudovulval induction or suppressing vulval development altogether [40,51,52,85,86]. Genetic suppressors that decrease the penetrance of *let-60(n1046)* Muv phenotype (Figure 2.2, blue bar) are orthologous to putative oncogenes. Conversely, genetic enhancers that, when targeted with RNAi, increase the penetrance of *let-60(n1046)* Muv phenotype (Figure 2.2, pink bar) are orthologous to putative tumor-suppressor genes. Therefore, screening for genes that increase or decrease the penetrance of Muv worms in *let-60(n1046)* background may identify putative Ras-cooperating driver genes.

RNAi is a very powerful tool to potently and rapidly perturb gene functions in *C. elegans*. Systemic RNAi can be introduced by feeding the worms with bacteria expressing double stranded RNA against the genes of interest. This allows for assaying for in vivo RNAi phenotypes in the context of a whole animal within a span of 1 – 2 generations. In addition, genetic interactions can be identified by inducing RNAi in genetic backgrounds of interest. The bacterial dsRNA library is available with ~90% coverage of the *C. elegans* genome, also referred to as the Ahringer library [24,25]. Therefore, in this chapter, I use RNAi to screen a
subset of this library (408 genes) for knockdowns that affect the penetrance of the Muv phenotype in the worms.

Previously, Wilson and colleagues carried out a screen through *C. elegans* kinome and identified 3 novel attenuators of Ras signaling, including *H37N21.1*, an *NRBP1* ortholog. They were able to confirm the role of pseudokinase *NRBP1* as a Ras-cooperating TSG in mice and cell transformation assays [9]. Loss of *NRBP1* increases Sall4 levels, and results in activation of Wnt signaling pathway. However, when *NRBP1* is mutated, tumorigenesis does not occur unless constitutively activated Ras is present. This represents an example of conserved genetic cooperation in tumorigenesis. In addition, this example postulates that additional new tumor-suppressor genes or new genetic interactions with activated ras can be discovered with similar quantitative RNAi screening in let-60(n1046).

In the RNAi screening by Wilson et al (2012), 100 - 200 worms were manually scored in triplicate for each RNAi construct assayed in the screen in order to achieve robust statistical power [9]. This makes it impractical to carry out a whole-genome screen. Thus, I used a candidate approach that focuses on two subsets of genes that may be enriched in putative ras-cooperating cancer genes.

The Tumor Sequencing Project (TSP) curated a list of 623 known cancer genes as the first genes to be resequenced in lung tumor DNA samples [12]. This list of genes is highly enriched in genes that are known to interact with activated Ras either in *C. elegans* or in mammals. Therefore, I used the dsRNA library of *C. elegans* orthologs (193 clones listed in Supplementary Table 1, henceforth referred to as the TSP library) to these human genes as part of this candidate RNAi screen.

A recent catalog of cancer gene mutations identified genes encoding kinases and transcriptional regulators as two groups of genes that are most likely to carry driver mutations [87]. Cancer cells often harbour altered epigenetic signatures, and these epigenetic changes are often themselves driver mutations. In addition, several transcriptional regulators, such as ETS LIN-1, Winged Helix LIN-31, RB1 LIN-35, and Hox LIN-39 are known to genetically interact with
Ras signaling and regulate vulval development in *C. elegans* [41,88-91]. Therefore, in order to systematically identify chromatin factors that may modify gain of function Ras phenotype, I used an RNAi library of chromatin factors (215 clones listed in Supplementary Table 1) as part of this candidate RNAi screen.

In this chapter, I describe an *in vivo* RNAi screen for putative Ras-cooperating driver gene orthologs in *C. elegans*. First, I assessed for the sensitivity and specificity of this assay using known suppressors and enhancers of *let-60(n1046)* phenotype. Then, I describe the screening protocol for identifying these genes using a candidate approach, and a pipeline for validating these positives from the screen. Finally, I discuss the positives that were identified from the screen.

2.3 Methods

2.3.1 Ortholog matching with the TSP list

The TSP listed 623 genes as starting point for resequencing lung tumor samples [12]. These 623 genes are enriched in genes with implicated roles in cancer including many that function in the EGF-RTK-Ras pathway. I wrote a Perl script to match the genes from the TSP list with the *C. elegans* orthologs, using the ortholog-matching output pairs from Inparanoid 6 [92]. From this list, 359 genes match with at least one *C. elegans* ortholog. *C. elegans* Gene IDs were downloaded from Wormbase (Version 170). Since some *C. elegans* genes match with more than one human orthologs, the final list of *C. elegans* genes include 198 genes. Out of these, 187 were present in the Ahringer RNAi library, 7 additional, including *lin-45, age-1, air-2, daf-2, jnk-1, chk-1, and phc-2* were successfully cloned and screened in parallel. These RNAi constructs were re-gridded in 96-well plate formats for the candidate RNAi screen. This library of 194 genes is referred to as the TSP library (Supplementary Table 1).

2.3.2 RNAi screening

*C. elegans* were maintained on OP50-seeded NGM agar at 20°C. Screening was done in 12-well agar plates as previously described [9] (Figure 2.3). This RNAi protocol has been shown
to generate the strongest and most consistent knockdown [93]. Mixed stage worms were washed off the NGM plates and filtered through 11 µm mesh to synchronize for L1 worms. L1 worms were then placed back on fresh OP50-seeded NGM plates. 24 hours later, L3 worms were washed off the OP50 plates and placed in the first column (W0) of the 12-well NGM agar plates (containing 1 mM IPTG and 50 ug/ml carbenicillin) pre-seeded with dsRNA expressing HT115 E. coli. 48 hours later when the worms reach adulthood, single viable adult worms were transferred to the three other wells. Worms were allowed to lay eggs for 24 hours before the adults were removed, and embryonic phenotypes scored 24 hours later. Vulval phenotypes and other adult phenotypes were manually scored 48 hours later. To screen for RNAi clones that give rise to increased or decreased penetrance of the Muv phenotypes, Muv and non-Muv worms were counted for each well, up to at least 20 worms per well. Because it took ~5 minutes to score 1 gene (3 wells), I was able to count up to ~36 wells per hour or ~96 RNAi clones per day.

Each of these screening rounds produced data from 3 independent clones of worms. Each dsRNA construct was assayed at least 3 times on independent experimental setups during different times. Data were analyzed in comparison to worms fed with 20 replicates of negative control dsRNA on the same experimental setup.

To rule out the possibility that the RNAi clones may themselves produced a Muv phenotype in wildtype background, I relied on previously published RNAi phenotype data which assayed for viable and post-embryonic phenotype using the entire Ahringer library [72]. In addition, mutant strains of positives were examined for possible inherent Muv phenotypes.

2.3.3 Data analyses

Some of the RNAi knockdowns may result in phenotypes that prevent quantitative comparison of adult phenotypes, i.e. if the RNAi resulted in embryonic or larval lethality, low brood size or sterility. Therefore, I eliminated the data points with consistently less than 2 data points that contained at least 20 worms each in each day of the screen before starting data analyses.

The Muv phenotype of let-60(n1046) was highly variable between experimental replicates. Therefore, I standardized the data from each day of the screen into the percentage of
enhancement or suppression of the Muv phenotype relative to the median value of \textit{let-60(n1046)} worms fed with negative control RNAi on each day (Figure 2.4).

For each round of the screen, normalized percentage suppression/enhancement scores were ranked. Receiver Operating Characteristic (ROC) plots were then estimated from the positive and negative controls that were included in the screen. Positives are defined as genes that are at least 2 standard errors of the mean from the median of the negative controls, and are ranked within less than 10% false positive rate as estimated by the inclusion of the negative clones. These genes were selected for genetic validation by generating double mutants, when possible. Subsequently, a Student’s \( t \)-test with Bonferroni correction was used as a post-hoc analysis to confirm that the distribution of the Muv phenotypes of the suppressors and enhancers were significantly different from the negative controls.

\section*{2.3.4 Validation of screen data}

RNAi clones that were positives in the screen were then re-tested at least twice in follow-up experiments. There is, however, the possibility that the RNAi phenotypes were due to the use of RNAi pathway, enhanced RNAi sensitivity of the knockdowns, or off-target effects. To rule out this possibility, I obtained hypomorphic or null alleles of these positives and crossed them to \textit{let-60(n1046)} to generated double mutants. If the double mutants recapitulate the RNAi phenotype, then these genes are considered to be true candidate Ras-pathway interacting genes. Otherwise, these genes may have been recovered as positives due to RNAi artifacts. This validation focused on genes with viable mutants that are not linked to \textit{let-60}, i.e. genes that are not located on chromosome IV.

To generate double mutants between \textit{let-60(n1046)} and genes that were identified as positives in the RNAi screen, I crossed mutants of these positives with \textit{let-60(n1046)}. PD4792 worms were heat-shocked to generate male progenies by placing parafilm-wrapped plates in 30°C water bath for 6 – 8 hours. In the next generation, male PD4792 worms were crossed with \textit{let-60(n1046)} hermaphrodites. Subsequently, heterozygous males were mated to the mutant allele of the gene to be validated, and segregants were isolated and genotyped for the presence of the desired allele in F1 and F2 generation. At least two independent clones were isolated for each genetic cross. Transgenes with GFP expression were followed by fluorescent microscopy to
ensure transmission of the GFP transgenes. Deletion mutations were followed using single
worm PCR. Point mutations were followed by single worm PCR and DNA sequencing through
the Center for Applied Genomics at SickKids Hospital. DNA primers were designed using
Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) to generate PCR products under 1.2 kb and
checked for no duplicate PCR products the C. elegans genome using Wormbase BLAST.

To prepare templates for single worm PCRs, each genotyped worm was placed in 12 μl of
single worm lysis buffer (50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM MgCl2, 0.45% Nonidet
P40, 0.45% Tween 20, 0.01% Gelatin, and 0.12 mg/ml Proteinase K) in a PCR tube, which was
then incubated at -80°C for 10 min, 60°C for 60 min and 95°C for 15 min to lyse the worms.
Subsequently, the worm lysate was added as template as 1:10 dilution in genotyping PCR
reactions. For all genetic crosses, parental strains were always lysed and genotyped in parallel
as PCR positive controls.

2.3.5 Estimating the false positive and true positive rate –
assessing the performance of the screen to identify true
positives

In order to assess the performance of the screen to identify true positives, I included 2 identical
replicates of 6 positive controls for suppressors, including isw-1 [94], ksr-1 [51], lin-45 [95],
mig-15 (Andy Fraser, personal communication), sur-2 [52] and sur-6 [86]; 3 positive controls
for enhancers, including blmp-1, dgk-2 (Andy Fraser, personal communication) and egr-1 [96];
and 3 genes that are known to function in vulval development, including lin-1 [97], lin-12 [98]
and lin-31 [42] to screen in parallel with the TSP library screens both at 20°C and at 16°C. In
addition, the TSP library includes other genes that are known to enhance or suppress the Muv
phenotypes from the chromatin factors library. The screens also included 12 clones of negative
control RNAi. The rate at which the screen identified negative controls at a certain cut off was
used to estimate the false positive rate. Whereas, the rate at which the screen identified true
positive controls at a certain cutoff was used to estimate the true positive rate. This data is then
used to generate ROC plots.
2.4 Results

In this RNAi screen, \textit{let-60(n1046)} worms were fed bacteria expressing dsRNA against genes of interest, and subsequently manually scored for penetrance of the Muv phenotype. In order to establish the screening conditions, I will first describe properties of \textit{let-60(n1046)} Muv phenotypes and then test the ability of this RNAi assay to identify known suppressors and enhancers. Next, I will describe my optimization of screen conditions and assessment of the screen data. Finally, I will discuss the validation of the positives identified from the screen.

2.4.1 The phenotype of \textit{let-60(n1046)}

Penetrance of \textit{let-60(n1046)} Muv phenotype is variable between experimental replicates and is temperature sensitive (Figure 2.5). At higher temperature, \textit{let-60(n1046)} worms exhibit higher Muv penetrance than at lower temperature. At 20°C, the median penetrance is \(~\text{60 – 80\%}~\) while at 16°C, the median penetrance is \(~\text{22 – 50\%}~\) (Figure 2.5). Overall, the distribution of \textit{let-60(n1046)} Muv phenotype follows a normal distribution (Figure 2.6), therefore statistical tests that assume a normal distribution can be applied.

Typically, an RNAi screen requires at least 3 experimental replicates to ensure reproducibility of the results and achieve statistical power. Given the inherent variability of \textit{let-60(n1046)} Muv phenotype, a large number of worms need to be scored. I increased the number of replicates to 9 as opposed to the usual 3. In addition, the data is standardized as percentage enhancement or suppression relative to \textit{let-60(n1046)} for each replicate of the screen as described in Materials and Methods (Figure 2.4). For example, if the median of the negative controls is 68\% and worms knocked down against a gene of interest is 85\% Muv, then this RNAi produced \((85\% - 68\%)/(100\% - 68\%) = 53\%~\) enhancement. In the next section, I discuss testing the sensitivity and specificity of this protocol to identify known suppressors and enhancers of the \textit{let-60(n1046)} Muv phenotype.
2.4.2 Assessing the accuracy of the RNAi assay to identify previously identified suppressors and enhancers of *let-60(n1046)* Muv phenotypes

To assess sensitivity and specificity of this RNAi assay, I carried out this assay using non-targeting RNAi, genes that were previously identified as modifiers of *let-60(n1046)* Muv phenotype, and genes that function in vulval development.

2.4.2.1 Testing for the performance of the RNAi assays to identify known suppressors of *let-60(n1046)* Muv phenotypes

Known characterized suppressors of *let-60(n1046)* Muv phenotypes include vulval development genes, including *ksr-1, let-23, sur-2* and *sur-6* which are required for wildtype vulval development [40,51,52,85,86]. Whereas, *isw-1(RNAi)* leads to a 20 – 40% penetrant lethal phenotype but is not required for wildtype vulval development [94]. With this screening protocol, most progenies of worms targeted with *sur-2* and *sur-6* by RNAi were 100% embryonic or larval lethal, so the suppression effects on the remaining worms that survived to adulthood were not observed (Figure 2.7). I found that although there were enough adult progenies of *ksr-1, isw-1* and *let-23* knockdowns to score vulval phenotype, these RNAi clones did not lead to significantly decreased penetrant Muv phenotypes comparing to negative controls (Figure 2.7). Therefore, this protocol (described in Materials and Methods section 2.3.2) could not accurately identify known suppressors of *let-60(n1046)* Muv phenotype.

2.4.2.2 Testing for the performance of the RNAi assays to identify known enhancers of *let-60(n1046)* Muv phenotypes

For enhancers, I tested *lin-1* and *lin-12* as they have negative roles downstream of Ras in vulval development fate [97,98]. Also, I included known RNAi enhancers including *nhl-2, egl-27, egr-1*, and *H37N21.1* [9,37,63,66].

All four known enhancers consistently recapitulated published results. *let-60(n1046)* worms fed with positive control RNAi for enhancers had significantly higher penetrant Muv phenotypes (p<0.05 for *egl-27* and p<0.01 for *nhl-2, egl-27, egr-1* and *H37N21.1*) in comparison to worms fed with negative control or non-targeting RNAi (Figure 2.7). *lin-1(RNAi)* also resulted in
significantly higher penetrance Muv worms (p<0.05), whereas lin-12 resulted in a marginally significant increase in penetrance Muv phenotypes (p = 0.07) (Figure 2.7).

In conclusion, I found that this RNAi protocol (Figure 2.3, methods) performed better in identifying known enhancers than suppressors. Therefore, although the RNAi protocol could technically identify both enhancers and suppressors, this screen is likely ideal for identifying enhancers. Possibly, this is because the RNAi suppressors that were tested resulted in lethal phenotypes under the RNAi conditions that produced the strongest and most consistent knockdowns. In the next section, I discuss the modification of various parameters of the screen protocol to dampen the knockdown effects may increase its performance for identifying suppressors, many of which are essential genes.

2.4.3 Optimizing the screen conditions to uncover hypomorphic RNAi phenotypes of essential genes

The RNAi protocol used in this study has been shown to generate the strongest and most consistent RNAi phenotype [93]. Also, I found that this screen protocol is optimized to perform well in identifying enhancers of let-60(n1046) Muv phenotypes. For many genes assayed, the strong RNAi knockdowns result in highly penetrant lethal or sterile phenotypes, precluding the possibilities of recovering these genes as positive. Notably, many of these essential genes function in Ras signaling or suppress let-60(n1046) Muv phenotype. Therefore, I tested whether slight modifications of the protocol would increase the recovery rates of these genes by 1) varying the stages at which worms are fed with dsRNA-expressing bacteria and 2) varying the temperatures of the screen. To test this modified protocol, I used a list of genes that are either known to function in vulval development or have previously shown to modify vulval phenotype in RNAi (Table 2.1).

To assess the performance of the RNAi assays, the recovery of false positive rates was plotted against true positive rates in a receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) estimates the probability that the assay will correctly classify true positives from false positives. An AUC of 50% would indicate that the assay does not classify true positives better than by random chance, whereas a higher AUC closer to 100% would indicate that the assay performs well to correctly classify true positives from false positives.
2.4.3.1 Varying the stages in which worms are fed dsRNA-expressing bacteria

Kamath et al (2001) reported that it is important to start the RNAi feeding from the L4 or earlier stage in the generation prior to the generation that would be scored, in order to knock down maternal effect transcripts [93]. At 22°C, Worms need to be fed dsRNA-expressing bacteria for at least 40 hours prior to phenotyping to ensure maximal knockdown. For quantitative changes in \textit{let-60(n1046)} Muv phenotypes, I find that knocking down worms starting at L3 stage (24 hours post L1 synchronization and 7 days before scoring) resulted in stronger phenotypes than starting at L4 stage (48 hours post L1 synchronization and 6 days before scoring). Therefore, in order to generate weaker knockdowns of essential genes to elicit vulval phenotypes, I modified the protocol by starting feeding from a later stage. When worms were fed with dsRNA-expressing bacteria from L1 stage of the generation to be assayed, most RNAi that targeted essential genes now result in a higher percentage of viable worms because the maternal gene products were available during embryonic and larval development. However, the RNAi knockdowns were still able to elicit vulval phenotypes. As a result, this protocol modification resulted in the RNAi assay that accurately identified known suppressors as such. Particularly, \textit{sur-2, sur-6, lin-25, eor-1} and \textit{mek-2} were identified as strong suppressors, and \textit{mpk-1} and \textit{lin-45} as weak suppressors. The AUC of the ROC curve for the suppressor was then improved to 83.49\% (Figure 2.8). Most of the enhancers included in this assay were not recovered as positives in this condition, except for \textit{egr-1}. The AUC for the enhancers were low at 40.70\% (Figure 2.8).

The improved performance of the assay to identify modifiers of \textit{let-60(n1046)} Muv phenotype appeared to be gene-dependent rather than a generalized effect, since some genes may require knocking down of maternal effect gene products to elicit phenotypes. For this small list of genes, starting dsRNA feeding during L1 stage of the generation to be screened significantly improved the power of the RNAi screen to identify suppressors. However, because the levels of knockdowns required to elicit suppression effects and lethality could vary from gene to gene, screening from L1 versus L3 stage will yield somewhat different but overlapping lists of positives.
2.4.3.2 Varying the temperature of the screen

Because *let-60(n1046)* worms are highly penetrant (60 – 80%) Muv at 20°C and less so at lower temperatures, I tested if screening at 16°C would enhance the power of the screen to identify true positives and reduce the false positive rates.

At 16°C, 25 – 50% of worms are Muv. This shift in the median of the population increased observation range for the enhancers and reduced the observation range for the suppressors, which could affect the screen performance. According to ROC curve AUC analysis, the screen for enhancers performed slightly better at 16°C than at 20°C with AUC ~83.14% at 20°C, and 86.76 and above at 16°C (Figure 2.9). The screen for suppressors, however, performed much worse at 16°C than at 20°C, with AUC 69.55% at 20°C and ~50% at 16°C (Figure 2.9). Therefore, decreasing the screen temperature to 16°C significantly decreased the screen performance for the suppressor screen, but improved the performance the enhancer screen.

In conclusion, I found that targeting essential genes by feeding from L1 stage of the generation to be scored may be less likely to produce lethal phenotypes. This may allow several of known suppressors of *let-60(n1046)* Muv phenotypes to be identified when they would not be identified with the standard protocol of feeding from the L3 stage of the prior generation. However, this modified RNAi protocol did not perform well to identify known enhancers of *let-60(n1046)*. In addition, reducing the temperature of the screen to 16°C increased the observation range that improved the performance of the screen to identify enhancers.

Because the standard RNAi screening protocol that perform well at identifying enhancers produced the strongest and most consistent knockdowns, I carried out the screen as described in Figure 2.3, and Materials and Methods. I started RNAi feeding at L3 stage and carried out the screen at 20°C. In the next section, I describe the assessment of screen performance and reproducibility of the screen data.

2.4.4 Assessment of screen performance and reproducibility of the screen data

The screen was carried out by manually counting Muv and non-Muv *let-60(n1046)* worms that are fed with RNAi against genes of interest. A total of 408 genes were assayed, with 3
replicates of more than 20 animals each on 3 independent rounds of the screen. Therefore, a total of at least 180 animals were screened for each gene, resulting in over 75,000 animals being assayed for the Muv phenotype. I was able to recapitulate 95% of previously published embryonic lethal and sterile RNAi phenotypes [25,93], suggesting that the RNAi inductions were highly effective.

Assessment of screen performance was done using a receiver operating characteristic (ROC) plot for positive controls that were included in the screen. The standardized data was then plotted as sensitivity (percent positive controls recovered) versus noise (percent negative controls recovered) in a receiver operating characteristic (ROC) plot (Figure 2.9). At 10% false positive rate, the screen condition identifies true enhancers well at ~80 - 100% true positive rate; but only identifies suppressors ~20% true positive rate. This is in agreement with AUC analysis. The AUC of the ROC plots for enhancers ranged from 83.14% to 99.18% for enhancers, suggesting that the RNAi screen performs very well to identify true enhancers of let-60(n1046) Muv phenotype. Therefore, I opted to focus on validating and characterizing the enhancers.

2.4.5 Validation of enhancers recovered from the RNAi screen

The RNAi screens recovered all four previously published enhancers that were included in the screen, including the TRIM-NHL protein nhl-2, and two MTA1 homologs egr-1, and egl-27 and cyclin-dependent kinase inhibitor cki-2 [64,66,96], together with 20 new RNAi enhancers of let-60(n1046) Muv phenotype (Table 2.2 and Figure 2.10).

While RNAi is a very powerful method to rapidly perturb gene function, validation steps are crucial to rule out potential nonspecificity and RNAi artifacts. Therefore, I sought to validate these positives by obtaining hypomorphic or null alleles and creating double mutants with let-60(n1046). If these genes were true enhancers of activated Ras, then the double mutant should recapitulate the RNAi phenotype with significantly higher penetrance Muv than the let-60(n1046) single mutant alone.

This validation focused on viable mutants that are not linked to let-60. Out of 7 double mutants I generated, 5 confirmed as true enhancers. Of these, nhl-2 and cki-2 were previously published
as enhancers of *let-60(n1046)* Muv phenotype [64,66]. PDK3 *pdhk-2* and Smad *sma-4* were weak enhancers of Muv phenotype, with \%Muv consistently >80% (Table 2.2). In addition, I was able to confirm that the PRDM homolog *blmp-1* was a true enhancer because *blmp-1(tm548);let-60(n1046)* worms were nearly 100% Muv (Figure 2.11, Table 2.3). In my hands and according to the whole-genome RNAi dataset by Kamath and colleagues [72] these worms are not Muv as single mutants, but targeting these genes by RNAi increase penetrance of the Muv phenotype in *let-60(n1046)* background (Table 2.2).

### 2.4.5.1 BLIMP1 homolog *blmp-1* is a novel enhancer of *let-60(n1046)* Muv phenotype

*blmp-1* was consistently identified as an enhancer in my RNAi screens, and the double mutant *blmp-1(tm548);let-60(n1046)* were 100% Muv (Figure 2.11). Interestingly neither *blmp-1(tm548)* nor *blmp-1(RNAi)* were Muv (Table 2.3).

*blmp-1* encodes the *C. elegans* ortholog of mammalian BLIMP-1 (*B-lymphocyte-induced maturation protein 1*) or PRDM1, which has a SET domain and 5 C2H2 Zinc fingers. In mammals, BLIMP-1 is expressed in B cells as they commit to differentiation to become terminal plasma cells. This cell fate commitment is a terminal differentiation process. BLIMP-1 transcriptionally represses expression of proliferative genes, and class-switching genes [99]. Loss of BLIMP-1 has been identified as a genetic signature in leukemia and lymphoma, suggesting that it functions as a tumor suppressor gene [14,83]. However, BLIMP-1 has never been studied in the context of gain of function Ras, and is relatively uncharacterized in the worms. Therefore, I have decided to further investigate on characterizing BLMP-1 in *C. elegans* (Chapter 3).

### 2.4.5.2 *sma-4*, TGF-β, recapitulates its role as a Ras-cooperating tumor suppressor gene

*sma-4* is a *C. elegans* ortholog of DPC4 tumor suppressor gene which is known to be mutated in 55% of pancreatic cancer [100]. *C. elegans* Sma (small) mutants, as well as other mutants with downregulated TGF-β signaling are much smaller than wildtype worms [101]. *Sma-4* and DPC4 encode TGF-β-like molecules which are typically responsible for differentiation rather than proliferation, which is consistent with its role as a tumor suppressor gene. In addition,
concomittent activated KRAS expression and DPC4 haploinsufficiency has been shown to induce pancreatic neoplasm, as well as to accelerate the neoplasia process [102,103]. Therefore, this cooperation between activated Ras and DPC4 appears to be conserved between worms and mammals.

2.4.5.3 pdhk-2, a PDK ortholog, enhances let-60(n1046) Muv phenotype

*pdhk-2* is a *C. elegans* ortholog of mammalian pyruvate dehydrogenase kinase, an enzyme localized in the mitochondrial membrane. In *C. elegans*, *pdhk-2* deficiency has been associated with greater preservation of residual fats and hence survival during dauer state [104]. In humans, reduced PDK isozyme activity in some tissues is associated with metabolic syndrome and diabetes [105]. In cancer cells, Hif-1α-induced PDK1 overexpression causes the Warburg effect, a hallmark of cancer when cells produce energy mostly from glycolysis followed by lactic acid fermentation rather than through pyruvate oxidation in the mitochondria [106]. Thus, PDK isozymes in mammals are oncogenes, but paradoxically mutating *pdhk-2* enhances *let-60(n1046)* phenotype. Possibly, *pdhk-2* also interacts with Ras at another stage that is not dependent on hypoxia or increased glycolysis. Additional studies will be required to understand how *pdhk-2* cooperates with Ras signaling in *C. elegans*.

2.4.6 Validation of the strongest suppressors

Although the RNAi assay of positive controls demonstrated that the screen performed much better to identify enhancers than suppressors, several RNAi clones consistently ranked as the lowest penetrance Muv in all rounds of the screen (Table 2.4 and Figure 2.12). Notably, while many positive controls of the suppressors that were used to test this assay were essential genes, these putative suppressors recovered in the screen were viable as RNAi knockdowns. Therefore, by generating double mutants, I validated four out of seven genes that were strongest and most consistent RNAi suppressors of *let-60(n1046)*. I was able to validate *akt-1*, and *lin-35* as true positives, while BRCA1 *brc-1*, STK32C *M03C11.1* and MLH1 *mlh-1* were not validated by this method (Figure 2.13). *pha-1(e2123ts)* marginally significantly suppressed the Muv of *let-60(n1046)* phenotype (p=0.05 without Bonferroni correction) at permissive temperature.
2.4.6.1 *akt-1*(lf) was confirmed as a suppressor of Muv phenotype, consistent with its role in tumorigenesis.

*akt-1* encodes a worm homolog of AKT, which is an effector of the PI3K/PTEN/AKT pathway. Although *akt-1*(RNAi) was a weak positive suppressor of *let-60(n1046)* Muv phenotype, *akt-1*(mg306);*let-60(n1046)* were only ~13% Muv, comparing to ~60 – 80% of *let-60(n1046)* (Figure 2.13). This is consistent with the role of AKT as a proto-oncogene in mammals. These two pathways often synergize in tumorigenesis and combination therapy that targets both Ras/Raf/MEK/ERK and PI3K/PTEN/AKT pathways are frequently used in cancer treatments [107]. Although the insulin signaling pathway has not been established as a downstream target of Ras signaling in *C. elegans*, several genetic interactions exist between Ras signaling and IGF-1 signaling in aging and dauer formation in worms [108,109]. IGF-1 signaling was also found to promote Ras signaling in the germline of *C. elegans* [110]. The PI3K homolog *age-1* had lethal loss of function and RNAi phenotype, so the suppression/enhancement effect of this gene was not available from the screen. However, Nakdimon et al (2012) showed that viable homozygous *age-1*(lf) progenies of heterozygous parents did not significantly modify the *let-60(n1046)* phenotype[108], although those animals could still be expressing AGE-1 from maternal effect transcripts. *aap-1*, a *C. elegans* PI3K adaptor/regulatory subunit also weakly suppressed the Muv phenotype in the RNAi screen (30.92% Muv, n = 335, - 17.83% suppression). Curiously, Nakdimon et al (2012) found that a deletion mutant of *akt-1* had no effect on the ectopic vulval induction of *let-60(n1046);daf-18(ok480)* background. Possibly, the suppression effect by *akt-1* might requires *daf-18*. Also, the difference in alleles or strain background could account for these differing observations.

2.4.6.2 *lin-35*, the retinoblastoma ortholog and a *synMuv* gene paradoxically suppresses *let-60(n1046)* Muv phenotype

*lin-35* encodes the *C. elegans* ortholog of the mammalian retinoblastoma (RB1) gene. *lin-35* was originally identified as a *synMuv* B gene in *C. elegans*, which function redundantly to prevent ectopic pseudovulval induction with other groups (A and C) of *synMuv* genes [58]. *lin-35* also mediates cell cycle through G1/S inhibition [111]. In addition, *lin-35* mutants exhibit enhanced RNAi sensitivity [112]. While RB1 is understood to be a tumor suppressor gene, Williams et al (2006) found that the RB1 protein is required for Ras-induced oncogenic
transformation as loss of RB1 prevents cell transformation in murine 3T3 cells with activated H-Ras [113]. Therefore, it is possible that RB1 is required at some steps of cell transformation but functions as a tumor-suppressor gene at later steps. Interestingly, Cui et al (2006) showed that lin-3 mRNA levels are reduced in lin-35 mutants comparing to wildtype worms [61], which could account for my finding that lin-35 mutants suppresses let-60(n1046) Muv phenotype. Additional characterizations would be required to understand how lin-35 interacts with activated Ras in C. elegans.

2.4.6.3 pha-1 is a suppressor of let-60(n1046) Muv phenotype

Although pha-1 sequence is similar to a bZIP family transcription factor, the protein is largely cytoplasmic [114-116]. Aside from a few other Caenorhabditis species, pha-1 does not have other metazoan orthologs. However, strong loss of function alleles of pha-1 result in lethal phenotype, suggesting that it is an essential gene. While pha-1 does not have previously implicated roles in vulval development, it functions redundantly with synMuv genes like lin-35 and efl-1 in pharyngeal morphogenesis [116].

The E2 ubiquitin conjugating enzyme UBCH7 ubc-18 appears to function in the same genetic circuit as lin-35 and pha-1. ubc-18;lin-35 double mutant phenocopies pha-1 mutant in terms of pharyngeal development defect. In addition, ubc-18;lin-35 compound mutants as well as compound mutants between ubc-18 and several other synMuv B genes phenocopy pha-1 pharyngeal defect phenotype [116]. Therefore, I tested whether ubc-18 would have the same effect on let-60(n1046) Muv phenotype by generating ubc-18(ku354);let-60(n1046) double mutant and found that ubc-18 is a strong suppressor of let-60(n1046) Muv phenotype (Figure 2.14). It is thus possible that similar genetic circuit that regulates pharyngeal development may also regulate vulval development.
2.5 Discussion

2.5.1 Advantages and disadvantages of RNAi screening in *C. elegans* to identify genetic interactions among driver gene homologs

A key question in cancer research involves distinguishing driver from passenger mutations and understanding how cooperation between driver mutations occur *in vivo*. Therefore, beyond identifying the cancer genes, it is also critical to understand how the mutations contribute to cell transformation. Because many genes and pathways that are perturbed in tumorigenesis are highly conserved among metazoa, several such pathways have been elucidated in model organisms.

There are several advantages to quantifying *C. elegans* Muv phenotype as a system for identifying cancer genetic interactions. First, vulval development involves the conserved EGF-RTK-Ras signaling between two cell types, the anchor cell and vulval precursor cells *in vivo*. The vulva is a well-characterized system for understanding how different cell types communicate and orchestrate growth. Second, when matching *C. elegans* orthologs of the TSP gene list, I find that for cancer-related signaling pathways, *C. elegans* is a much simpler system because many human gene orthologs that are paralogous to each other match with a single or a few *C. elegans* homologs. As a result, a subset of 359 human genes only matched to 198 *C. elegans* genes. Therefore, worms are less likely to have multiple paralogs that compensate for the same functions, which may make it easier to elicit phenotypes from RNAi knockdowns. Third, many genetic tools available like RNAi, genetic mutants, fosmid and cDNA libraries, genomic sequences and functional genomic data make it easier to validate and characterize the positives. Lastly, many relevant pathways are remarkably well conserved between worms and mammals, and are well characterized in vulval development.

This system, however, is not without drawbacks. First, since the Muv phenotype was to be scored during adulthood, it was not possible to assay for effects of essential gene knockdowns on *let-60(n1046)* Muv phenotype with RNAi. Several of these essential genes have important function in vulval development, especially genetic suppressors of *let-60(n1046)* Muv phenotype and some *synMuv* genes. Therefore, the majority of genes identified as positives
were limited to genes with viable adult RNAi phenotypes. I found that starting feeding dsRNA-expressing bacteria from as late as L1 stage may uncover some essential genes, especially suppressors, if knocking down of maternal effect gene products were not required to suppress the Muv phenotype.

I have not been able to automate the detection of the Muv phenotype for a high-throughput RNAi screen. I found that vulval-specific fluorescent reporters typically do not express in all ectopic pseudovulvae and may not express bright enough signals to be consistently detected by high content fluorescent microscopy or a Biosorter. In addition, because *C. elegans* transgenic reporters are typically transformed at high concentrations, high copy numbers of vulval-specific promoters may sequester some relevant transcription factors and sensitize the strain background for false screen results. Therefore, the screen required manual quantification of the Muv phenotype, rendering it mid-throughput, at best.

The inherently variable Muv phenotype of *let-60(n1046)* means that the screen raw data have substantial noise. However, I have been able to validate many novel positives from the screen, including *blmp-1*. In addition, the human orthologs of *nhl-2* and *H37N21.1* were shown to function as tumor suppressor genes in mammalian systems following their characterization as enhancers of *let-60(n1046)* Muv phenotypes [9,66]. Therefore, while the frequency of the Muv phenotype is quantitative, the analyzed data is more useful semi-quantitatively to indicate whether the gene is relevant as being a suppressors or an enhancer. The power of this screen, however, is that it can identify novel modifiers of activated Ras phenotype that have not been identified by other forms of genetic screening. This advantage could be because this screen is carried out *in vivo* which allows for screening for a process that involves communications between multiple cell types (e.g. AC and VPCs), and because reverse genetic screens can often uncover positives that were not previously identified using forward genetic screens.

2.5.2 Statistical analysis of the screen data

The variable nature of *let-60(n1046)* Muv phenotype presented a challenge for statistical analyses as significant overlaps were found between positive and negative clones, making it difficult to classify biologically significant positives from negatives. Therefore, I used multiple statistical means to—first—assess the sensitivity and specificity of the screen data and then
classify biologically significant positives from other clones. To address the day-to-day variation of *let-60(n1046)* Muv phenotype, the data was standardized to the median of the negative control for each round of the screen. Then, the data was then ranked based on this standardized score.

A Receiver Operating Characteristic plot was estimated by plotting the rate at which negative controls were recovered as positives against the rate at which positive controls (genes that were previously shown to modify *let-60(n1046)* Muv phenotype) were recovered as positives for various cutoffs. From these plots, I found that the best sensitivity versus specificity ratio was achieved at approximately 10% false positive rate, as about 80% of true enhancers were recovered. Therefore, I used 10% false positive rate cutoff, or where 10% of negative control clones were recovered, as cutoffs for classifying the genes as positives from the screen. For both screens, this top 10% cutoff coincided with 12 genes. Therefore, genes with the sum of ranks among the top 12 were determined as positives, given that their enhancements of Muv phenotype were more than two standard errors of the mean (SEM) from the median of the negative controls. By taking into account the SEM value, this rules out the possibility of recovering RNAi clones that have high between-replicate or well-to-well variation as positives, so only RNAi clones that robustly suppressed or enhanced the Muv phenotype were recovered.

Several viable suppressors of *let-60(n1046)* Muv phenotypes have been identified by RNAi either in *let-60(n1046)* background or *lin-15AB(n765)* background [8]. If those genes had been included as positive controls in the ROC plot estimation, perhaps the ROC plot would demonstrate a much better sensitivity/specificity ratio. Interestingly, three suppressors from the chromatin factor screen were previously identified as suppressors of *lin-15AB* Muv phenotype, including *dpy-30, isw-1* and *htz-1* (Table 2.4) [8]. In addition, I also identified many of these genes in another RNAi screen for suppressors of *blmp-1(tm548);let-60(n1046)* Muv phenotype (Chapter 3, Table 2.4). Thus, it is possible that the ROC curve for the suppressor screen underestimated the power of the screen to identify viable suppressors. The *let-60(n1046)* suppressors which were also identified as suppressors of *lin-15AB* and *blmp-1(tm548);let-60(n1046)* Muv phenotype are likely to be biologically significant. Therefore, a screen for suppressors in a genetic background with 100% penetrance Muv may be a much more effective and streamlined way to uncover suppressors than in *let-60(n1046)* background.
2.5.3 Knockdowns that affect viability may obscure the actual enhancement or suppression effects of the RNAi clones

Among the RNAi clones assayed, I was able to recapitulate 95% of sterile or embryonic lethal RNAi phenotypes previously annotated Kamath et al (2003) [24]. Since the Muv phenotype is typically scored during adulthood, the severity of the knockdowns may have hindered identification of their genetic interactions with let-60(n1046). Unfortunately, several of these genes were implicated with roles in vulval development and were thus likely to be modifiers of let-60(n1046) Muv phenotypes. These include genes that function in RTK-RAS-MAPK signaling, and synMuv genes like lin-53 and trr-1 [59,117]. Adjustments of screen conditions to dampen the RNAi knockdown effects may therefore provide useful results for these genes.

2.5.4 Adjustments of screen conditions to dampen the levels of knockdowns may allow for assaying phenotypes from essential genes

It may be possible to adjust the RNAi screening conditions to create hypomorphic and viable RNAi phenotypes for essential genes. Kamath et al (2001) titrated the IPTG concentration and tested the phenotypes of unc-37, which is an essential gene. Hypomorphic mutants of unc-37 display a variety of phenotypes [93]. They found that unc-37(RNAi) was completely lethal when the bacteria were induced at 1 mM IPTG, but 48% lethal and 100% uncoordinated when the feeding bacteria were induced at 1 μM IPTG. However, this may not be the case for all genes, and the ideal screen condition to identify the maximum number of true positives among essential genes remain to be determined.

Another way to dampen the degree of RNAi knockdown achieved for any target gene would be to provide the dsRNA-expressing bacteria at a later life stage. Because many genes express from maternally supplied transcripts, it is typically necessary to feed the worms in the generation prior to the generation to be assessed for phenotypes. Although Kamath et al [93] didn’t find differences when feeding at stages earlier than L4, I did find that feeding worms starting L3 stage resulted in stronger changes in Muv phenotypes than at L4 or young adult stages. The weaker phenotypes at later stages could be due to either lower penetrance of the
RNAi phenotype or from weaker knockdown effects. When dsRNA bacteria was fed from L1 stage of the generation to be phenotyped, several more essential gene knockdown worms survived until adulthood, and their suppressor phenotypes were recapitulated. However, this effect may be gene-dependent, as some genes are essential during larval development, and other genes will not elicit consistent RNAi phenotypes if the maternal effect gene products were not knocked down.

While the original RNAi screen protocol was well optimized to identify enhancers of the \textit{let-60(n1046)} Muv phenotype, additional optimization and testing would be required in order for the screen to perform well as a suppressor screen.

### 2.5.5 Confirmation that the enhancement and suppression phenotypes are not due to inherent RNAi phenotypes

Several genes that were targeted in these RNAi screens have inherent Muv RNAi phenotypes, such as \textit{lin-12} and \textit{lin-1}. Therefore, ideally, to confirm that the RNAi clones identified from the screen were true enhancers and suppressors of \textit{let-60(n1046)} Muv phenotypes, these RNAi clones should also be fed to wildtype worms in parallel. Because this RNAi assay involved several steps of preparation and manual manipulation of the animals, I opted to take this consideration at the follow up steps rather than as a screening step. First, I examined previously published data for RNAi phenotypes in wildtype background from the Ahringer library [72]. Second, during the validation steps, I obtained null or hypomorphic mutants and examined for their inherent Muv phenotypes before generating double mutants. However, for genes that would be further followed-up, such as \textit{blmp-1}, an RNAi by feeding to wildtype worms to confirm that the RNAi clone alone does not result in Muv phenotype would be necessary (Table 2.3).

### 2.5.6 Novel enhancers from the RNAi screen

The RNAi screen condition was optimal for identifying viable enhancers of \textit{let-60(n1046)} Muv phenotype. All previously published enhancers, including \textit{nhl-2}, \textit{nrbp-1}, \textit{egl-27} and \textit{egr-1} were consistently identified in the screen [9,63,66]. The screen also identified several novel enhancers which I did not validate either because the mutant alleles were not available or the
genes were linked. With the recent development of CRISPR mutagenesis in *C. elegans* [118], it may be possible to validate these genes by targeted mutagenesis. So far, I was able to confirm three genes as novel enhancers of *let-60(n1046)* Muv phenotype. These genes include PRDM1 *blmp-1* from the chromatin factor screen, and Smad *sma-4* and PDK3 *pdhk-2* from the TSP library screen.

2.5.7 Novel suppressors from the RNAi screen

Although the RNAi screen protocol was optimal for identifying enhancers, several genes consistently came up as the lowest penetrance Muv (Table 2.4). By generating double mutants, I was able to genetically validate the RB1 gene *lin-35* and AKT *akt-1* as strong suppressors, and *pha-1* as weak suppressor of *let-60(n1046)* Muv phenotype. Since I found that *akt-1* is a suppressor of activated Ras in the worms, it is possible that PI3K signaling may function downstream of Ras in *C. elegans* as they do in mammals. Alternatively, the suppression of the *let-60(n1046)* Muv phenotype by *akt-1* mutant could also imply that *akt-1* signaling acts in a parallel pathway which converges onto common target genes with Ras or MAPK signaling. Additional analyses would be required to fully understand the interactions between *akt-1* and *let-60*.

2.5.7.1 Some suppressors of *let-60(n1046)* also suppress Muv phenotypes in other genetic backgrounds, including the synMuv mutant *lin-15AB* and *blmp-1(tm548);let-60(n1046)*

Three suppressors of *let-60(n1046)* were previously identified as synMuv suppressors, including *dpy-30, htz-1* and *isw-1*; with *isw-1* also previously confirmed as a suppressor of *let-60(n1046)* [8]. Single mutants of these genes are neither Muv nor Vul. Null mutants of *dpy-30* and *htz-1* are not viable. I therefore did not genetically validate *dpy-30* and *htz-1*. However, because these genes suppress Muv phenotypes in synMuv, *let-60(n1046)* and *blmp-1(tm548);let-60(n1046)* worms, they may be generally required for ectopic pseudovulval induction. Additional characterization of such pathways would be required to understand the precise mechanism underlying how these genes suppress Muv phenotype in multiple genetic backgrounds.
2.6 Conclusion and significance

In summary, I used in vivo RNAi screening in C. elegans to identify putative Ras-cooperating tumor-suppressor genes. The RNAi screen uncovered blmp-1, sma-4 and pdhk-2 as enhancers, and lin-35, ubc-18, pha-1 and akt-1 as a suppressors of let-60(n1046) Muv phenotype. Because the mammalian homolog of blmp-1, PRDM1, is a known tumor suppressor gene that has not been previously shown to cooperate with activated Ras, I chose to further characterize blmp-1. I will describe my detailed investigation of blmp-1 in vulval development and its interaction with Ras signaling in Chapter 3.
Figure 2.1: The principle behind using a quantitative screening for modifiers of Muv phenotype in let-60(n1046) to identify putative Ras-cooperating cancer genes.
A. A simplified model of how multiple driver mutations cooperate to transform cells into malignant tumors.
B. Top: Sequence alignment demonstrating that activated Ras in let-60(n1046) is homologous to the V12 activated Ras found in human cancers. Bottom: how genetic screening for suppressors and enhancers of the Muv phenotype in let-60(n1046) background may identify homologs of proto-oncogenes and tumor-suppressor-gene respectively.
Figure 2.2: An example distribution of Muv phenotype in *let-60(n1046)* worms.

*let-60(n1046)* worms were fed with negative control RNAi (clear white bar), an example of a suppressor (blue bar, *lin-35*) and an example of an enhancer (pink bar, *nhl-2*). X-axis denotes the proportion of each clone that are Muv, whereas Y-axis denotes the percentage of clones that were assayed.
Figure 2.3: Quantitative RNAi screening timeline and protocol for enhancers and suppressors of the Muv phenotype
Figure 2.4: Data analysis workflow for the RNAi screen data.
To account for the inherent variability of let-60(n1046) Muv phenotype, weighted average data from each RNAi clone was centered to median of let-60(n1046) negative controls (top panel). The observation range on each side was then re-scaled as 100% on each side (negative for suppression and positive for enhancement). The median-centered data was then ranked against all genes in the same library within the same round of RNAi screening. Genes that pass the top 10% cutoff (12 genes for each category) which are more than 2 SEM away from the negative controls were then selected for further validation.
Figure 2.5: *let-60(n1046)* Muv phenotype varies between replicates and is also temperature-dependent.
Distribution of penetrance of the Muv phenotype of worms fed with negative control RNAi from different replicates of the RNAi screen. A and B were from screens conducted at 20°C. C and D were from screens conducted at 16°C. Median Muv penetrance of *let-60(n1046)* worms fed with negative control RNAi were 77.78%, 61.82%, 50.00 and 22.22% for A, B, C and D, respectively.
Figure 2.6: Histogram displaying median-centered data from the RNAi screen

A. chromatin remodeling factors and B. Tumor Sequencing Project library. Transparent bar represents let-60(n1046) worms fed with negative control RNAi. Purple bar represents data from worms fed with screening RNAi.
Figure 2.7: Box plot displaying changes in the penetrance of Muv worms in *let-60(n1046)* worms targeted with RNAi against known suppressors and enhancers of *let-60(n1046)* Muv phenotype, as well as genes that are known to function in vulval development. Horizontal axis denotes relative proportion of Muv phenotype in comparison to *let-60(n1046)* targeted with negative control RNAi clones. Vertical axis denotes the corresponding RNAi enhancers.

*p*.01 and #p*.05 difference from negative controls, 2-tailed t-test with Bonferroni correction. This assay was done using the standard conditions as described in Figure 2.3.
Figure 2.8: ROC curves for the classification of suppressors and enhancers in the RNAi assay that starts by applying dsRNA-expression bacteria from L1 stage.
Blue line - the ROC curve for suppressors, AUC 83.49%. Red line - the ROC curve for enhancers, AUC 40.70%.
When the screen protocol is modified by starting feeding the dsRNA-expressing bacteria from L1 stage, the RNAi screen can more accurately identify known suppressors, but less accurately identify known enhancers.
Figure 2.9: Assessment of screen performance from different replicates of the screen, with RNAi feeding starting from L3 stage.

Top 4 panels: ROC plots from positive controls included in different replicates of the screen.

Table: screen conditions and ROC area under the curve (AUC) for suppressors and enhancers identified in each screen.

When the screen is carried out by start feeding from L3 stage of the parental generation, the screen can accurately identify enhancers, but less accurately identify suppressors. Screening at lower temperature decreases the accuracy of the screen to identify suppressors, but increases the accuracy of the screen to identify enhancers.
Figure 2.10A: Changes in the penetrance of Muv worms in let-60(n1046) targeted with RNAi against enhancers from the chromatin factor screen.
Horizontal axis denotes relative proportion of Muv phenotype in comparison to let-60(n1046) targeted with negative control RNAi. Vertical axis denotes the corresponding RNAi enhancers. *p<.01 and # p<.05. Student’s t-test with Bonferroni correction.
Figure 2.10B: Changes in the penetrance of Muv worms in *let-60(n1046)* targeted with RNAi against enhancers from the TSP library screen.
Horizontal axis denotes relative proportion of Muv phenotype in comparison to *let-60(n1046)* targeted with negative control RNAi. Vertical axis denotes the corresponding RNAi enhancers.
* indicates p<.01 and #p<.05. Student’s t-test with Bonferroni correction.
Figure 2.11A: Histogram displaying enhancement of the let-60(n1046) Muv phenotype by blmp-1(RNAi). Pink bar – let-60(n1046);blmp-1(RNAi). White bar - let-60(n1046) treated with negative control RNAi.

Figure 2.11B: Histogram displaying enhancement of the let-60(n1046) Muv phenotype by blmp-1(tm548). Pink bar – let-60(n1046);blmp-1(tm548). White bar - let-60(n1046).
Figure 2.12A: Changes in the penetrance of Muv worms of let-60(n1046) targeted with RNAi against suppressors from the chromatin factor screen.
Horizontal axis denotes relative proportion of Muv phenotype in comparison to let-60(n1046) targeted with negative control RNAi. Vertical axis denotes the corresponding RNAi enhancers. *p<.01 and #p<.05. Student’s t-test with Bonferroni correction.
Figure 2.12B: Changes in the penetrance of Muv worms of \textit{let-60(n1046)} targeted with RNAi against suppressors from the TSP library screen.

Horizontal axis denotes relative proportion of Muv phenotype in comparison to \textit{let-60(n1046)} targeted with negative control RNAi. Vertical axis denotes the corresponding RNAi enhancers.

* $p<.01$ and # $p<.05$. Student’s t-test with Bonferroni correction.
Figure 2.13: Changes in the penetrance of *let-60(n1046)* Muv phenotype in double mutants between *let-60(n1046)* and mutants of genes identified as RNAi suppressors. Full genotypes include (top to bottom) *let-60(n1046), let-60(n1046);pha-1(2123), let-60(n1046);lin-35(n745), let-60(n1046);M03C11.1(tm4066), let-60(n1046);brc-1(tm1145), let-60(n1046);akt-1(mg306), and let-60(n1046);mlh-1(ok1917). Horizontal axis denotes relative proportion of Muv phenotype in comparison to *let-60(n1046)* worms.

* p<0.01 for *lin-35* and *akt-1*, 2-tailed Student’s t-test assuming different distribution with Bonferroni correction. p = 0.05 for *pha-1* (without Bonferroni correction). n > 300 for each mutant.
Figure 2.14: Changes in the penetrance of Muv worms in double mutants of genes identified as RNAi suppressors.

Full genotypes include (top to bottom) let-60(n1046), let-60(n1046);pha-1(2123ts), let-60(n1046);lin-35(n745), and let-60(n1046);ubc-18(ku354). Horizontal axis denotes relative proportion of Muv phenotype in comparison to let-60(n1046) worms. Vertical axis denotes the corresponding mutant gene.

* p<0.01 for lin-35 and ubc-18, 2-tailed Student’s t-test assuming different distribution with Bonferroni correction. p = 0.05 for pha-1 (without Bonferroni correction). n > 300 for each mutant.

lin-35(n745) and ubc-18(ku354) are suppressors of let-60(n1046) Muv phenotype. pha-1(e2123ts) is a weak (marginally significant) suppressor of let-60(n1046) at permissive temperature.
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<td>MED23 mediator subunit ortholog, functions downstream of Ras [52]</td>
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<td>Novel protein, with positive roles in vulval development [121]</td>
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<td>lin-45</td>
<td>suppressor</td>
<td>Raf [95]</td>
</tr>
<tr>
<td>mpk-1</td>
<td>suppressor</td>
<td>MAPK [124]</td>
</tr>
<tr>
<td>ksr-1</td>
<td>suppressor</td>
<td>Kinase suppressor of Ras (KSR) [51]</td>
</tr>
<tr>
<td>lin-39</td>
<td>suppressor</td>
<td>Homeoprotein required for midbody region cell fate specification, including</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VPC competence [89]</td>
</tr>
<tr>
<td>mig-15</td>
<td>suppressor</td>
<td>Nck-interacting kinase, a known suppressor of let-60(n1046) Muv phenotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Personal communication, Andy Fraser)</td>
</tr>
<tr>
<td>lin-31</td>
<td>-</td>
<td>ETS Transcription factor downstream of let-60 Ras, mutants are 67% Muv and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33% Vul [42]</td>
</tr>
<tr>
<td>bar-1</td>
<td>suppressor</td>
<td>Beta catenin, required for VPC competence [125]</td>
</tr>
<tr>
<td>let-23</td>
<td>suppressor</td>
<td>EGFR upstream of Ras, required for vulval development [40]</td>
</tr>
<tr>
<td>lin-1</td>
<td>enhancer</td>
<td>Winged-helix transcription factor downstream of let-60 Ras, mutants are ~100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Muv [97]</td>
</tr>
<tr>
<td>egr-1</td>
<td>enhancer</td>
<td>MTA1 homolog, a synMuv gene [63,96]</td>
</tr>
<tr>
<td>egl-27</td>
<td>enhancer</td>
<td>MTA1 homolog, a synMuv gene, paralogous with egr-1 [63,96]</td>
</tr>
<tr>
<td>gap-2</td>
<td>enhancer</td>
<td>RasGAP [126]</td>
</tr>
<tr>
<td>dgk-2</td>
<td>enhancer</td>
<td>Diacyl glycerol kinase, a known enhancer of let-60(n1046) [9]</td>
</tr>
<tr>
<td>H37N21.1</td>
<td>enhancer</td>
<td>NRBPI pseudokinase homolog, a known enhancer of let-60(n1046) Muv</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phenotype [9]</td>
</tr>
</tbody>
</table>
Table 2.2: List of enhancers identified from the RNAi screens.

<table>
<thead>
<tr>
<th>Locus name</th>
<th>public name</th>
<th>RNAi % Muv</th>
<th>RNAi % enhancement</th>
<th>Validated as double mutant</th>
<th>Double mutant Percent Muv (n)</th>
<th>RNAi Muv in WT [24]</th>
<th>Single Mutant Muv (n &gt; 200) - allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>F26F4.7</td>
<td>nhl-2</td>
<td>97.49</td>
<td>91.72</td>
<td>Yes - Pos. [66]</td>
<td>92.65% (292)</td>
<td>No</td>
<td>No - ok818</td>
</tr>
<tr>
<td>F25D7.3</td>
<td>blmp-1</td>
<td>96.01</td>
<td>88.49</td>
<td>Yes</td>
<td>98.07% (589)</td>
<td>No</td>
<td>No - tm548</td>
</tr>
<tr>
<td>F58A4.11</td>
<td>gei-13</td>
<td>95.25</td>
<td>84.95</td>
<td>ND - NA</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>T27C4.4</td>
<td>egr-1</td>
<td>92.26</td>
<td>71.12</td>
<td>ND - Pos. [63]</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>F22F1.1</td>
<td>hil-3</td>
<td>86.12</td>
<td>62.40</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C32F10.5</td>
<td>hmg-3</td>
<td>88.9</td>
<td>62.33</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C09G4.4</td>
<td>mes-6</td>
<td>90.31</td>
<td>59.20</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C44F1.2</td>
<td>gmeb-3</td>
<td>83.98</td>
<td>49.94</td>
<td>ND - NA</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C44B9.4</td>
<td>athp-1</td>
<td>82.18</td>
<td>46.39</td>
<td>ND</td>
<td>ND</td>
<td>No</td>
<td>No - tm4223</td>
</tr>
<tr>
<td>C04A2.3</td>
<td>egl-27</td>
<td>82.3</td>
<td>45.13</td>
<td>ND - Pos. [63]</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C28H8.9</td>
<td>dpff-1</td>
<td>82.79</td>
<td>44.91</td>
<td>ND</td>
<td>ND</td>
<td>No</td>
<td>No - tm4287</td>
</tr>
<tr>
<td>F32E10.6</td>
<td>cec-5</td>
<td>81.75</td>
<td>42.42</td>
<td>ND - NA</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C09G4.5</td>
<td>mes-6</td>
<td>82.24</td>
<td>42.24</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C07G1.3</td>
<td>pet-1</td>
<td>74.15</td>
<td>63.60</td>
<td>ND - Linked</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>F59A6.1</td>
<td>nsy-1</td>
<td>75.52</td>
<td>59.92</td>
<td>No</td>
<td>65.23% (1404)</td>
<td>No</td>
<td>No - ok593</td>
</tr>
<tr>
<td>R12B2.1</td>
<td>sma-4</td>
<td>76.6</td>
<td>55.45</td>
<td>Yes</td>
<td>78.78% (665)</td>
<td>No</td>
<td>No - e729</td>
</tr>
<tr>
<td>ZK370.5</td>
<td>pdhk-2</td>
<td>70.14</td>
<td>48.12</td>
<td>Yes</td>
<td>81.72 (2605)</td>
<td>No</td>
<td>No - tm3075</td>
</tr>
<tr>
<td>C16B8.1</td>
<td>lin-18</td>
<td>69.53</td>
<td>46.32</td>
<td>No</td>
<td>30.51% (757)</td>
<td>No</td>
<td>Yes - e620</td>
</tr>
<tr>
<td>C32D5.2</td>
<td>sma-6</td>
<td>73.52</td>
<td>45.90</td>
<td>ND</td>
<td>ND</td>
<td>No</td>
<td>No - ok2894</td>
</tr>
<tr>
<td>T24F1.3</td>
<td>rsf-1</td>
<td>74.39</td>
<td>44.70</td>
<td>ND - NA</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>T05A6.2</td>
<td>cki-2</td>
<td>68.35</td>
<td>43.85</td>
<td>Yes, Pos. [64]</td>
<td>76.9 (1956)</td>
<td>No</td>
<td>No - ok2105</td>
</tr>
<tr>
<td>C04A11.3</td>
<td>gck-4</td>
<td>71.33</td>
<td>40.36</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C02F4.1</td>
<td>ced-5</td>
<td>66.39</td>
<td>36.10</td>
<td>ND - Linked</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>Y50D7A.2</td>
<td>xpd-1</td>
<td>65.97</td>
<td>34.08</td>
<td>ND - NA</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = no data. NA = no allele publicly available. Let = mutant allele has a lethal or sterile phenotype. Linked = gene is linked to let-60. Pos. = positive controls, which are genes that have been previously published as enhancers of let-60(n1046) Muv phenotype.

Table 2.3: Average percent Muv of blmp-1(tm548), let-60(n1046) and blmp-1(tm548);let-60(n1046) worms.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>20°C Average % Muv ± SD</th>
<th>n</th>
<th>16°C Average % Muv ± SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>blmp-1(tm548)</td>
<td>0.00 ± 0.00</td>
<td>272</td>
<td>0.00 ± 0.00</td>
<td>437</td>
</tr>
<tr>
<td>let-60(n1046)</td>
<td>77.5 ± 11.8</td>
<td>202</td>
<td>24.4 ± 19.0</td>
<td>721</td>
</tr>
<tr>
<td>blmp-1(tm548);let-60(n1046)</td>
<td>99.5 ± 1.00</td>
<td>589</td>
<td>89.7 ± 15.0</td>
<td>595</td>
</tr>
<tr>
<td>N2</td>
<td>0.00 ± 0.00</td>
<td>&gt;1000</td>
<td>0.00 ± 0.00</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>blmp-1(RNAi)</td>
<td>0.00 ± 0.00</td>
<td>923</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

SD = Standard deviation. n = total number of worms assayed. ND = no data.
Table 2.4: Suppressors of *let-60*(n1046) Muv phenotype identified in the RNAi screen.

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Public name</th>
<th>RNAi % Muv</th>
<th>RNAi % suppression</th>
<th>Validated as double mutant</th>
<th>Double mutant percentage Muv (n)</th>
<th>RNAi Muv detected in WT [24]</th>
<th>Single Mutant Muv (n &gt; 200) - allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>E03A3.3</td>
<td>his-59</td>
<td>25.69</td>
<td>-62.01</td>
<td>ND - NA</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C32F10.2</td>
<td>lin-35</td>
<td>30.15</td>
<td>-56.61</td>
<td>Yes</td>
<td>18.76% (3127)</td>
<td>No - n745</td>
<td></td>
</tr>
<tr>
<td>Y48A6C.5</td>
<td><em>pha-1</em></td>
<td>28.42</td>
<td>-57.25</td>
<td>Marginal*</td>
<td>50.63% (776)</td>
<td>No</td>
<td>No - fdl1, e2123</td>
</tr>
<tr>
<td>ZK63.6</td>
<td>dpy-30</td>
<td>30.42</td>
<td>-54.67</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C47D12.1</td>
<td><em>trr-1</em></td>
<td>17.78</td>
<td>-59.92</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>F23F1.1</td>
<td><em>nyc-1</em></td>
<td>30.64</td>
<td>-55.04</td>
<td>ND</td>
<td>No</td>
<td>No - tm4264</td>
<td></td>
</tr>
<tr>
<td>F37A4.8</td>
<td>isw-1</td>
<td>41.56</td>
<td>-32.96</td>
<td>ND - Pos. [94]</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>F45E1.6</td>
<td>his-71</td>
<td>21.42</td>
<td>-53.42</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>R08C7.3</td>
<td><em>hrz-1</em></td>
<td>34.16</td>
<td>-49.02</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>Y116A8C.22</td>
<td>athp-3</td>
<td>42.90</td>
<td>-36.59</td>
<td>ND - Linked</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>F21G4.3</td>
<td><em>phf-34</em></td>
<td>41.85</td>
<td>-36.29</td>
<td>ND - NA</td>
<td>No</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>K03A1.6</td>
<td><em>his-50</em></td>
<td>28.34</td>
<td>-32.05</td>
<td>ND - NA</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>M03C11.1</td>
<td>M03C11.1</td>
<td>31.94</td>
<td>-42.85</td>
<td>No</td>
<td>51.83% (2488)</td>
<td>No</td>
<td>No - tm4066</td>
</tr>
<tr>
<td>Y18D10A.19</td>
<td>fkb-2</td>
<td>37.70</td>
<td>-34.45</td>
<td>ND</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C10H11.8</td>
<td>C10H11.8</td>
<td>28.73</td>
<td>-42.96</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C36A4.8</td>
<td><em>brc-1</em></td>
<td>32.25</td>
<td>-29.87</td>
<td>No</td>
<td>51.71% (1456)</td>
<td>No</td>
<td>Yes** - tm1145</td>
</tr>
<tr>
<td>R03G5.2</td>
<td><em>sek-1</em></td>
<td>38.95</td>
<td>-22.93</td>
<td>ND</td>
<td>No</td>
<td>No – km4</td>
<td></td>
</tr>
<tr>
<td>Y53G8AR.3</td>
<td><em>ral-1</em></td>
<td>39.72</td>
<td>-25.29</td>
<td>ND - Let</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>F14B4.2</td>
<td>F14B4.2</td>
<td>33.03</td>
<td>-46.14</td>
<td>ND - NA</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C32F10.2</td>
<td><em>lin-35</em></td>
<td>34.68</td>
<td>-31.87</td>
<td>Yes</td>
<td>18.76% (3127)</td>
<td>No</td>
<td>No - n745</td>
</tr>
<tr>
<td>Y41D4B.13</td>
<td><em>ced-2</em></td>
<td>43.14</td>
<td>-16.44</td>
<td>ND</td>
<td>ND</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>C12D8.10</td>
<td><em>akt-1</em></td>
<td>39.28</td>
<td>-20.35</td>
<td>Yes</td>
<td>12.97 (1928)</td>
<td>No</td>
<td>No - mg306</td>
</tr>
<tr>
<td>T28A8.7</td>
<td><em>mlh-1</em></td>
<td>28.09</td>
<td>-32.76</td>
<td>No</td>
<td>63.94 (1091)</td>
<td>No</td>
<td>Yes** - ok1917</td>
</tr>
</tbody>
</table>

ND = no data. NA = no allele publicly available. Let = mutant allele has a lethal or sterile phenotype. Linked = gene is linked to *let-60*. Pos. = positive controls, which are genes that have been previously published as suppressors of *let-60*(n1046) Muv phenotype.

*At permissive temperature, *pha-1*(2123ts) validated as a marginally significant weak suppressor, with 24.69% suppression relative to *let-60*(n1046) on the same day, with two-way t-test p = 0.05.

** Because BRCA homolog *brc-1* and MLH homolog *mlh-1* both function in DNA repair, mutations in these genes result in increased spontaneous mutation rates. Therefore, it is likely that the Muv phenotypes in these mutant backgrounds are caused by spontaneous mutations in other genes rather than from *brc-1* and *mlh-1* mutations themselves. Correspondingly, the Muv phenotypes of these worms appear in some clones of worms that were isolated, and not others, suggesting that these may resulted from spontaneous mutations.
Chapter 3

Characterization of *C. elegans blmp-1* as an attenuator of Ras signaling

This work is unpublished. I performed all the work associated with this chapter.
Chapter 3
Characterization of C. elegans blmp-1 as an attenuator of Ras signaling

3.1 Summary
The PRDI-BF1/PRDM1/Blimp1 ortholog blmp-1 was identified as a genetic enhancer of the activated Ras Muv phenotype in C. elegans in an RNAi screen (Chapter 2). The blmp-1 single mutant is not Muv. blmp-1 mutant worms have wildtype vulval cell division but abnormal vulval morphogenesis. However, blmp-1(tm548);let-60(n1046) worms are 100% Muv, and each worm has more and larger ectopic pseudovulvae than let-60(n1046) worms. Since loss of blmp-1 enhances the gain of function Muv phenotype of let-60(n1046), blmp-1 may be an attenuator of vulval induction signals or may act in parallel pathway to negatively regulate vulval cell induction.

PRDI-BF1/PRDM1/Blimp1 was previously characterized as a marker of B cell terminal differentiation and a tumor suppressor gene in activated-B cell diffuse large B cell lymphoma (ABC-DLBCL) [13,14,99]. While B-cell signaling is a Ras-dependent process and activated Ras may be a driver mutation in a subset of ABC-DLBCL [127], Blimp1 was never characterized as a Ras-cooperating cancer gene.

Since this study was the first characterization of blmp-1 in the context of activated Ras, in this chapter, I sought to understand how blmp-1 interacts with Ras and the EGF-RTK-Ras-MAPK pathway. I found that blmp-1(tm548) attenuated the reduced vulval induction phenotype in EGF-RTK-Ras-MAPK signaling Vul mutants, but did not completely restore vulval induction to wildtype levels. In addition, blmp-1(tm548) resulted in increased MAPK activation, as evidenced by ectopic egl-17 expression. Next, I examined spatial and temporal expression pattern of blmp-1 and found that blmp-1 expression is downregulated in daughters of induced VPCs. Finally, I tested different hypotheses on how loss of blmp-1 enhances let-60(n1046) phenotype. First, I carried out an RNAi screen for suppressors of blmp-1(tm548);let-60(n1046) Muv phenotype to potentially identify key downstream targets of blmp-1. These suppressors may be required for the high penetrant Muv phenotype of blmp-1(tm548);let-60(n1046). The
RNAi screen identified the transcription factor *pax-3* as one such gene, as well as *lin-39* and several other moderate suppressors. Second, I examined how *blmp-1* is downregulated in daughters of induced VPCs, and whether that is necessary and sufficient for vulval or pseudovulval induction.

I found that *blmp-1* is expressed in multiple tissues in addition to the vulva and *blmp-1* worms have multiple phenotypes, suggesting that *blmp-1* has pleiotropic functions. Consistent with the finding that *blmp-1* loss of function enhances activated Ras phenotype, multiple lines of evidence suggest that *blmp-1* is a weak attenuator of EGF-RTK-Ras-MAPK signaling. *blmp-1* expression is downregulated upon VPC induction downstream of Ras in the vulval cells, and this downregulation is important for wildtype vulval development. This downregulation in vulval cells is likely to be through transcriptional regulation via the *blmp-1* promoter rather than by miRNA.

In conclusion, this study was the first characterization of *blmp-1* in the context of activated Ras, which may shed light on our understanding of tumors which are driven by loss of PRDM1, such as ABC-DLBCL.

### 3.2 Introduction

#### 3.2.1 *blmp-1* encodes an ortholog of PRDM1/PRDI-BF1/Blimp1, a highly conserved protein with highly conserved regulatory circuits

PRDM1 or PRDI-BF1 (humans), Blimp1 (mice, zebrafish and flies), and *blmp-1* (worms) encode a ca 800 amino acid transcription factor. It has a SET domain and 5 Krüppel-type C2H2 Zinc fingers (Figure 3.1A). It has been speculated that Blimp1 represses transcription by recruiting other transcription factors because experimental evidence thus far fail to detect its intrinsic methyltransferase activities [128]. Among other transcription factors that it recruits include Groucho proteins (TLE1/TLE2, Grg5), G9a and HDAC1 [128-130]. Only the first two zinc fingers are required for DNA binding [131].
3.2.2 All *blmp-1* metazoan orthologs have pleiotrophic functions, mainly to orchestrate gene expression for cell fate specification.

Orthologs of *blmp-1* (henceforth referred to as Blimp1) are highly conserved in metazoans. In all model systems characterized, Blimp1 has dynamic expression patterns and pleiotropic functions. The expression of this gene typically signifies cell fate specification. In a variety of vertebrate systems, Blimp1 functions in smooth muscle cell fate specification and limb bud development [132-134]. In addition, in mouse models, Blimp1 functions to specify the fates of primordial germ cells (PGC) and their migrations [134]. In *Drosophila*, Blimp1 specifies ecdysone-mediated development and terminal tracheal differentiation [135,136].

The best-characterized roles of Blimp1 are in the immune system, including as a key transcription factor in the terminal differentiation of B cells to become antibody-secreting plasma cells [137]. Blimp1 levels are upregulated as B cells mature into antibody-secreting plasma cells after B-cell receptor (BCR) activation by antigens. B cells and plasma cells have distinct gene expression programs. Transcription factors that are active in B cells, such as Myc, Pax5, CIITA and Ets1, are pro-growth transcription factors and are direct repression targets of Blimp1. Activation of these B cell-specific transcription factors lead to cell division and suppress terminal differentiation; whereas, transcriptional regulation in plasma cells represent growth-suppression and lead to cell fate commitment. Gene expression in B cells keep the cells ready to respond to antigenic signaling to the BCR by cell division (clonal expansion) rather than to terminally differentiate into plasma cells. In the absence of BCR activation, Pax5 represses Blimp1 expression, thus maintaining the B cell fate [138]. Upon BCR activation by a compatible antigen, MAPK cascade is activated leading to Pax5 phosphorylation by ERK1/2, eventually derepressing Blimp1 expression and allowing the initiation of plasma cell gene expression program [139,140]. Activated B cells differentiating into lymphoblasts express intermediate levels of Blimp1, Pax5 and other proliferative genes undergo rapid proliferation. Subsequently, Blimp1 levels rise to repress expression of many B-cell transcriptional regulators, including Myc and Pax5, rendering the cells postmitotic and committed to the terminal plasma cell fate [99].
3.2.3 The paradoxical roles of Blimp1 in two distinct hallmarks of cancer: antiproliferation and metastasis-promoting can be both modeled with two different phenotypes in C. elegans

Blimp1 seems to contribute differently to two different hallmarks of cancer, depending also on the tissue of origin of the cancer. Blimp1 generally promotes terminal cell fate and prevents uncontrolled proliferation in response to cell-fate specifying extracellular signals. Therefore, loss of Blimp1 function may contribute to the first hallmark of cancer, which is the self-sufficiency in growth signals. Paradoxically, since Blimp1 is also important for correct survival and migration of PGCs, its expression appears to be important for metastatic potential of some cancers.

3.2.3.1 Blimp1 is a tumor suppressor gene that may cooperate with Ras in white blood cell malignancies

In lymphoid malignancies, Blimp1 is a tumor suppressor gene [83]. Previous reports showed that loss of function mutations of Blimp1 were found in up to 24% of ABC-DLBCL, which is the more common but less curable subtype of non-Hodgkin Lymphomas [13,141]. In a comprehensive profiling of Blimp1 mutations in DLBCL, perturbations of Blimp1 were detected in up to 53% of ABC-DLBCL [14]. The same study also found that mice deficient in Blimp1 specifically in B cells developed lymphoproliferative disorders reminiscent of ABC-DLBCL. Although this demonstrated that loss of Blimp1 is likely a driver mutation of DLBCL, the authors of this study believed that the latency and clonality of the cancer indicated that Blimp1 on its own was not sufficient to drive tumorigenesis [14]. Rather, additional mutations were acquired as the lymphoproliferative disorder developed into lymphoma. Mutations that inactivated the NF-κB pathway and FOXP1 may be driver mutations that cooperate with Blimp1 perturbation [142]. In addition to B cells, Blimp1 was shown to function as a tumor suppressor gene in natural killer cell lymphomas and anaplastic large T-cell lymphomas [143-145].

BCR activation, a Ras dependent process, initially results in proliferation of activated B cells before Blimp1 is upregulated as the cells differentiate into plasma cells [137]. In addition, acute perturbation of Blimp1 promoted B cell proliferation [142]. However, the tumorigenic
cooperation between Blimp1 and activated Ras in blood cancers have not been elucidated. Activated Ras may be a driver mutation in a small subset of ABC-DLBCL [127]. Furthermore, drugs that target Ras signaling are being used in the treatments of DLBCL [146]. Therefore, elucidating how perturbation of Blimp1 may cooperate with activated Ras may shed light on diagnosis, treatment and prognosis determination of ABC-DLBCL.

3.2.3.2 Perturbations of Blimp1 cooperates with oncogenic Ras to drive metastasis in many epithelia-derived Ras-driven cancers.

Interestingly, in oncogenic Ras-driven epithelial-derived cancers such as lung and breast cancers, the roles of Blimp1 appear to be contrary to its roles in leukemias and lymphomas. For epithelial-derived cancers, metastatic spread represents poorer prognoses and is typically the cause of cancer deaths. Blimp1 expression in epithelial-derived cancer cell lines seems to be important for their metastatic phenotypes in combination with oncogenic Ras. The Sonenshein group demonstrated that Blimp1 expression is upregulated downstream of Ras signaling through AP-1 transcription factors in glioma, lung, and pancreatic cancer cell lines; and higher expression of Blimp1 correlated with more metastatic phenotypes [147,148]. It is possible that Blimp1 has regulatory targets that are important for cell migration, since it is important for primordial germ cell migration during embryonic development [134]. However, these studies showing that Blimp1 is upregulated in metastatic cells were carried out using cancer cell lines [147,148]. In addition, although Blimp1 was upregulated in these cell lines, they did not test whether the upregulated Blimp1 proteins had wildtype functions. Therefore, additional in vivo evidence would be required to confirm the roles of Blimp1 in metastasis and perhaps poorer prognoses for these tumors. Although Blimp1 has roles during mammary gland development, it was not shown whether the re-activation of Blimp1 expression as a driver mutation occurs early or late in the carcinogenesis process [147]. Thus, much about roles of Blimp1 in epithelial-to-mesenchymal transition and metastasis in epithelial cancers remains to be elucidated.

In *C. elegans*, the roles of *blmp-1* in cell migration are evidenced in its distal tip cell (DTC) migration defective phenotype. Each *C. elegans* gonad has a single distal tip cell, whose migration is spatiotemporally regulated [149]. Distal tip cells not only have to sense the right matrix cues, but also have to match with the developmental timing of the rest of the animals [150]. BLMP-1 functions to inhibit precocious *unc-5* transcription so *blmp-1(lf)* mutant worms
have precocious DTC migration due to precocious and increased unc-5 expression. BLMP-1 protein level is downregulated by the F-Box protein DRE-1, zinc finger transcription factor LIN-29 and steroid hormone receptor DAF-12. In wildtype worms, this downregulation of BLMP-1 protein levels leads to de-repression of unc-5 transcription [149]. Given that the genes and processes involved in DTC migration of unc-5 transcription are conserved between worms and mammals, it is possible that characterization of blmp-1 functions will shed light on the roles of Blimp1 in cell migration or metastasis.

3.2.3.3 The parallels between B-cell receptor signaling and vulval induction

Both B-cell differentiation and vulval development are Ras-dependent processes where extracellular signals trigger cell fate determination and cell proliferation before post-mitotic terminal differentiation. Low levels of Blimp1 expression correlate with a proliferative, multipotent cell fate. Whereas increased Blimp1 expression typically signifies an antiproliferative and terminally differentiated cell fate, which occurs after the cell receives extracellular growth signals.

Prior to vulval induction, gene expression program in VPCs maintains the VPCs’ multipotency. The VPCs can assume three different cell fates depending on whether or not it receives extracellular pro-growth LIN-3 (EGF) signals from the anchor cell. Once the cells receive the inductive signal, they undergo cell divisions and terminal differentiation according to their assumed cell fate. Similarly, B cells are potent to proliferate upon receiving B cell receptor signal from a compatible antigen before differentiating into plasma cells. Blimp1 is not expressed in B cells and proliferating activated B cells, but is specifically upregulated as the cells differentiate into postmitotic plasma cells [137]. Loss of Blimp1 alone may not be sufficient to drive tumorigenesis in ABC-DLBCL [14], just as blmp-1 single mutants in C. elegans are not Muv. However, loss of Blimp1 in combination with other driver mutations can drive aggressive ABC-DLBCL and blmp-1;let-60 worms are 100% Muv.

3.2.3.4 Blimp1 and apoptosis

In mammals, the role of Blimp1 in apoptosis depends on the cell type and where they are in the
developmental process. If Blimp1 is absent when it is needed, then the cells undergo apoptosis. If Blimp1 is upregulated when it is not needed, the cells also undergo apoptosis. Blimp1 mutant embryos undergo massive apoptosis, especially in mesenchyme cells, which typically express high levels of Blimp1 [134]. During B-cell development, ectopic expression of Blimp1 before the cell is ready results in apoptosis. In plasma cell tumors such as in multiple myeloma, however, loss of Blimp1 results in apoptosis [151]. These apoptosis events are more likely the apoptotic cleanup of cells that are incorrectly specified or incorrectly divide rather than as a direct function of Blimp1. However, Yan et al (2007) reported that Blimp1 and TP53 reciprocally regulates each other’s transcription in an autoregulatory feedback loop in HCT116 colorectal cancer cell line [152]. Since the mechanisms and molecular circuitry of how each cancer clone acquires different hallmarks of cancer may be different, much about roles of Blimp1 in apoptosis remains to be elucidated.

3.2.4 Post-transcriptional regulation of Blimp1 may also be highly conserved.

Not only is Blimp1 highly conserved between worms and mammals, but the regulatory circuitry surrounding Blimp1 function, such as its transcription binding motifs and miRNA regulation, appears to be remarkably conserved. Blimp1 binding motif or the PRDI site, as determined by ChIP experiments are nearly perfect complementarity between C. elegans and mammals [140,153] (Figure 3.1B and C). Blimp1 is a known miRNA target and disruption of this miRNA regulation has been shown to contribute to lymphomagenesis [154,155]. Blimp1 is a target of miR-9 and let-7a in a Hodgkin lymphoma cell line and overexpression of these miRNAs result in lower levels [155]. Nie and colleagues also showed that Blimp1 is a target of let-7 family miRNAs and overexpression of let-7 is found in DLBCL [154].

In C. elegans, combined computational and biochemical miRNA site prediction suggest that blmp-1 is a heterochronic gene that may be a miRNA target [149,156,157]. Notably, there are four let-7 family miRNA predicted seed regions within the 3’UTR of blmp-1 (Table 3.1) [156]. In addition, blmp-1 is a target of DRE-1 mediated ubiquitination both in worms and mammals [157].
3.2.5 *blmp-1* has pleiotropic functions in *C. elegans*.

In *C. elegans*, *blmp-1* was also named *odd-3* (Drosophila odd-skipped like) and *dpy-24*. *blmp-1* single mutants have dumpy, darker in color, defective distal tip cell migration, abnormal vulva eversion and uncoordinated movement phenotypes, suggesting that *blmp-1* has pleiotropic functions. Based on these phenotypes, *blmp-1* may have roles in dosage compensation, collagen production, spatiotemporal cell migration, fat metabolism as well as neuromuscular development. Despite having multiple important roles, this gene was relatively uncharacterized in *C. elegans* until the first modENCODE dataset, which included results from BLMP-1 ChIP-Seq data, was released in 2009. BLMP-1 was included among the first 23 transcription factors to be assayed for binding sites by the modENCODE project and it stood out as the transcription factor with the most binding sites with 6833 peaks across the *C. elegans* genome [153]. In addition, BLMP-1 binding motif (TTTCACTTT) has the highest occurrence among all transcription factor binding motifs predicted by the initial modENCODE study in 2010. In 2015, the modENCODE project has assayed 300 *C. elegans* transcription factors and found that some transcription factors have more binding sites than BLMP-1.

Consistent with its pleiotropic roles, *blmp-1* is dynamically expressed in many cell types. These include the vulval, hypodermal cells, seam cells, intestinal cells, the somatic gonads, tail cells, pharyngeal cells, head neurons and hypodermis cells, as well as in the embryos [157,158].

*blmp-1* has been characterized as a late-functioning heterochronic gene in distal tip cell migration and hypodermal cells, including alae formation [149,157]. Expression profiling by RNA-Seq of whole worm *blmp-1* mutants showed an enrichment of genes that function in molting and cuticle development, sugar and aminosugar metabolism, and protein modification [157]. Consistent with its role in hypodermal cell development, *blmp-1* worms were found to have defective mail tail morphogenesis phenotype due to precocious initiation of the tail morphogenesis in L3 [159]. Interestingly, *blmp-1* worms were also observed to have pharyngeal muscles that do not attached to each other, and to be hypersensitive to hypoxia [160,161].
3.2.5.1 Previous characterizations of \textit{blmp-1} functions in vulval development

\textit{blmp-1} Evl phenotype may be partly due to heterochronic defects in seam cell and gonad development [149,157]. In addition, the zinc finger transcription factor \textit{bed-3} was described as a key regulatory target of \textit{blmp-1} [162]. \textit{bed-3} encodes a zinc-finger transcription factor that is required for the third and final round of vulval cell division. \textit{bed-3} worms have a Pvl phenotype due to reduced vulval cell division [163].

\textit{SynMuv} genes are defined by their genetic interactions: mutations in these genes in combination with a \textit{synMuv} gene from a different group result in Muv worms (see Chapter 1). Because \textit{blmp-1} encodes a nuclear protein with a SET domain, it was included in comprehensive surveys for genes that interact with \textit{synMuv} genes [8]. Because SET domain proteins typically have histone methyltransferase (HMT) activities, \textit{blmp-1} was included in a comprehensive survey of 38 \textit{C. elegans} HMTs for their roles in vulval development and found that \textit{blmp-1} was not a \textit{synMuv} gene, nor was it a \textit{synMuv} suppressor [164].

3.2.6 Open questions and goals for this chapter

\textit{blmp-1} was identified in my RNAi screen for genetic enhancers of activated Ras Muv phenotype. I found that \textit{blmp-1} loss of function was a strong genetic enhancer of \textit{let-60(n1046)} Muv phenotype. \textit{blmp-1} on its own, although slightly protruded, does not have Muv phenotype. This is congruent with the tumor suppressor role of Blimp1 in ABC-DCLBL, that lymphomagenesis requires other driver mutations to cooperate with the loss of Blimp1 [14]. However, Blimp1 as an anti-proliferative tumor suppressor gene was never elucidated in cooperation with activated Ras, although it has been characterized as a pro-metastatic gene in Ras-driven epithelial cancers [147,148].

Interestingly, although \textit{blmp-1} is a transcription factor that seems to function as an attenuator of Ras signals in \textit{C. elegans}, it is not a \textit{synMuv} gene or a \textit{synMuv} suppressor. For this thesis, I focus on understanding how loss of \textit{blmp-1} cooperates with gain of function Ras and how \textit{blmp-1} functions in vulval development. Key questions include 1) how and where \textit{blmp-1} is expressed, and whether \textit{blmp-1} functions cell autonomously in vulval development 2) how
blmp-1 interacts with Ras or other pathways in vulval development 3) whether there are key downstream transcriptional regulation targets of blmp-1 in vulval development, and 4) how blmp-1 functions are regulated in coordination with Ras signaling. Because several aspects of blmp-1 functions are remarkably conserved between worms and mammals, it is likely that these findings will shed light into our understanding of how Blimp1 may function as a Ras-cooperating driver gene.

3.3 Materials and Methods

3.3.1 RNAi screening and assays

RNAi screening and assays are carried out as described in Chapter 2 using the Ahringer RNAi library of 16,757 RNAi clones, which covers ~90% of the C. elegans genome [72,74]. RNAi suppressor screen for blmp-1(tm548);let-60(n1046) Muv phenotypes are carried out in 12-well plates, whereas RNAi assays for microscopy are carried out in 6-cm or 12-cm plates using the same protocol as described in Chapter 2.

3.3.2 Worm strains

Mutant strains MT2124 let-60(n1046), MT4866 let-60(n2021), PS427 lin-45(sy96), CB1275 lin-1(e1275), MT301 lin-31(n301), HT1593 unc-119(ed3), NL746 pax-3(pk223), CB262 unc-37(e262), PS1839 let-23(sa62), MT2123 let-23(n1045), MT8189 lin-15A(n765), MT1806 lin-15A(n767)X, MT2495 lin-15B(n744)X and reporter strains PS3525 syIs59[egl-17::cfp + unc-119(+)], OP109 wgIs109[(WRM0622cE11)blmp-1::TY1::EGFP::3xFLAG + unc-119(+)], VT1153, VT1160, VT1189, VT1259 and VT1665 were obtained from the C. elegans Genetics Center. FX548 blmp-1(tm548) and FX1771 pax-3(tm1771) were obtained from the National Bioresource Collection (Japan). Worms were cultured and maintained at 22°C according to standard protocol [165]. Strain constructions were followed by either fluorescent reporter signal or PCR.

3.3.3 Microscopy

Observation of hermaphrodite phenotypes under the microscope using Nomarski optics was carried out as previously described [68]. Animals were mounted on 2% agarose pads melted in
distilled water. Worms were anesthetized in 10 μL of 1-10 mM sodium azide solution before imaging. All phenotypes were scored with the 40x objective lens. Worms were imaged at room temperature for fluorescent protein expression with Leica DMRE Epifluorescence/Phase/DIC microscope. Images were taken with the Hamamatsu ORCA-ER C4742-95 Camera with the HCI Image Live 3.0 program.

3.3.4 Generation of reporter transgenic constructs

Andy Fire plasmids, pRF4, pBlueScriptKS, as well as pPRRF162 and pCFJ90 (P\text{myo-3}::dsRED and P\text{myo-2}::mCherry, respectively) were generous gifts from Dr. Peter Roy. pKH22, which is a derivative of pB255, containing the complete ~9 kb lin-31 promoter sequence, 2 multiple cloning sites, an artificial intron and unc-54 3’UTR, was a generous gift from Meera Sundaram [166]. ORFeome pDONR-cDNA plasmids for \text{blmp-1}, \text{pax-3} and \text{unc-37} were obtained from Geneservice (UK). The plasmid pMM016 [167], which expresses \text{unc-119}, was a generous gift from the laboratory of Dr. Jim Dennis. The construction of individual plasmids is described in Supplementary Table 3. All PCR-amplified constructs were verified by DNA sequencing through the Center of Applied Genomics at SickKids Hospital (Toronto, ON).

All microinjections were performed using the Eppendorf Femtojet™ Microinjector and Femtotips™ Microinjection capillary tips. At least two independent transgenic lines were examined for each construct.

3.3.4.1 \text{blmp-1} transcriptional reporters

These reporters generated as described in Supplementary Table 3. PCRs to generate transcriptional reporters were carried out using Takara ExTaq. The PCR protocol used was 94°C for 30s; 98°C for 10 s, 52°C for 30s, 72°C for 1 min/kilobase of PCR product for 25 cycles; and 72°C for 15 minutes. All constructs generated by PCR were verified by DNA sequencing through the Centre for Applied Genomics at SickKids Hospital. For \text{blmp-1} promoter constructs, 5 – 10 ng of the reporter plasmids were co-injected with 5 ng/ul of pMM#016, 0.5 ng/ul pNW36 (dpy-7::dsRed) and pKS to make up to 120 ng/ul total DNA concentration into HT1593 \text{unc-119(-)} worms. For \text{pes-10Δ} minimal promoter constructs, up to 40 ng of the reporter plasmids were used in order to achieve visible fluorescent signals. In
addition to *unc-119* rescue, red signals from pNW36 were followed to ensure the presence of the transgene in the cells of interest.

### 3.3.4.2 Fosmid recombineering

All fosmid reporter constructs were generated using fosmid recombineering as previously described [168] to generate C-terminally tagged BLMP-1 and PAX-3 with fluorescent epitopes. Fosmids WRM0635aE10 and WRM067aB09 were used for *blmp-1* and *pax-3* respectively. A similar protocol is also done on the GFP-recombineered fosmid to swap out *blmp-1* 3’UTR with *unc-54* 3’UTR, to delete miR-84 miRNA site in *blmp-1* 3’UTR and to delete putative *blmp-1* binding site (II:10146449..10146862) in the *pax-3* promoter. 0.5 – 1 ng/ul of fosmids was coinjected with 5 ng/ul pMM#016 and pKS up to 120 ng/ul total DNA concentration into HT1593 *unc-119(-)* worms, except *pax-3::gfp* fosmid (1 ng/ul) was coinjected with 1 ng/ul of pRF4 and pKS up to 120 ng/ul into N2 worms.

### 3.3.4.3 *in vivo* recombination reporters

pNW8 and pNW9 contain ~2 kb sequence of *lin-31* promoter, which is not sufficient to drive expression in the vulva (Meera Sundaram, personal communication). In order to generate constructs expressing a cDNA in the vulva, additional intronic sequence of *lin-31* promoter is needed. Therefore, I generated P<sub>*lin-31*::ORF</sub> reporters using *in vivo* recombination (Figure 3.2). pKH22 was linearized with NcoI to expose ends with more than 300 bp homologous to pNW8 and pNW9 that were linearized with an enzyme that digest within *lin-31* promoter. pNW8 was digested with EcoRV and ApaI to isolate the 2.5 kb band containing P<sub>*lin-31*::gfp::unc-54 3’UTR</sub> band. pNW9 was digested with NsiI and EagI, and separated with agarose gel electrophoresis to isolate the 5034 bp band containing P<sub>*lin-31*::BLMP-1::gfp::unc-54 3’UTR</sub>. Linearized pKH22 and the isolated bands were co-injected at 10 ng/ul each, together with 0.5 ng/ul of pNW36, 5 ng/ul of pMM#016 and pKS up to 120 ng/ul. Transgenic lines with successful *in vivo* recombination events to generate vulval-specific expression transgenes were followed by GFP expression in the vulva at 1- and 2-cell stage.
3.3.4.4 Integration of worms carrying extrachromosomal arrays

High transmission transgenic lines carrying pNW5, pNW6 and pNW43 were integrated using UV irradiation as previously described [169]. For \textit{pax-3::gfp} fosmid and \textit{pax-3::mChOpti} fosmids, low transmission transgenic lines were integrated into the genome as previously described [170]. Stably integrated lines were then outcrossed at least 6 times before use in genetic analysis experiments.

3.3.5 Genetics

3.3.5.1 Generation of double mutants

Bristol N2 strains, \textit{blmp-1(tm548)} or PD4792 worms (for crosses with \textit{let-60} or other mutants on LG IV) were heat-shocked to generate males by placing parafilm-wrapped plates in 30°C water bath for 6 – 8 hours. For strains that were male mating defective and/or vulvaless, wildtype males were crossed to the strains with less than 100% penetrant vulval defects prior to mating. Transgenes with GFP expression were followed by fluorescent microscopy to ensure transmission of the GFP transgenes. Deletion mutations were followed using single worm PCR. Point mutations were followed by single worm PCR and DNA sequencing through the Center for Applied Genomics at SickKids Hospital. DNA primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) to generate PCR products under 1.2 kb and checked for no duplicate PCR products the \textit{C. elegans} genome using Wormbase BLAST.

To prepare templates for single worm PCRs, each genotyped worm was placed in 12 μl of single worm lysis buffer (50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM MgCl2, 0.45% Nonidet P40, 0.45% Tween 20, 0.01% Gelatin, and 0.12 mg/ml Proteinase K) in a PCR tube, which was then incubated at -80°C for 10 min, 60°C for 60 min and 95°C for 15 min to lyse the worms. Subsequently, the worm lysate was added as template as 1:10 dilution in genotyping PCR reactions. For all genetic crosses, parental strains were always lysed and genotyped in parallel as positive controls.

3.3.5.2 Balancing of \textit{pax-3(tm1771)}

Since \textit{pax-3(tm1771)} worms are embryonic or larval lethal, the balancer strain DR1785 \textit{mIn1[dpy-10(e128)]/unc(e120) II} was crossed to worms heterozygous for \textit{pax-3(tm1771)} to
generate balanced strains heterozygous for \textit{mIn1} and \textit{pax-3(tm1771)}. Heterozygous worms have wildtype body length, and throw Dpy homozygous progenies.

### 3.3.5.3 Confirmation that the recombineered fosmids rescued the deletion mutant phenotypes

For \textit{blmp-1}, OP109 worms was heat-shocked to generate males and mated to \textit{blmp-1(tm548)}. The presence of \textit{blmp-1::gfp} fosmid was followed using both fluorescent microscopy and by PCR with oligos nw70 and nw71. After self-fertilizing for two generations, the homozygotic presence of \textit{tm548} deletion was confirmed with at least 12 F3 progenies still carrying the deletion.

For \textit{pax-3}, balanced heterozygous \textit{pax-3(tm1771)/mIn1[dpy-10(e128)]II} worms, which appear to be superficially wildtype were injected with 1 ng/ul of \textit{pax-3::gfp} fosmid, 1 ng/ul of pRF4 and pKS up to 120 ng/ul. Non-Dpy Rol GFP(+) single transformants were then isolated until they no longer threw Dpy, then genotyped for \textit{tm1771} deletion using oligos nw72+nw73. Subsequently, to confirm that only GFP-tagged \textit{pax-3} is present, I sought to recapitulate \textit{pax-3} mutant phenotypes by feeding GFP(RNAi) bacteria to specifically knock down \textit{pax-3::gfp} transcript expressing from the recombineered reporter.

### 3.3.5.6. Phylogenetic footprinting

To identify transcription factor binding sites in highly conserved regions of \textit{blmp-1} promoter, I queried the Cis-BP database to identify transcription factor motifs [171]. The identified transcription factor motifs were then superimposed onto the 5-species DNA sequence alignment from the UCSD browser to identify highly conserved putative transcription factor binding sites.

### 3.4 Results

#### 3.4.1 \textit{blmp-1} is a novel genetic enhancer of activated ras phenotype in \textit{C. elegans}.

From the RNAi screen described in Chapter 2, I found that targeting \textit{blmp-1} by RNAi enhanced the activated Ras phenotype. While \textit{let-60(n1046)} worms are ~60 – 80% Muv, I found that let-
60(n1046);blmp-1(RNAi) worms were consistently over 90% Muv, and the double mutant let-60(n1046);blmp-1(tm548) were nearly 100% Muv (Figure 2.11 and Table 2.3). In addition, let-60(n1046);blmp-1(tm548) worms have higher degrees of vulval induction and more VPC cells induced than let-60(n1046) worms (Figure 3.3, Table 3.2). Therefore, blmp-1 loss of function enhances the activated ras phenotype in C. elegans.

3.4.2 blmp-1 has pleiotropic phenotypes.

I was able to acquire two different deletion alleles of blmp-1: tm548 and s71. Both strains and blmp-1(RNAi) worms have similar superficial phenotypes (Figure 3.4 for tm548 and data not shown for s71 and RNAi). blmp-1 worms are smaller, dumpy (shorter body), darker in color, have uncoordinated movements, distal tip cell migration defects and slow growth. I also observed that blmp-1 is expressed in vulval, hypodermal cells, seam cells, intestinal cells as well as head and tail neurons (Figure 3.5).

3.4.3 blmp-1 worms are not Muv but have Evl phenotype.

All blmp-1(tm548) worms observed do not develop ectopic pseudovulva (Table 3.2 and 2.3), but they do have a slightly protruded vulva (Figure 3.6), which can lay eggs a like wildtype vulva. This was also observed for blmp-1(RNAi), and blmp-1(s71) (data not shown). blmp-1(tm548) vulval phenotype is rescued with the transgene wgIs109 which harbours a functional copy of blmp-1::gfp (Figure 3.7). These blmp-1 worms have wildtype vulval cell division that result in 22 cells like a wildtype vulva. However, blmp-1 worms have somewhat abnormal vulval morphogenesis past mid-L4 stage, resulting in protruded vulva (Figure 3.6). Since the Pvl phenotype was originally coined for vulval developmental defects due to reduced vulval cell division, whereas the Everted vulva phenotype was coined to describe worms with abnormal vulval morphogenesis or attachment to the gonad [38,39]. Because blmp-1 have normal vulval cell division, I would characterize its vulval phenotype as due to vulval eversion defect (Evl) rather than due to reduced vulval cell division. An egg-laying defective (Egl) phenotype, typically characterized by accumulation of eggs inside the uterus, was not observed, although this could reflect a reduction of brood size. These phenotypes, together with other noted gross phenotypes (Dpy, Unc, darker color) are completely rescued in the presence of the fosmid-recombineered blmp-1::gfp transgene (Figure 3.7).
3.4.4 *blmp-1* is a weak attenuator of EGF-RTK-Ras-MAPK signaling.

In order to determine whether *blmp-1* attenuates EGF-RTK-Ras-MAPK signals, I examined expression of *egl-17*, whose expression is activated downstream of MAPK activation [57]. Typically, during vulval induction *egl-17* expression appears graded, being higher in the P6.p cell than in P5.p and P7.p cells. Later on during the 1-cell stage, the EGF-RTK-Ras-MAPK activation is counteracted by Notch signaling in P5.p and P7.p cells, such that *egl-17* reporter expression disappears in these cells and continues to be absent during 2-cell stage. Because EGF-RTK-Ras-MAPK signaling is not counteracted by Notch signaling in P6.p cell, *egl-17* reporter expression continues to be expressed only in P6.p daughters at 2-cell stage.

Perturbations of genes that attenuate EGF-RTK-Ras-MAPK signaling result in increased percentage of worms that ectopically express *egl-17* in P5.p or P7.p at 2-cell stage. Scoring increased frequency of ectopic *egl-17* expression in P5.p or P7.p cell has been commonly used to categorize genes or pathways as attenuators of MAPK signaling in vulval induction [9,43,75]. These attenuators include *lin-12* Notch and its downstream targets, as well as RasGAP *gap-1* [43]. Like *blmp-1*, many of these genes are not Muv on their own, but may be synthetic Muv with each other. In addition, these genes are genetic enhancers of *let-60(n1046)* Muv phenotype. Therefore, I examined vulval *egl-17::*gfp reporter expression in *blmp-1* mutants at 2-cell stage, in comparison to wildtype and *gap-1* deficient worms. I find that *blmp-1(tm548)* worms have 42% ectopic expression in comparison to wildtype (12%), but less than in *gap-1* worms which have 87% ectopic expression (Figure 3.8). Therefore, *blmp-1* worms have moderately increased percentage of worms with ectopic *egl-17* expression, suggesting that *blmp-1* is a weak attenuator of EGF-RTK-Ras-MAPK signaling.

Several of these EGF-RTK-Ras-MAPK attenuators are not Muv as single mutants, but are Muv in combination with each other. Therefore, I tested whether *blmp-1(tm548);gap-1(ga133)* are Muv. I find that very low percentage of *blmp-1(tm548);gap-1(ga133)* worms do exhibit Muv phenotype (<0.1%, Figure 3.9). Therefore, although *blmp-1* worms are not Muv, BLMP-1 weakly attenuates EGF-RTK-Ras-MAPK signaling.
The synMuv genes encode nuclear proteins that redundantly negatively regulate ectopic pseudovulval induction (described in Chapter 1). The three groups of synMuv genes: A, B and C are only synthetic Muv between groups, but not within groups. In order to test whether blmp-1 is a synMuv gene, I either targeted blmp-1 by RNAi in synMuv mutants or crossed blmp-1(tm548) with synMuv mutants (Table 3.3). As expected, known synMuv gene pairs resulted in penetrant Muv phenotypes. However, perturbation of blmp-1 in the presence of any synMuv mutations did not result in Muv worms, suggesting that blmp-1 is not a synMuv gene. This replicated the results previously published by Anderson et al (2007) [172]. Notably, I did observe that blmp-1(tm548);lin-15B worms have exaggerated cuticle defects and penetrance Egl phenotype (Figure 3.10), suggesting that blmp-1 may genetically interact with lin-15 in other developmental processes.

Collectively, these findings support that blmp-1 may weakly attenuates vulval inductive signaling either downstream of EGF-RTK-Ras-MAPK pathway, or as a member of another pathway. It likely does so in a manner that is different from how other known attenuators function. I then aimed to understand how blmp-1 functions in the vulva and how it interacts with Ras signaling.

3.4.5 Expression of blmp-1 in the vulva is downregulated upon vulval induction, possibly downstream of Ras signaling.

To gain insight into how blmp-1 is spatiotemporally regulated, I sought to characterize blmp-1 expression by examining transcriptional reporters and a fosmid-based reporter [173]. Because the C. elegans genome is relatively compact, most cis-regulatory elements of a gene lie within a few kilobase pairs of the start codon, so the inclusion of 2 – 3 kb in a transcriptional reporter typically suffices to characterize gene expression patterns [174]. Whereas, the fosmid reporter of blmp-1 (wgIs109) includes the entire blmp-1 locus, as well as 18.8 kb upstream and 7 kb downstream sequence. I found that the transcriptional reporter containing 2 kb upstream of the start codon exhibited similar downregulation patterns in P5.p – P7.p daughters as did the fosmid-based reporter, although the transcriptional reporter expressed much stronger overall signals, with a few other subtle differences (Figure 3.5). All 6 vulval precursor cells expressed the same levels of blmp-1 (Figure 3.5D). After the first cell division, at 2-cell stage, blmp-1
levels are downregulated in daughters of P5.p – P7.p cells (Figure 3.5F-G and 3.11). The downregulation is stronger in P6.p cells than in P5.p cells and P7.p cells, suggesting that this downregulation correlates with the degree of vulval cell induction (Figure 3.11). After the vulval cells complete three rounds of cell division, the fosmid-based reporter signals increase in the vulval cells and persist in the anchor cell (Figure 3.7H), but remain invisible for the transcriptional reporter (Figure 3.7I).

I also observed several other differences between blmp-1 transcriptional reporter and fosmid-based reporters. For example, in the fosmid-based reporter, I observed expression in the somatic gonad prior to the first vulval cell division (Figure 3.5D, asterisk) and the expression persists in the anchor cell in subsequent stages (Figure 3.5F-H). Seam cells and lateral hypodermal expression is quite weak from the fosmid reporter, typically only detectable in-focus with the confocal microscope (Figure 3.5C, orange triangles), but are very brightly visible from the transcriptional reporter (Figure 3.5E, orange triangles). Intestinal cells expression is detectable at a low level from the fosmid reporter, but is the most prominent signal from the transcriptional reporter (Figure 3.5E, G).

3.4.6 blmp-1 downregulation upon vulval induction is downstream of Ras activation.

Because blmp-1 levels are downregulated in the VPC daughters during 2-cell stage after vulval induction (Figure 3.11), it is possible that EGF-RTK-Ras-MAPK pathway is required for this downregulation. Therefore, I crossed these reporters to mutants in this pathway or targeted these genes by RNAi. These include Muv mutants let-60(n1046), lin-1(e1275) and lin-31(n301), and Vul mutants i.e. lin-39(RNAi), lin-3(RNAi), lin-45(RNAi) (Figure 3.12).

In all Vul worms, blmp-1 reporters are not downregulated in any of the 12 VPC daughters (Figure 3.12, E-G), suggesting that vulval induction and activation of the EGF-RTK-Ras-MAPK pathway is required for blmp-1 downregulation.

In let-60(n1046) worms, I observed that the downregulation of blmp-1 in induced VPC daughters correlated with vulval induction as well as ectopic pseudovulval induction (Figure
Let-60(n1046) worms have ~60 – 80% penetrant Muv phenotype where at least one of P3.p, P4.p or P8.p assume vulval cell fate and develop ectopic pseudovulvae (Figure 3.12A). The remaining 20 – 40% of worms seem to have only P5.p - P7.p assuming the vulval cell fate and develop wildtype vulva. For let-60(n1046) worms that would go on to develop wildtype vulva (n = 5), blmp-1 expression is only downregulated in P5.p – P7.p daughters, similarly to wildtype worms (Figure 3.12B and Figure 3.5G). Whereas, let-60(n1046) worms that would go on to develop ectopic pseudovulva from P3.p, P4.p or P8.p cells (n = 20), blmp-1 expression is downregulated in the corresponding VPC cell daughters (Figure 3.12A).

Lin-1 and lin-31 encode heterodimeric transcription factors that have been characterized as terminal effector of the vulval induction pathway, as the dimer is disrupted by MAPK phosphorylation of LIN-31 downstream of Ras activation [41]. lin-1(e1275) worms are 100% Muv at 20°C with all VPCs induced [175]. lin-31(n301) are ~66% Muv with smaller ventral protrusions and ~33% Vul because lin-31 has both positive and negative roles in vulval development [175]. I found that vulval expression of blmp-1 in both lin-31(n301) and lin-1(e1275) backgrounds is lower than in wildtype background, while expression in other tissues remain the same (Figure 3.12C and D, compare with Figure 3.5G). blmp-1 signals in lin-1(e1275) background were uniform across all 12 VPC cell daughters at 2-cell stage (Figure 3.12D). However, blmp-1 expression in lin-31(n301) background had rather non-uniform and punctate patterns regardless of the eventual vulval phenotype (n = 17 worms) (Figure 3.12C). Therefore, lin-31 and lin-1 may either be required for wildtype patterns of blmp-1 expression or blmp-1 downregulation.

In conclusion, blmp-1 downregulation requires vulval induction, which is dependent on EGF-RTK-Ras-MAPK activation. Because blmp-1(tm548) worms are not Muv, the downregulation of blmp-1 alone is not sufficient to induce vulval induction. In the next section, I test whether the downregulation of blmp-1 expression in the vulva is required for correct vulval induction.
3.4.7 Forced expression of blmp-1 in vulval cells result in reduced vulval induction, leading to Pvl phenotype

In order to determine whether the downregulation of blmp-1 is required for vulval induction and pseudovulval induction, I forced BLMP-1 expression in the vulva cells by driving expression of BLMP-1 cDNA C-terminally tagged with eGFP under vulval-specific lin-31 promoter ($P_{lin-31}::BLMP-1::GFP::unc-54$ 3’UTR). As a negative control, I used lin-31 promoter driving expression of the same eGFP expression ($P_{lin-31}::gfp::unc-54$ 3’UTR). With both reporters, I observed expression in the vulval cells (Figure 3.13).

$P_{lin-31}::gfp$ reporter signal was observed to express in all vulval cells (Figure 3.13C and D). $P_{lin-31}::BLMP-1::GFP::unc-54$ 3’UTR reporter gene product appears to have detectable nuclear-localized expression in the vulva up until late L4 stage, and the reporter signal became no longer detectable during adulthood of the same animal (Figure 3.13A and B). These differences in observed reporter signals were likely due to nuclear-localization and post-translational modification of BLMP-1, even though lin-31 promoter remains active. After observing several worms with these expression patterns (n = 12 in wildtype background and n = 25 in let-60(n1046) background), I found that $P_{lin-31}::BLMP-1::GFP::unc-54$ 3’UTR consistently reduced vulval induction, whereas worms carrying $P_{lin-31}::gfp$ consistently had wildtype vulva (Figure 3.14). Therefore, forced expression of blmp-1 in induced VPC cells clearly perturbs vulval development.

The transgene $P_{lin-31}::BLMP-1::GFP::unc-54$ 3’UTR did not decrease the penetrance levels of the Muv phenotype in let-60(n1046) in comparison to $P_{lin-31}::gfp$ (Figure 3.14B). However, I observed significantly decreased vulval cell division in the endogenous vulva of let-60(n1046) similarly to the patterns observed in wildtype background (Figure 3.14B).

Interestingly, in 3 out of 16 let-60(n1046);blmp-1(tm548) worms but not in blmp-1(tm548) or let-60(n1046) worms, I observed that some vulva cells may undergo another round of vulval cell division beyond the wildtype vulva (Figure 3.15, Table 3.2). Therefore, because over expression of blmp-1 reduced endogenous vulval cell division, and loss of function of blmp-1 in the presence of let-60(n1046) weakly increased the number of endogenous vulval cell
division, it is possible that *blmp-1* may have roles in the regulation of vulval cell division in the worms. However, since these subtle changes in cell division due to overexpression *blmp-1* did not abolish vulval induction, nor did loss of *blmp-1* result in Muv phenotype, it is likely that *blmp-1* functions late in the regulation of vulval development. In the next section, I continue to elucidate the genetic interactions between *blmp-1* and other components of RTK-EGFR-Ras signaling pathway.

### 3.4.8 Genetic interactions between *blmp-1* and mutants in EGFR signaling pathway.

To explore the genetic interactions between *blmp-1* and other components of the RTK-Ras-MAPK signaling pathway, I targeted *blmp-1* with RNAi in Muv mutants, including RTK *let-23(sa62 gf)*, ETS transcription factor *lin-1(e1275)* and Winged Helix transcription factor *lin-31(n301)* (Figure 3.16). With the exception of *lin-31(n301)* which is a null allele, these Muv mutants involve hypomorphic alleles and their Muv phenotypes are temperature sensitive (Table 3.4). Also, I generated compound mutants between *blmp-1(tm548)* and known vulvaless mutants, including *lin-3(e1417)*, *let-60(n2021)* and *lin-45(sy96)*.

As positive controls for *let-60(n1046)* Muv enhancer and suppressor, respectively, I also included *nrbp-1(RNAi)* and *lin-39(RNAi)* in this experiment. *nrbp-1*, an ortholog of NRBP-1, was previously identified in a similar RNAi screen for kinases that modify *let-60(n1046)* phenotype [9]. Perturbation of *NRBP1* appears to lead to perturbation of Wnt and Notch signaling pathways in mammals [9]. *lin-39* encodes Hox transcription factor responsible for VPC competency, which is upstream of vulval induction [176].

#### 3.4.8.1 *blmp-1(RNAi)* weakly enhanced the Muv phenotype of *let-23(sa62 gf)* but not *lin-1* or *lin-31*

While *blmp-1(RNAi)* significantly enhanced the penetrance of *let-60(n1046)* Muv phenotype, it did not significantly enhance *lin-1(e1275)* and *lin-31(n301)* Muv phenotypes at 20°C (Figure 3.16A, B and C). However, *blmp-1(RNAi)* very weakly enhanced the Muv phenotype of *let-23(sa62 gf)* (Figure 3.17D).
**nrbp-1(RNAi)**, however, marginally enhanced the Muv phenotype of **let-23(sa62)**, and only slightly enhanced the Muv phenotype of **lin-1(e1275)** (Figure 3.16D). These differences may be because **blmp-1** has stronger RNAi phenotype, than **nrbp-1** or that **blmp-1** and **nrbp-1** enhance **let-60(n1046)** Muv phenotype through different mechanisms. Additional experiments would be required to understand how **blmp-1** interacts with **lin-1**, **lin-31** and **let-23**.

### 3.4.8.2 **blmp-1(tm548)** does not rescue the Vul phenotype in hypomorphic Vul mutants in EGFR-Ras-RTK signaling pathway

Since BLMP-1 attenuates vulval induction signals, it is possible that perturbation of **blmp-1** may rescue phenotypes of hypomorphic mutants in vulval induction pathways. Therefore, I first targeted **let-60** and **lin-3** with RNAi in **blmp-1** and N2 worms. I observed that **blmp-1(tm548);let-60(RNAi)** and **blmp-1(tm548);lin-3(RNAi)** developed ventral protrusions when examined under dissecting microscope (Figure 3.17). Next, I scored vulval induction index of double mutants between **blmp-1** and hypomorphic mutants in EGFR-Ras-RTK signaling pathway, including **lin-45(sy96)**, **let-60(n2021)** and **lin-3(e1417)** (Table 3.5). Wildtype worms have 3 VPC cells P5.p – P7.p completing wildtype cell divisions and therefore the vulval induction index of 3.0, whereas hypomorphic mutants in the EGFR-Ras-MAPK signaling typically have vulval induction index of less than 3.0, and gain of function mutants more than 3.0 (see Chapter 1 and Figure 1.1).

The EGFR-Ras-MAPK signaling pathway is essential for many developmental processes, so mutants of genes in this pathway are often subviable. About 1% of **let-60(n2021)** worms die as larvae [177] and 15% are Vul. 91% of **lin-45(sy96)** worms die as larvae, while of the remaining, 8% are Vul, 7% have wildtype vulva and the remainder have some degree of reduced vulval induction [95]. **lin-3(e1417)** is a hypomorphic allele with a mutation in the anchor-cell specific enhancer, rendering its phenotype more vulval specific and more viable than other mutants [178]. ~89% of **lin-3(e1417)** worms are Vul and 15% of these worms have a single ventral protrusion [175].

Loss of **blmp-1** function did not alleviate the subviability of these mutants (data not shown). Rather, **blmp-1(tm548);let-60(n2021)** and **blmp-1(tm548);lin-45(sy96)** have even less fecundity
than either of the parental strains, as only less than 10% of worms survive to become adults and more than 50% of adults are sterile. Therefore, for these compound mutants, I was only able to recover ~12 viable L4 animals in 3 months. However, the \textit{blmp-1(tm548)} mutation did not seem to significantly affect fecundity in \textit{lin-3(e1417)} background.

Regardless of their original vulval phenotypes, the \textit{blmp-1(tm548)} mutant and all \textit{blmp-1} compound mutants analyzed have single ventral protrusions, suggesting that the Evl phenotype is due to vulval morphogenesis that happened following vulval cell division. Indeed, even double mutant worms that had wildtype vulval cell division (vulval induction index 3.0) all had vulval morphogenesis defects.

Based on the vulval induction index, \textit{blmp-1(tm548);lin-45(sy96)} had slightly decreased vulval induction compared to \textit{lin-45(sy96)} alone (Table 3.5). Therefore, \textit{blmp-1(tm548)} did not rescue the decreased vulval induction phenotype in \textit{lin-45(sy96)} background. \textit{blmp-1(tm548)} slightly increased the vulval induction index in \textit{let-60(n2021)}, and significantly increased the vulval induction index in \textit{lin-3(e1417)} background, although average vulval induction of \textit{lin-3(e1417);blmp-1(tm548)} was still less than that of wildtype worms (Table 3.5).

This result implies that although \textit{blmp-1} attenuates Ras signaling and may be downregulated downstream of Ras activation, loss of \textit{blmp-1} does not bypass the requirement RTK-EGF-Ras-MAPK pathway component in vulval induction. In hypomorphic mutants of RTK-EGF-Ras-MAPK signaling where there are some low levels of vulval induction, loss of \textit{blmp-1} did appear to partially restore vulval induction. More animals would need to be scored as the penetrance of vulval induction may be skewed by low viability of the mutants, but for strains that did not affect viability like \textit{lin-3(e1417)}, the increased vulval induction was significant.

\subsection*{3.4.8.3 RNAi screen for suppressors of \textit{blmp-1;let-60} Muv phenotype identified paired box transcription factor \textit{pax-3}}

Because mammalian Blimp1 functions primarily as a transcriptional repressor [179], it is possible that targeting its key transcriptional repression targets by RNAi may reverse the enhanced Muv phenotype. Therefore, I carried out an RNAi screen to identify suppressors of
*blmp-1(tm548);let-60(n1046)* Muv phenotype. This was relatively straightforward as the double mutant worms were ~100% Muv.

While scoring the phenotype was simple – by looking for knockdowns that result in non-Muv worms, the screen still required manual setups and individual manipulation of animals. Therefore, I considered a candidate RNAi screen. Because transcriptional networks govern cell fate both in B cell maturation and vulval development, I chose to start by screening with transcription factors and chromatin remodeling factors.

The suppressor screen was done through a library of 711 chromatin remodeling factors and transcription factors. The screen identified one strong positive, *pax-3*, (in addition to *lin-39* and *let-60*, which are anticipated positive controls) and 18 moderate positives (Table 3.6). Strong positives consistently resulted in an abundance of non-Muv worms, whereas the moderate positives consistently resulted in a significant number of non-Muv worms, although less than 50%. Some borderline positives were also evident, but were not pursued further.

The single strong hit was *pax-3*. *pax-3* strongly suppressed both *let-60(n1046)* and *blmp-1(tm548);let-60(n1046)* Muv phenotype (Figure 3.18). In the following section, I will discuss evidences which suggested that *pax-3* might be a key target of *blmp-1* in its cooperation with activated Ras, which had led me to further follow up on this gene.

*pax-3* encodes a paired-box gene in the Pax3/7 family, which is typically involved in muscle and melanoma cell fate specification in vertebrates [180]. Generally, Pax transcription factors are part of gene expression programs for growth, so Pax genes are proto-oncogenes [181].

*pax-3(RNAi)* worms are constipated, have body morphology defects, egg laying defects, with a ventral protrusion (Figure 3.19) and a penetrant early larval lethal phenotype, suggesting that it is a pleiotropic gene which is essential for early development. In *Pristionchus pacificus*, a closely related nematode to *C. elegans*, *pax-3* is involved in vulval formation and is a target of *lin-39* [182]. Computationally predicted interactions and a synthetic genetic interaction dataset indicate that *pax-3* genetically interacts with a series of vulval development genes including
sos-1, let-23, lin-39, bar-1 and sem-5 [64,183]. Therefore, pax-3 likely regulates vulval development in C. elegans.

To test whether pax-3 is also required for ectopic pseudovulval induction in let-60(n1046) worms, I targeted pax-3 with RNAi in let-60(n1046). We found that pax-3 also suppressed let-60(n1046) Muv and dgk-2(gk124);let-60(n1046) Muv phenotypes (Figure 3.18 and Andy Fraser, personal communication). Therefore, pax-3 may be required for wildtype vulval or pseudovulval development. Interestingly, other moderate positives from this screen were also identified as suppressors in the screen for suppressors of let-60(n1046) phenotype (Table 3.6 and Table 2.4), suggesting that genes that are essential for vulval formation are required for blmp-1(tm548);let-60(n1046) Muv phenotype.

3.4.9 Does blmp-1 regulate pax-3 expression via Groucho similarly to how PRDM1 regulates Pax5 expression via Groucho in mammals?

Interestingly, the modENCODE ChIP-Seq data identified a BLMP-1 binding peak near pax-3 start codon (Figure 3.20). This appeared reminiscent of Pax5 repression by Blimp1 involving corepression by Groucho (TLE1/TLE2) [129]. In addition, in Drosophila, Groucho is phosphorylated by MAPK downstream of EGFR-RTK-Ras signaling, and this phosphorylation attenuates Groucho-mediated transcriptional repression [184,185]. Pax5, however, also functions through interaction with the Groucho corepressor complex [186]. Therefore, I hypothesized that BLMP-1 may also transcriptionally repress pax-3 via Groucho (unc-37) in C. elegans (Figure 3.21).

unc-37 is an essential gene in C. elegans [187]. Although its ortholog has been characterized with ubiquitous roles, unc-37 has been characterized in embryonic and neuronal cell fate specification [188,189]. Its hypomorphic alleles have not been characterized for its roles in vulval development. I found that the hypomorphic mutant unc-37(e262) exhibit enhancement of the Muv phenotype of let-60(n1046) (Figure 3.18), such that blmp-1 may function with unc-37 to repress pax-3 expression.
3.4.10 *pax-3* is expressed in the vulva, with vulval-specific *cis*-regulatory elements in its introns.

To elucidate *pax-3* function, I sought to examine its *in vivo* expression pattern initially through transcriptional reporter. Unlike *blmp-1* where the 2 kb sequence upstream of the start codon was sufficient to drive the reporter expression, the transcriptional reporter constructs of *pax-3* containing 2 kb and 3 kb of upstream promoter sequence had no detectable fluorescent expression when worms were examined with a compound microscope (Figure 3.22).

To determine whether other regulatory sequences of *pax-3* are necessary for its expression, I created a fosmid-based reporter by fosmid recombineering fluorescent tags before the stop codon of the *pax-3* gene. The fosmid reporter contains the entire gene locus and ca 19 kb of sequence up and downstream of the gene. The recombineered fosmid rescued the lethal and all post-embryonic phenotypes of worms homozygous for the *pax-3(tm1771)* deletion allele, such that homozygous *pax-3(tm1771)* worms carrying the recombineered fosmid look superficially wildtype (Figure 3.23). Therefore, the fosmid-based transgene appears to encode fully functional PAX-3 protein and recapitulate wildtype expression of endogenous *pax-3*.

I observed very low but detectable embryonic and vulval expression from the fosmid reporter, but no detectable expression in other tissues or during other stages (data not shown and Figure 3.24). This was surprising given that *pax-3* is considered to be an essential gene with pleiotropic functions and that the fosmid-based construct completely rescued the mutant phenotypes. Possibly, the compound microscope may not be sufficiently sensitive to detect *pax-3* expression in other tissues, especially when fosmid-based reporter had to be transformed into the worms at very low concentrations in order to achieve viable transgenic lines. Potentially, antibody staining or another method of visualizing expression would be more accurate or sensitive.

PAX-3::GFP as expressed from the fosmid-based reporter localizes to the nucleus, which is consistent with the fact that PAX-3 is a transcription factor. During 1-cell stage, I observed *pax-3* expression only in the VPC cells (P3.p – P8.p cells), with levels higher in P6.p than in other cells (Figure 3.24A). After the first cell division, *pax-3* expression is only weakly
detectable in P6.p daughters (Figure 3.24B). From the second cell division onward, pax-3 expression level is detectable in P6.p descendants (vulE and vulF), but is upregulated in P5.ppx and P7.pax and their descendants (vulC and vulD) (Figure 3.24C-E, 3.25C).

In the let-60(n1046) single mutant background, I observe pax-3 upregulation in P6.p as well as in other cells during 1-cell stage (Figure 3.25A, 3rd row). In addition, pax-3 levels remain present in those cells at 2-cell stage (Figure 3.25B, 3rd row). Therefore, pax-3 may be upregulated in response to vulval induction or involved in the vulval induction process.

pax-3 has an unusually large intron 5 (3038 bp), which suggests that the introns may contain some regulatory elements (Figure 3.22 and 3.26). In order to test this hypothesis, I created transgenes containing the intron 5 or other introns of pax-3 together with sequences 3 kb upstream of its start codon (Figure 3.22). I found that the construct containing intron 5 of pax-3 was sufficient to drive expression of pax-3 in vulC and vulD cells. Whereas, the construct containing introns 1-4 of pax-3 was sufficient to drive expression in vulE and vulF cells (Figure 3.22).

3.4.11 Does BLMP-1 negatively regulate pax-3 expression by transcriptional repression?

In order to further test the hypothesis that BLMP-1 represses expression of pax-3, I compared fosmid reporter signals between worms in wildtype and blmp-1, as well as in let-60(n1046) and blmp-1(tm548);let-60(n1046) (Figure 3.25). I find that pax-3 reporter signals in the vulva were not significantly higher in blmp-1 mutants, so it is unlikely that blmp-1 actually regulates pax-3 expression.

In order to test whether BLMP-1 ChIP-Seq peak in the pax-3 promoter is essential for pax-3 expression or repression, I excised out the 413 bp region from pax-3 promoter in the pax-3 fosmid using fosmid recombineering. Instead of upregulating pax-3 expression, this completely abolished pax-3 reporter expression. Thus, although the promoter region of pax-3 upstream of its start codon is not sufficient for pax-3 expression, the region annotated as BLMP-1 binding
site by the modENCODE project is necessary for pax-3 expression, together with its intronic cis enhancer elements.

3.4.12 Validation of pax-3 phenotypes with independent RNAi constructs and mutant alleles

To rule out potential off-targeting effects, I sought to validate the pax-3(RNAi) phenotype using a non-overlapping RNAi construct (Figure 3.26), a viable mutant and by generating a tissue-specific knockdown of pax-3. The original construct from the Ahringer library (Figure 3.26) [24], as well as the plasmid L4440 with full-length pax-3 cDNA suppressed the Muv phenotype. A non-overlapping construct recapitulated this phenotype, albeit to a lesser extent than the original construct (Figure 3.27). Other phenotypes of pax-3, including body morphology defects, egg laying defects, protruding vulval, sterile progeny and larval lethality are also observed with this construct.

3.4.13 The downregulation of blmp-1 is unlikely to occur through let-7 family miRNA in the vulva, although blmp-1 may be a miRNA target.

Several lines of evidence suggest that the downregulation of C. elegans blmp-1 downstream of Ras signaling may happen through let-7 family miRNA. Firstly, PRDM1 is a known target of let-7 family miRNA in Hodgekin’s Lymphoma and DLBCL [154,155]. let-7 family miRNA is a protooncogene known to be overexpressed in these lymphomas. let-7 family miRNA negatively regulates Ras signaling both in C. elegans and in cell lines [65]. In C. elegans, let-7 family miRNAs include let-7, miR-48, miR-84 and, miR-241. Notably, mir-84 overexpression suppresses the Muv phenotype of a let-60(ga89), a gain of function mutant [65]. In addition, blmp-1 3’UTR is ~1 kb, which is unusually large for a C. elegans 3’UTR, suggesting that it may contain regulatory elements. miRWIP, which combines computational and biochemical data up to 90% accuracy for miRNA, predicted several let-7 family miRNA and other miRNA sites within blmp-1 3’UTR (Table 3.1) [156].

To test whether global perturbation of miRNA affect BLMP-1 protein levels, I targeted alg-2 with RNAi in worms carrying blmp-1 fosmid-based reporter. I found that alg-2(RNAi)
attenuated the downregulation of the reporter signals (Figure 3.28). Therefore, *blmp-1* transcript may be a miRNA target.

Interestingly, *C. elegans* *let-7* family miRNAs, particularly *miR-84* and *let-7*, have complementary expression patterns at 2-cell stage as do *blmp-1* reporter expression levels (Figure 3.29). *P_{let-7}::gfp* is expressed evenly in all 6 VPC cells and is upregulated in P5.p – P7.p at 2-cell stage before expression upregulates in all vulval cells again. *P_{miR-84}::gfp* is more highly expressed in P6.p than in P5.p and P7.p and their descendants at all stages, but most prominently at 2-cell stage. Therefore, in order to test whether miRNA is responsible for the downregulation of *blmp-1*, I created a transcriptional reporter of *blmp-1* with *blmp-1* 3’UTR, and *blmp-1* 3’UTR deleted for a predicted *miR-84/let-7* seed match region, position 95 – 110 base pairs downstream of the stop codon (Figure 3.30). However, I observed that the *P_{blmp-1}::gfpNLS::unc-54* 3’UTR have similar vulval expression as *P_{blmp-1}::gfpNLS::blmp-1* 3’UTR or the same reporter deleted for predicted *miR-84* site within *blmp-1* 3’UTR (Figure 3.31). Therefore, the downregulation of *blmp-1* downstream of vulval induction is likely to occur transcriptionally and perhaps the effect of *alg-2*(RNAi) on *blmp-1* expression may be indirect.

In the next sections, I characterized *blmp-1* promoter region to identify key transcription factors responsible for the downregulation of *blmp-1* in induced VPC cells.

### 3.4.14 Deletion constructs and phylogenetic footprinting identified regions of *blmp-1* promoter that are important for epidermal and intestinal expression

In order to understand the regulation of *blmp-1* expression, I sought to determine the upstream DNA sequence elements that regulate *blmp-1* expression, I deleted segments of *blmp-1* promoter in 200 bp intervals (pNW12 – pNW20), except for pNW21 which harbors a 295 bp deletion immediately upstream of the start codon (Figure 3.32). I was able to categorize the expression of these reporters into three different outcomes:

1) Deletion resulted in lost vulval and hypodermal expression pattern but retained promoter activity in the intestine (Figure 3.33A). For this category, there is one deletion, pNW13, which deletes the -1696..-1895 bp region of *blmp-1* promoter.
2) Deletion completely abolished reporter expression so that no reporter expression was observed in all tissues (Figure 3.33B). These deletions include pNW17 and pNW21, which delete the -896..-1095 and -1..-295 bp, respectively.

3) Deletion had no observable effect on the reporter expression (Figure 3.33C), so the observed expression pattern is similar to wildtype, with vulval, hypodermal and intestinal cell expression. These deletions include all other 200 bp deletion constructs, i.e. pNW12, pNW14, pNW15, pNW16, pNW18, pNW19, pNW20.

Based on these results, the key transcriptional regulatory elements that govern vulval expression are likely in regions #2(-1895..-1696), #5(-1295..-1096) and #10(-295..1). To further narrow down the sequence elements that are necessary for blmp-1 expression, I created another series of 50 bp deletions within the #2(-1895..-1696), #5(-1295..-1096) and #10(-295..1) regions, which correspond to pNW22 – pNW35, respectively (Figure 3.32). I found that the pNW22 recapitulated the expression pattern of the pNW13 (Figure 3.33A), whereas all other 50 bp deletions drove the same expression patterns as wildtype blmp-1 promoter (Figure 3.33C). Therefore, this region (-1895..-1846) that is deleted in pNW22 contains a sequence element that is required for expression of blmp-1 in the hypodermal and vulval cells, but not in the intestinal cells. I was not able to see loss of promoter activity in any of pNW26 – pNW35, which are 50 bp deletions within regions #5(-1295..-1096) or #10(-1..-295). Worms carrying pNW26 – pNW35 all have reporter expression in the vulval, hypodermal and intestinal cells like wildtype worms.

The #10(-1..295) bp region on its own (pNW40) recapitulates the same expression patterns as pNW13 and pNW22 (Figure 3.34C and 3.33A). All 3 plasmids lack the 50 bp region (-1895..-1846), suggesting that this region may provide elements required for vulval and hypodermal cell expression. In addition, it is likely that the #10(-295..1) region harbors transcription start sites of blmp-1. The actual transcription start sites of blmp-1 within its promoter remain to be experimentally confirmed.

Next, I tried to investigate whether the 200 and 50 bp subregions are individually sufficient to drive blmp-1 expression in the vulval or hypodermal tissue. Typically, this can be tested by
cloning candidate enhancer DNA fragment upstream of the pes-10Δ minimal promoter using the plasmid pPD122.53 from the Fire library.

To determine whether #2(-1895..-1696) and #5(-1295..-1096) subregions of blmp-1 promoter are sufficient to drive blmp-1 expression, I generated minimal expression vectors containing these subregions upstream of the pes-10Δ minimal promoter (pNW37 and pNW38, Supplementary Table 3 and Figure 3.34A). Also, since pNW22 recapitulated the same expression pattern as pNW13, I generated vectors with the (-1895..-1846) 50 bp region of blmp-1 promoter upstream of pes-10Δ minimal promoter (pNW39, Figure 3.34B).

The #5(-1295..-1096) region upstream of the minimal pes-10Δ promoter (pNW38) did not produce any detectable expression signal (data not shown). This region is a poorly conserved region of blmp-1 promoter (Figure 3.35). In addition, the 50 bp subdeletions pNW26 – pNW29 did not recapitulate the expression pattern of pNW16 but maintain similar expression patterns as pNW5 (wildtype). Therefore, I decided not to further characterize this 200 bp region and instead focus on the 2(-1895..-1696) and 10(-295..1) regions instead.

The 10(-295..1) region of blmp-1 promoter (pNW40) is sufficient to drive blmp-1 expression in the gut cells on its own, but not in the vulval or hypodermal cells (Figure 3.34C). Whereas, region #2(-1895..-1696) and the first 50 bp sub-region were sufficient to drive vulval and hypodermal cells, but not gut cells expression (Figure 3.34A pNW37 and 3.34B pNW39).

Therefore, it appears that the cis-regulatory elements required for intestinal expression is located within region #10(-295..1), and for vulval and hypodermal cell expression is located within the first 50 bp of #2(-1895..-1696).

In order to reconstruct a minimal promoter of blmp-1 which contains only necessary elements, I also prepared pNW41 and pNW42, which respectively contain #2(-1895..-1696) and its first 50th bp upstream of region #10(-295..1) (Figure 3.34D and 3.34E). pNW41 fully recapitulates wildtype expression pattern of blmp-1 (Figure 3.34E, compare with Figure 3.5G), whereas pNW42 exhibited less consistent expression in the VPC daughters (Figure 3.34D). Therefore, although the (-1845..1696) region of blmp-1 promoter may not be sufficient for blmp-1 expression in the vulva, it may contain a sequence element necessary for precise downregulation of blmp-1 in daughters of induced vulval precursor cells. It appears that most
important expression elements that comprise blmp-1 promoter lie within regions 2(-1895..-1696) and 10(-295..1).

Based on DNA sequence alignment of the blmp-1 promoter from 4 species of Caenorhabditis, the blmp-1 promoter is highly conserved between -2095..-1551 bp upstream of the start codon, and another region ca -0..-323 bp upstream of the start codon (Figure 3.34). Interestingly, these regions overlap with the regions that were identified to be important for blmp-1 expression. Using the Cis-BP database, I scanned for putative transcription factor binding sites in these regions (Figure 3.36 and Table 3.7) [190]. Predicted transcription factor binding sites that overlap with highly conserved regions are annotated in Figure 3.36A and B including regions #2(-1895..-1696) and #10(-295..0) respectively. The candidate transcription factors are listed in Table 3.7.

In summary, by generating a series of deletions within the 2095 bp upstream blmp-1 promoter, I identified regions that are necessary for vulval, hypodermal and intestinal cell expression. By testing these regions in combination with the pes-10Δ minimal promoter, I demonstrated that the region 2(-1895..-1696) contains an enhancer element that is sufficient to drive vulval expression when a basal promoter is present. In fact, region 2(-1895..-1696), together with 10(-295..0) comprise a minimal blmp-1 promoter which contains all cis elements required for vulval, intestinal and hypodermal cell expression similarly to a full 2095 bp blmp-1 promoter (Figure 3.34E and 3.5G).

3.5 Discussions

The PRDM1/Blimp1 ortholog blmp-1 was identified in the RNAi screen as a genetic enhancer of activated Ras Muv phenotype. This was the first time that blmp-1 has been characterized as a putative Ras-cooperating tumor-suppressor gene. Genetic evidences showed that blmp-1 does not function in the same pathway as other known genetic enhancers of activated Ras, suggesting that blmp-1 cooperates with Ras through a different, perhaps novel, mechanism. I found that blmp-1 is a weak attenuator of EGF-RTK-Ras-MAPK signaling. In addition, it is downregulated in vulval cells with Ras activation, in a manner that correlates with Ras activation. While loss of blmp-1 function alone did not lead to Muv phenotype in wildtype background, ectopic expression of blmp-1 in vulval cells reduced vulval induction. Although
there are several miRNA sites within blmp-1 3’UTR and several of these miRNAs are upregulated in the vulval cell upon vulval induction, I could not establish that blmp-1 is a miRNA target in the vulva. Instead, I observed that the blmp-1 transcriptional reporter which lacks the endogenous blmp-1 3’UTR also exhibits downregulation in induced vulval cells, suggesting that the downregulation of blmp-1 happens mainly through transcriptional regulation. Analysis of blmp-1 promoter identified regions necessary and sufficient for blmp-1 expression in the vulval, hypodermal and intestinal cells, and pointed to key transcription factors that may be responsible for the downregulation of blmp-1 in the vulva.

In order to identify key transcriptional repression targets of BLMP-1 through which BLMP1 may attenuate EGF-RTK-Ras-MAPK signaling, I carried out an RNAi screen to find suppressors of blmp-1(tm548);let-60(n1046) Muv phenotype. The screen covering 711 genes identified the paired-box gene pax-3 as the only strong suppressor.

In this section, I will discuss how these findings fit in with the tumor-suppressor roles of Blimp1 in mammals, especially for ABC-DLBCL. Then, I discuss how my findings about blmp-1 and pax-3 contribute to our present understanding of vulval development.

3.5.1 Blimp1 may be a ras-cooperating tumor-suppressor gene, especially in ABC-DLBCL

blmp-1(tm548) enhances gain of function Ras phenotype in C. elegans, suggesting that Blimp1 may coordinate with gain of function Ras in ABC-DLBCL. In B cells and ABC-DLBCL, Ras functions downstream B Cell Receptor and NF-κB. Although activated ras is not itself identified as a frequent mutation in ABC-DLBCL, in a small number of cases, activated Ras has been implicated as a driver mutation [127]. In addition, drugs with targets in the Ras signaling pathway are being used to treat ABC-DLBCL [146]. For example, tipifarnib, a farnesyltransferase inhibitor which inhibits Ras, has some efficacy in about 17% of patients with refractory aggressive B-cell lymphoma [191]. Therefore, elucidating the interactions between blmp-1 and Ras signaling pathway may help to reveal additional details about signaling pathways that are perturbed in ABC-DLBCL.
In an assay that tests for attenuator of Ras signal, I found that *blmp-1* was a weak positive attenuator of Ras signaling. This parallels a comparable model that *blmp-1* may be downregulated downstream of Ras transcriptionally. In fact, in B-cells, Pax5 represses Blimp1 expression by cooperating with Groucho [186]. Upon B-cell activation activation, Pax5 is phosphorylated downstream of Ras, leading to de-repression of Pax5 expression targets including Blimp1 [139]. There is also evidence in *Drosophila* that Groucho is phosphorylated downstream of Ras signaling, which downregulate its repressive functions [184,185]. In addition, *blmp-1* transcriptional regulation targets may also cooperate with Ras, just as Blimp1 seem to regulate targets like Myc and p53, or other pro-growth genes in mammals.

### 3.5.2 Genetic interactions between *blmp-1* and mutants in Ras signaling pathway suggests that *blmp-1* functions late in vulval development.

My microscopy data suggested that *blmp-1* is downregulated in response to vulval induction. In hypomorphic Vul mutants, the downregulation does not occur (Figure 3.12E-G). In *let-60(n1046)* worms, the downregulation occurred in cells that would assume the vulval cell fate (Figure 3.12A and B). In addition, *blmp-1(tm548)* and *blmp-1(RNAi)* worms have wildtype vulval induction (Figure 3.6). Therefore, *blmp-1* is downregulated downstream of Ras signaling.

In *lin-31* (winged helix) and *lin-1* (Ets) mutants, the downregulation of *blmp-1* reporter signal did not seem to correlate with the degree of vulval induction (Figure 3.12C and D). In addition, when I targeted *blmp-1* with RNAi in other Muv mutants, I found that *blmp-1* enhanced the Muv phenotype of *let-60(n1046)* and *let-23(sa62)* but not of *lin-1(e1275)* and *lin-31(n301)* (Figure 3.16). Therefore, it is possible that *blmp-1* functions downstream of Ras signaling but may be upstream of or at the same step as *lin-1* and *lin-31*.

Interestingly, in B-cell terminal differentiation, Ets1 directly binds to Blimp1 and this physical interaction inhibits Blimp1 binding to DNA such that there is an activation of genes that are typically repressed by Blimp1 [192]. If similar interactions are conserved in worms, this would be consistent with *blmp-1* not enhancing the phenotypes of *lin-1* mutants. However, while *lin-1* is epistatic to Ras signaling with both vulval development and larval viability phenotypes [41],
\textit{blmp-1} is not. This suggests that \textit{blmp-1} and \textit{lin-1} may not function together in the same manner as do Blimp1 and Ets1, or that there are other functions of \textit{lin-1} that are more critical to vulval development and larval viability. Further biochemical characterizations would be necessary to understand the interactions between \textit{lin-31}, \textit{lin-1} and \textit{blmp-1} in \textit{C. elegans}.

3.5.3 Expression dynamics of \textit{blmp-1} is likely due to post-transcriptional and post-translational modification

In all model systems in which Blimp1 functions were characterized, Blimp1 has pleiotropic phenotypes and dynamic expression levels that rise and fall with various stages of development. Blimp1 expression level is regulated at many levels, i.e. transcriptionally, through miRNA and through ubiquitination. Notably, the regulation of BLMP-1 levels by SCF E3 Ubiquitin ligase DRE-1/FBXO11 is conserved [157]. Blimp1 is regulated by \textit{let-7} family and \textit{miR-9} miRNAs, and this interaction is important in Reed-Sternberg cells and DLBCL [154,155]. In addition, Blimp1 protein deleted for endogenous PEST motifs leads to stabilization and extended half life [192]. These findings are consistent with low, albeit ubiquitous and dynamic expression of \textit{blmp-1} from the fosmid-based expression reporter (Figure 3.5), since BLMP-1 levels from the fosmid may be subject to post-translational regulation, while GFP levels from the transcriptional reporter are not.

Since Blimp1 is regulated by miRNA through its 3’UTR, and miRNA regulations are often remarkably conserved, I sought to determine whether \textit{blmp-1} was a miRNA target. However, as I found that \textit{blmp-1} transcriptional reporters with either \textit{blmp-1} or the heterologous \textit{unc-54} 3’UTR have similar vulval expression patterns, I did not find evidence that \textit{blmp-1} was a miRNA target in the vulva (Figure 3.30). It was possible that I did not detect any expression change because \textit{blmp-1} transcriptional reporter signals were low in the vulva regardless of the 3’UTR since the downregulation occurs through \textit{blmp-1} promoter. In addition, the expression of \textit{blmp-1} in induced vulval cells relative to the hypodermal cells were higher with the fosmid-based reporter compared to the transcriptional reporter (Figure 3.5). Therefore, was possible that an enhancer or other elements outside of the 2.1 kb promoter was required for persistent vulval expression and post-transcriptional regulation responsible for \textit{blmp-1} downregulation in the vulva. A different vulva-specific promoter other than \textit{blmp-1} could be used to confirm
whether \textit{blmp-1} is subjected to miRNA regulation in the vulva. In addition, it is possible that \textit{blmp-1} is a miRNA target in other tissues, especially in the seam cells.

3.5.4 Modulatory \textit{cis}-regulatory elements regulate tissue-specific \textit{blmp-1} expression dynamics

Since \textit{blmp-1} expression from the 2 kb upstream regions yielded very weak signals in induced VPC cells at 2-cell stage, I hypothesized that the majority of \textit{blmp-1} expression in the vulva was regulated transcriptionally via this promoter segment. Serial deletions of \textit{blmp-1} promoter across the 2.1 kb region did not identify a specific single transcriptional element that is responsible for the downregulation of \textit{blmp-1} in the vulva (Figure 3.32-3.34). The 50 bp region of \textit{blmp-1} promoter (-1846..-1895) was sufficient to drive expression in the vulval and hypodermal cells in a non-uniform manner, suggesting that \textit{cis}-repressor elements may be present in the promoter, most likely in the 150 bp region immediately 3’ to this 50 bp region. Interestingly, 50-bp deletions in the region immediately 3’ to the 5’th 50 bp region did not result in reporter signals that were upregulated in induced VPC daughters, suggesting that there may be multiple redundant repressor elements within the 150 bp region. Phylogenetic footprinting via motif finding in highly conserved regions identified putative regulatory sites by many transcription factors, including ones with implicated roles in vulval development, such as \textit{lin-11}, \textit{lin-31}, \textit{egl-5}, \textit{lin-39}, \textit{cog-1}, \textit{efl-2} and \textit{mab-5} (Table 3.7) \cite{41,90,193}. Additional genetic and biochemical experiments would be necessary to determine the roles of these transcription factors in regulation of \textit{blmp-1} expression.

I also find that different regions of \textit{blmp-1} reporters are responsible for tissue-specific expression of \textit{blmp-1}. The 2\textsuperscript{nd} 200 bp of \textit{blmp-1} promoter (-1696..-1895) is necessary for vulval and hypodermal cell expression, including in the head, tail and seam cells. The 295 bp immediately upstream of the start codon is responsible for transcriptional initiation as well as intestinal cell expression. While the transcriptional reporter signals are strong in the seam and intestinal cells, the fluorescent signals from the fosmid-based reporter are weak in these cells. Therefore, it is apparent that \textit{blmp-1} expression is regulated at multiple levels. It would also be interesting to elucidate the phenotypes of worm BLMP-1 protein that is deleted for PEST motifs or ubiquitination sites. In fact, it is possible that the mutant BLMP-1 protein would be stabilized and may fully prevent vulval induction when overexpressed in the vulva.
3.5.5 *blmp-1* single mutant Evl phenotype could be due to defective seam cells.

*blmp-1* worms have wildtype uterus and connection to the gonad but incomplete or retarded alae formation [157]. The *C. elegans* vulva (vulE and UTSE cells) hangs on to seam cells, which fuse into alae in adult worms, for stability [36]. Because *blmp-1* expresses in the seam cells and also have heterochronic alae formation defects (Figure 3.5E and [157]), the *blmp-1* vulval eversion defective phenotype could be due to seam cell defects as well as the failure to synchronize between the gonads and the vulval development.

I did not detect expression of *blmp-1* in the gonads with the transcriptional reporter (Figure 3.5G and I), but some somatic gonad expression was detected with the fosmid-based reporter (Figure 3.5D and F). *blmp-1* expression was observed in the gonad prior to VPC induction (Figure 3.5D), and subsequently decreases after the first VPC induction (Figure 3.5D). Generally, anchor cell expression was detected up until the L4 stage (Figure 3.5F). Therefore, it appears that regulatory elements outside of *blmp-1* promoter may be responsible for expression in the gonad. In addition, *blmp-1* may also play a role in gonad development outside of the vulval cells.

3.5.6 *blmp-1* may cooperate with activated Ras differently than *synMuv* genes or other enhancers of *let-60(n1046)*.

Although *blmp-1* was a chromatin factor that attenuated vulval induction signals (Figure 3.8), it is not a *synMuv* gene [8]. In addition, it does not have a synthetic Muv phenotype in combination with most known negative Regulators of ras signaling that are downstream targets of Notch, except for a very low (<0.1%) penetrant Muv phenotype in combination of gap-1(*ga133*) (Figure 3.9). Therefore, it is likely that *blmp-1* cooperates with gain of function Ras through some alternate mechanism. I also observed that forced expression of *blmp-1* result in reduced terminal vulval cell division (Table 3.2) and in some *blmp-1(tm548);let-60(n1046)* worms, some vulval cells were found to undergo an additional round of cell division (Figure 3.15). Thus, *blmp-1* may regulate heterochronicity of vulval cell division, in addition to gonad and seam cell development. Additional investigation would be required to confirm this hypothesis.
3.5.7 Genetic evidence suggests that \textit{blmp-1} functions late in vulval development

\textit{blmp-1} expression appears to be downregulated downstream of Ras signaling as \textit{blmp-1} expression is reduced during 2-cell stage, after vulval induction. Loss of \textit{blmp-1} leads to an exaggerated activated Ras Muv phenotype in \textit{let-60(n1046)}, but does not rescue Vul phenotypes of Vul mutants (Table 3.5). Therefore, it is possible that \textit{blmp-1} functions as a gatekeeper of cell proliferation in vulval development. However, forced expression of \textit{blmp-1} in the vulva did not lead to Vul phenotype or reduced the penetrance Muv phenotype in \textit{let-60(n1046)} background. Instead, forced expression of \textit{blmp-1} results in decreased vulval induction or the partially Vul phenotype that results in protruded vulva in adults. Therefore, the downregulation of \textit{blmp-1} is required for correct vulval cell fate specification during the 2\textsuperscript{nd} and 3\textsuperscript{rd} rounds of VPC cell divisions. This places \textit{blmp-1} in the same genetic circuit as genes that regulate late vulval development, including \textit{cog-1, egl-38 and bed-3} [163,193].

Interestingly, Yang et al (2015) found that \textit{blmp-1(s71)}, a point mutation of \textit{blmp-1}, led to under-induced vulva similarly to \textit{bed-3} mutants [162]. In my hands, I find that worms overexpressed with \textit{blmp-1} have reduced vulval induction patterns similar to \textit{bed-3} mutants, as well as \textit{cog-1} and \textit{lin-29} (Table 3.3) [149,163]. These differing observations could possibly be either because \textit{blmp-1} represses \textit{bed-3} expression so perhaps overexpression of the full-length isoform of \textit{blmp-1} results in reduction in \textit{bed-3} function. Unfortunately, no \textit{bed-3} RNAi vectors were included in the Ahringer library that I used to screen for modifiers of Muv phenotype of both \textit{let-60(n1046)} or \textit{blmp-1(tm548);let-60(n1046)} mutants. Additional characterization would be necessary to understand how \textit{blmp-1} interacts with the genetic circuitry that regulates late vulval cell specification and morphogenesis.

3.5.8 Genes that are required for VPC competence upstream of vulval induction, as well as genes that function downstream may be identified in suppressor screens for Muv phenotype of \textit{blmp-1(tm458);let-60(n1046)}.

In order to identify transcriptional targets that are regulated by \textit{blmp-1} in its cooperation of activated Ras, I carried out an RNAi screen for suppressors of \textit{blmp-1(tm548);let-60(n1046)}
Muv phenotype. Consistently, the positive controls *let-60* and *lin-39* were recovered as positives in the screen. Since *let-60* and *lin-39* both have functions upstream of *blmp-1*, it is possible that the genes that function upstream of *blmp-1* would also be recovered as positives in this proposed this screen. Therefore, additional evidence would be required to determine whether any positives were indeed the transcriptional regulation targets of BLMP-1.

While the modENCODE ChIP-Seq data indicated that BLMP-1 may bind to *pax-3* promoter, the recently published RNA-Seq data demonstrated that *pax-3* was not differentially regulated in *blmp-1* worms than in wildtype worms [157]. In addition, I did not observe that *pax-3* reporter signals were significantly upregulated in *blmp-1* mutants (Figure 3.25). While I cannot rule out the possibility that *pax-3* may transcriptionally regulate *blmp-1*, *pax-3* is likely not a direct transcriptional regulation target of *blmp-1*.

Interestingly, several moderate positives from my suppressor screen of *blmp-1(tm548);let-60(n1046)* overlapped with the suppressors identified in the screen for modifiers of the *let-60(n1046)* Muv phenotypes (Chapter 2). These weak suppressors were not noted in a screen for suppressors of *lin-15AB(n765)* Muv phenotype [8]. Since the screen did not require extensive manual counting as nearly 100% of the worms were Muv, the suppressors could readily be identified in a less labor-intensive manner. In addition, since these suppressors suppressed the Muv phenotype without causing a Vul phenotype, it is likely that these positives function downstream of vulval induction. Therefore, RNAi screens for suppressors of highly penetrant Muv phenotypes compound mutants that include *let-60(n1046)* may be powerful and less labor-intensive strategy to identify putative Ras-cooperating protooncogenes.

3.5.9 *pax-3* may function to regulate development prior to VPC formation in *C. elegans* as well as late in vulval cell division

*pax-3* deficient worms that reach adulthood have single ventral protrusion but typically no developed vulval tissue, as well as very strong Egl phenotype (Figure 3.19), suggesting *pax-3* may have roles prior to vulval induction. In fact, Thomson and colleagues recently characterized *pax-3* as a gene that regulates the ventral hypodermal cells, which later become VPCs [194]. In addition, since *pax-3* is expressed in the VPC cells, dividing VPC cells and is most highly expressed after VPC cell divisions are complete, it is likely that *pax-3* also
functions late in vulval development. It is important to note, also, that expression patterns of pax-3 from the fosmid-based reporter is remarkably similar to that of egl-17 (Figure 3.37). egl-17 encodes a fibroblast growth factor-like protein and is a known downstream target of Ras signaling, partly through winged helix lin-1 and Ets lin-31 transcription factors [195]. Therefore, it is possible that pax-3 and egl-17 share similar transcriptional regulation circuits for their roles in vulval development. Because viable pax-3 hypomorphic mutants were not available and there were significant discrepancies in the RNAi phenotypes, stage- and tissue-specific perturbation of pax-3 would be necessary to understand the roles of pax-3 in vulval development. With the recent advent of CRISPR, it may be possible to generate targeted point mutations within the pax-3 gene in order to study viable post-embryonic phenotypes of pax-3.

3.6 Conclusion and significance

In this chapter, I characterized blmp-1 as a putative Ras-cooperating tumor suppressor gene. I found that blmp-1 is a genetic enhancer of activated Ras phenotype in C. elegans, although blmp-1 worms alone are not Muv. Therefore, this genetic interaction between let-60 and blmp-1 may be a good model for genetic cooperation between multiple mutations in cancers, such as in ABC-DLBCL. Genetic evidence suggests that during vulval development, blmp-1 is downregulated downstream of Ras signaling through transcriptional regulation, although blmp-1 may be regulated as a miRNA target in other tissues. Like other model organisms in which Blimp1 has been characterized, blmp-1 has pleiotropic functions in the C. elegans. blmp-1 weakly attenuates EGF-RTK-Ras-MAPK signals. Forced expression of blmp-1 in the vulval result in a partially Vul phenotype. My findings support that blmp-1 may function late in vulval development.
A.

Figure 3.1: BLMP-1 domain structures and conserved DNA binding motif
A. blmp-1 domain structures with the domain annotation for the C. elegans protein. BLMP-1 has a SET domain and 5 C2H2 Zinc fingers.
B. The binding motifs of BLMP-1 inferred from modENCODE ChIP-Seq data [153]
C. The alignment of putative murine Blimp1 binding site in promoters of its core target genes, as determined by ChIP and EMSA assays, adapted from [140]

B.

C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc</td>
<td>AAAGGGAAG</td>
</tr>
<tr>
<td>CIITA</td>
<td>GAAGTGAAAT</td>
</tr>
<tr>
<td>IFN-β</td>
<td>GAAGTGAAAG</td>
</tr>
<tr>
<td>Pax-5</td>
<td>AAAGTGAAATC</td>
</tr>
</tbody>
</table>

Figure 3.2: in vivo recombination reporters to generate constructs P_{lin-31}::gfp and P_{lin-31}::blmp-1::gfp to express BLMP-1 and GFP in the vulva
Figure 3.3: Superficial vulval phenotypes of let-60(n1046) (top) and blmp-1(tm548);let-60(n1046) (bottom).

blmp-1(tm548);let-60(n1046) worms have more ectopic VPC cell induction, a protruding true vulva, and larger pseudovulvae than let-60(n1046) worms.
Figure 3.4: Superficial phenotype of $blmp-1(tm548)$ worms (bottom), in comparison with wildtype adult worms (top).

$blmp-1$ worms are dumpy (shorter body), darker in color and have a protruded vulva.
Figure 3.5A-C: Tissue-specific expression of blmp-1 as assessed by fosmid-based and transcriptional fluorescent reporters.

Merged DIC and fluorescent images of early L3 worms, prior to the first VPC cell division, using fosmid-base reporter wgIs109 [blmp-1::TY1::EGFP::3xFLAG + unc-119(+)] (A and C) and transcriptional reporter Is[blmp-1promoter::gfpNLS::unc-54 3’UTR; unc-119(+)] (B).

A. and B. In the head and pharyngeal cells.

C. Lateral hypodermal cells in-focus. Orange triangles point to alae or ridge as seen in the middle of the worm. Seam cell nuclei (green fluorescence, in focus) that run along the ridge.

Green fluorescence marks the nuclei of cells that carry the transgene. Yellow fluorescence in A. and C. mark gut autofluorescence as signals are detectable in both green and yellow channels. Not all cells are in focus.
Figure 3.5D-E: Tissue-specific expression of blmp-1 as assessed by fosmid-based and transcriptional fluorescent reporters.

Merged DIC and fluorescent images of early L3 worms, prior to the first VPC cell division, using fosmid-base reporter wgIs109 [blmp-1::TY1::EGFP::3xFLAG + unc-119(+)] (D) and transcriptional reporter Is[blmp-1promoter::gfpNLS::unc-54 3’UTR; unc-119(+)] (E).

D. Expression in the VPCs, anchor cell (red arrow), gonad (asterisk) and tail cells

E. Expression in the intestinal cells, seam cells (orange triangles), pharyngeal cells and head cells

Because the reporters are integrated, all cells carry the transgene and green fluorescence marks the nuclei of cells that express blmp-1. Yellow fluorescence in E marks gut autofluorescence as signals are detectable in both green and red channels. Not all cells are in focus. Gut nuclei and pharyngeal expression is detected much more readily with the transcriptional reporter than with the fosmid reporter.
Figure 3.5(F-K): (legend continues on next page)
Figure 3.5(F-K): Tissue-specific expression of *blmp-1* after VPC cell divisions, as assessed by fosmid-based and transcriptional fluorescent reporters.

Merged DIC and fluorescent images of early L3 worms, prior to the first VPC cell division, using fosmid-base reporter *wgIs109*[ *blmp-1::TY1::EGFP::3xFLAG + unc-119(+)*) (F, H, J) and transcriptional reporter *Is*[ *blmp-1promoter::gfpNLS::unc-54 3’UTR; unc-119(+)*) (G, I, K).

F. and G. Before VPCs undergo a cell division. Downward arrows point to VPCs, whereas upward arrows point to anchor cell.

H. and I. After the first VPC cell division. Brackets denote VPC daughters and upward arrows point to anchor cell. The other fluorescing cells along the top (ventral) side of the worms are daughters of uninduced VPC cells that fuse with the hypodermal syncytium.

J. and K. After vulval cell division is completed and vulval morphogenesis process is ongoing. Upward arrow points to the anchor cell. Asterisks denote vulval lumen. Upward arrows point to anchor cell. Because the reporters are integrated, all cells carry the transgene and green fluorescence marks the nuclei of cells that express *blmp-1*. These include intestinal and lateral hypodermal cells (in focus in Figure 3.5E). Yellow fluorescence marks gut autofluorescence as signals are detectable in both green and red channels. Not all cells are in focus.
Figure 3.6: Vulval morphogenesis and eversion process in \textit{blmp-1(tm548)}, in comparison to wildtype worms.

Different rows for L4 depict different focal planes corresponding to midline (top), sub-lateral (middle) and lateral planes (bottom) to show all VPC descendants. \textit{blmp-1} vulval cell division is identical to wildtype, up until late L4 stage where the vulval lumen resembles a Christmas tree (also referred to as the Christmas tree stage). Subsequently, \textit{blmp-1} has a vulval eversion defect which results in slightly protruded vulva comparing to wildtype worms. Red letters in the top 2 rows are shorthand labels (e.g. A and B1 for vulA and vulB1, respectively) for the 7 cell types that are present in the L4 after the vulval cells finish dividing (see Figure 1.1).
Figure 3.7: *blmp-1* superficial phenotypes (Dpy, darker color, Unc, Evl) were rescued by the transgene *wgIs109 [blmp-1::TY1::EGFP::3xFLAG + unc-119(+)]*, which carries *blmp-1* fosmid recombineered to C-terminally tag with GFP and 3xFLAG tag.
Figure 3.8: Ectopic expression of egl-17::gfp in the VPC daughters at 2-cell stage.

Pink bar – percentage of the animals in each mutant background that have persistent ectopic egl-17::gfp in either P5.p or P7.p daughters at 2-cell stage. A representative photomicrograph of such ectopic expression in gap-1(ga133) background.

Blue bar – percentage of animals in each mutant background that have no ectopic egl-17::gfp in P5.p or P7.p daughters at 2-cell stage. A representative photomicrograph of wildtype-pattern egl-17 expression without expression in P5.p or P7.p daughters.

blmp-1 appears to be a weak attenuator of Ras signals because blmp-1 mutant increases the frequency of ectopic egl-17 expression in P5.p or P7.p daughters. Binomial test p<0.01 between blmp-1 and WT, and gap-1 and WT.
Figure 3.9: *blmp-1(tm548);gap-1(ga133)* Muv phenotype. <0.1% of *blmp-1(tm548);gap-1(ga133)* are Muv, with small ventral protrusions (red arrows). Blue arrow indicates actual vulva. n>3,000.

Figure 3.10: *blmp-1(tm548);lin-15B(n744)* worms’s penetrant Egl phenotype that was not present in either mutant alone.
~50% penetrant cuticle defects that result in small ventral bumps (red arrow) without vulval tissue. n > 100.
Figure 3.11: *blmp-1* fosmid reporter signals is downregulated more in P6.p than in P5.p and P7.p.

Top: quantified fluorescent reporter signals from fosmid-based reporter (n>10). At 2-cell stage. Bottom: A representative fluorescent microscopy image of worms carrying *blmp-1* fosmid reporter. Because the reporters are integrated, all cells carry the transgene and green fluorescence marks the nuclei of cells that express *blmp-1*. 
Figure 3.12A-C: *blmp-1* transcriptional reporter expression in *let-60(n1046)* and *lin-31(n301)* worms.

Merged DIC and fluorescent images of L3 worms after the first VPC cell division, using the transcriptional reporter Is[*blmp-1promoter::gfpNLS::unc-54 3’UTR; unc-119(+)].

A. *let-60(n1046)* worm that would become a Muv adult. These worms typically have downregulation of *blmp-1* reporter signals in P3.px, P4.px, or P8.px cells. For this representative animal, an absent of fluorescent signal in P4.px, and reduction of signal in P8.px are observed.

B. *let-60(n1046)* worm that would become a nonMuv adult have similar VPC expression patterns as do wildtype worms (Figure 3.5G). Expression levels are high in P3.px, P4.px and P8.px, and are low or absent in P5.px – P7.px.

C. Representative images of *lin-31(n301)* worms. These worms have reduced and punctate expression levels across all 12 Pn.px cells.

Because the reporters are integrated, all cells carry the transgene and green fluorescence marks the nuclei of cells that express *blmp-1*. VPC daughters and the AC are in focus.
**Figure 3.12D-G: blmp-1 transcriptional reporter expression in lin-1(e1275), lin-39(RNAi), lin-3(RNAi) and lin-45(RNAi) worms.**

Merged DIC and fluorescent images of L3 worms after the first VPC cell division, using the transcriptional reporter Is[blmp-1promoter::gfpNLS::unc-54 3’UTR::unc-119(+)].

D. Representative image of lin-1(e1275) worms, which have uniformly reduced blmp-1 expression across all 12 Pn.px cells.

E. Representative image of lin-39(RNAi) worms whose VPCs do not undergo the first VPC cell division, except for P6.p. blmp-1 expression remains persistently high across all vulval cells.

F. and G. Representative image of lin-3(RNAi) and lin-45(RNAi) worms, respectively, which develop to become Vul adults. blmp-1 expression remains persistently high across all 12 Pn.px cells. Because the reporters are integrated, all cells carry the transgene and green fluorescence marks the nuclei of cells that express blmp-1. Not all cells are in focus.
Figure 3.13: Vulval phenotypes of wildtype worms overexpressing BLMP-1 under in the vulva under lin-31 promoter.

DIC, fluorescent and merged images of worms carrying the transgenes \(Ex[P_{lin-31}::blmp-1::gfp::unc-54\ 3'UTR + P_{dpy-7}::dsRed + unc-119(+)]\) and \(Ex[P_{lin-31}::gfp::unc-54\ 3'UTR + P_{dpy-7}::dsRed + unc-119(+)]\). Red arrow points to the vulva. \(P_{dpy-7}::dsRed\) was used as a coinjection marker to follow worms carry the transgene in all of their vulval and hypodermal cells.
Figure 3.14: Vulval induction index of worms overexpressing BLMP-1 under lin-31 promoter in the vulva, in wildtype and let-60(n1046) backgrounds.

Vulval induction index was assessed under Nomarski optics as described in section 1.3.2 for worms carrying the transgenes overexpressing BLMP-1 (Ex[P\text{lin-31}::\text{blmp-1}::\text{gfp}::\text{unc-54 3'UTR} + P\text{dpy-7}::\text{dsRed} + unc-119(+)], yellow bars) and overexpressing GFP only (Ex[P\text{lin-31}::\text{gfp}::\text{unc-54 3'UTR} + P\text{dpy-7}::\text{dsRed} + unc-119(+)], orange bars) in wildtype and let-60(n1046) backgrounds. Background strains were included as controls (blue bars)

A. In wildtype background, only P5.p – P7.p displayed as no ectopic vulval induction of other VPCs were observed.

B. In let-60(n1046) background.

Error bars = 1 SEM. * indicates that the two values are over 2 SEM different, indicating 95% confidence intervals.
Figure 3.15: *blmp-1(tm548);let-60(n1046)* vulva cells may undergo another round of vulval cell division beyond wildtype vulva, such as the vulval cell indicated by red arrow. Image was taken at 40X magnification under Nomarski optics.
Figure 3.16: Relative changes in the penetrance of Muv phenotypes of various Muv mutants targeted by RNAi against known enhancers (blmp-1, unc-37 and nrbp-1) and suppressors (lin-39 and pax-3) of let-60(n1046).

Negative controls = worms fed with non-targeting RNAi.
Figure 3.17: Superficial adult phenotypes of *blmp-1* worms targeted with *let-60* and *lin-3* by RNAi
Figure 3.18: Changes in Muv phenotypes of \textit{blmp-1(tm548);let-60(n1046)} and \textit{let-60(n1046)} as a result of \textit{pax-3(RNAi)} and \textit{unc-37(e262)}.

RNAi assay and quantification of Muv phenotype were carried out as described in section 2.3.2. \textit{lin-39(RNAi)} and \textit{let-60(RNAi)} were included as positive controls. Like \textit{lin-39(RNAi)} and \textit{let-60(RNAi)}, \textit{pax-3(RNAi)} suppresses the Muv phenotype of both \textit{blmp-1(tm548);let-60(n1046)} and \textit{let-60(n1046)} (p<0.01, 1-tailed t-test with Bonferroni correction). \textit{unc-37(e262)} is a hypomorphic mutant of \textit{unc-37} (Groucho) that enhances \textit{let-60(n1046)} Muv phenotype of \textit{let-60(n1046)} (p<0.01). \textit{unc-37(e262)} worms are not Muv (n = 540). Untreated \textit{let-60(n1046)} worms were included to compare with \textit{let-60(n1046);unc-37(e262)}. 
Figure 3.19: Reduction of function phenotypes of \textit{pax-3}
Top 2 panels: Photomicrograph of \textit{pax-3(tm1771)} \textit{Ex[pax-3::gfp(WRM067aB09) + rol-6(su1006)]} worms that lost the extrachromosomal array.
Bottom panel: Wildtype worm.

Figure 3.20: \textit{blmp-1} binding peak in \textit{pax-3} promoter from modENCODE ChIP-Seq experiment, as generated by Wormbase GBrowse [153].
Figure 3.21: Hypothetical model that blmp-1 represses pax-3 via recruiting Groucho, which is de-repressed upon MAPK phosphorylation downstream of Ras.

A. In wildtype worms, pax-3 promoter is repressed.
B. In let-60(n1046) background, activated Ras results in attenuated pax-3 repression.
C. In let-60(n1046) background, loss of blmp-1 abolishes pax-3 expression, resulting in increased penetrance of Muv phenotype.
Figure 3.22: Promoter elements required for L4 vulval expression of *pax-3* may be located in its introns.

While *pax-3* fosmid reporter is sufficient to drive expression in vulC, vulD, vulE and vulF cells, the 2 kb sequence upstream of *pax-3* start codon is not sufficient to drive reporter expression. When intron 5 of *pax-3* is added upstream of the promoter, this restores strong expression in vulC and vulD cells. When the first four introns and exons are added, this restores weak expression in vulE and vulF cells.

Figure 3.23: *pax-3(tm1771)* carrying the fosmid-based reporter (*Ex[pax-3::gfp(WRM067aB09) + rol-6(su1006)]*) which expresses PAX-3::GFP are viable and look superficially wildtype.
Figure 3.24A-C: *pax-3* expression based on the fosmid reporter *Is/pax-3::gfp(WRM067aB09) + rol-6(su1006)* vulval expression pattern at different stages.

A. *pax-3* fosmid reporter is expressed in P4.p – P8.p and is highest in P6.p during 1-cell stage than in other VPC cells.

B. After the first VPC cell division, the signal is only detectable dimly in P6.p daughters and to a lower degree in P5.pp and P7.pa.

C. The reporter signals then upregulate in P6.p granddaughters, and daughters of P5.pp and P7.pa after the second vulval cell division. Not all cells are in focus.
Figure 3.24D-F: *pax-3* expression based on the fosmid reporter *Is[pax-3::gfp(WRM067aB09) + rol-6(su1006)]* vulval expression pattern at different stages.

D. After the third vulval cell division, the reporter signal is present in cells vulC, vulD, vulE and vulF, with the signals being highest in vulD.

E. (ventral view) and F. (lateral view) reporter signals are detectable in adult vulva.
Figure 3.25A: *pax-3* expression levels in the VPCs in wildtype worms, *blmp-1(tm548)*, *let-60(n1046)*, and *blmp-1;let-60* worms.  
Arrows point to individual VPCs. Asterisks denote anchor cells, which is immediately dorsal of P6.p cell. This *pax-3* expression is based on the fosmid reporter Is[*pax-3::gfp(WRM067aB09) + rol-6(su1006)].
Figure 3.25B: *pax-3* expression levels in the VPC daughters in wildtype worms, *blmp-1*(tm548), *let-60*(n1046), and *blmp-1;let-60* worms.

Brackets denote individual pairs of VPC daughters. Asterisks denote anchor cells, which is immediately dorsal of P6.p cell. This *pax-3* expression is based on the fosmid reporter *Is[pax-3::*gfp(WRM067aB09) + rol-6(su1006)].*
Figure 3.25C: *pax-3* expression levels in VPC great-granddaughters in wildtype, *blmp-1(tm548)*, *let-60(n1046)*, and *blmp-1;let-60* worms during L4 molt.

Asterisks denote anchor cells, which is immediately dorsal of P6.p cell and the middle of the vulva lumen. This *pax-3* expression is based on the fosmid reporter Is*[pax-3::gfp(WRM067aB09) + rol-6(su1006)].
Figure 3.26: non-overlapping RNAi constructs targeting *pax-3*. Construct A is the original construct in the Ahringer library. Not pictured is the RNAi construct C with the PCR product amplified from the cDNA plasmid, which only contains the coding sequence of *pax-3*. 
Figure 3.27: Changes of penetrance of the Muv phenotypes in let-60(n1046) worms fed with 3 different RNAi constructs targeting pax-3. 

blimp-1(RNAi) was included as a positive control for RNAi effectiveness.
Figure 3.28: *blmp-1* expression in VPC cells of worms knocked down for *alg-2*.

A. *alg-2*(RNAi);*WgIs109*  
B. *WgIs109* worms fed with non-targeting RNAi  
C. Quantification comparing fluorescence levels across n > 10 *WgIs109* worms fed with *alg-2* vs non-targeting RNAi. Error bars = 1 SD.
**Figure 3.29A:** Dynamic vulval expression patterns of miRNA reporters that were predicted to have binding sites in blmp-1 3’UTR: *let*-7 and *miR*-48.

*let*-7 is expressed in all VPCs, and subsequently upregulated in descendants of P5.p – P7.p after the first cell division.

*miR*-48 has no detectable expression, except for 2 vulval cells during L4 molt.
Figure 3.29B: Dynamic vulval expression patterns of reporters driven by promoters of miRNAs that were predicted to have binding sites in bimp-1 3’UTR: miR-84 and miR-241. miR-84 is expressed in the gonad, including the anchor cell. It is upregulated in P6.p and expressed at a lower level in P5.p and P7.p, and this difference in expression patterns persist in descendants of P5.p – P7.p through L4 molt.

miR-241 is expressed in the gonad and at a low level in all vulval and hypodermal cells through L4 molt.
Figure 3.29C: Dynamic vulval expression patterns of reporters driven by promoters of miRNAs that were predicted to have binding sites in blmp-1 3’UTR: miR-34 and miR-1. miR-34 is expressed in some muscle and hypodermal cells, but have no detectable expression in the vulva. miR-1 has detectable but low peripheral expression, but no detectable expression in the vulva.
Figure 3.30: Schematic for *blmp-1* constructs containing negative control heterologous *unc-54* 3’UTR, the entire *blmp-1* 3’UTR (ca 1 kb) and *blmp-1* 3’UTR deleted for *miR-84/let-7* seed match region (positions 95 – 110) as predicted by miRWIP [156].
Figure 3.31: Expression patterns from *blmp-1* transcriptional reporter transgenes with different 3’UTR.

Top: Is[blmp-1promoter::GFP::blmp-1 3’UTR]

Middle: Is[blmp-1promoter::GFP::blmp-1 3’UTR deleted for miR-84 site]

Bottom: Is[blmp-1promoter::GFP::unc-54 3’UTR]
Figure 3.32: Schematic for blmp-1 promoter deletion constructs.
Only blmp-1 promoter portion and GFP are displayed. All reporters in this figure have unc-54 3’UTR.
Full details about these constructs are listed in supplementary table 3.
A. pNW16 and pNW21

B. pNW13, pNW21 and pNW40

C. pNW5(WT), pNW12, pNW14-20, pNW23-35, pNW41-42

Figure 3.33: Representative expression of 3 categories of expression patterns for the corresponding deletion constructs.
A. No vulval or hypodermal cell expression, but retains gut cell expression as large bright green nuclei.
B. No detectable GFP signal.
C. Same pattern as intact blmp-1 promoter with expression in the gut, hypodermal cells and vulval cells. Images are representative of n > 10 for each transgene examined. A. and C. were taken at 2 s exposure, where as B. were overexposed (taken at 10 s exposure) to look for any possible weak GFP signal, hence grainy gut granule non-GFP background signal.
Figure 3.34: Constructs and representative images for testing sufficiency of blmp-1 promoter sequence elements.

pNW37, pNW39 and pNW40 were injected at 40 ng/µl and co-injected with 0.5 ng/µl hypodermal cell markers dpy-7::dsRed to confirm the presence of the extrachromosomal arrays in cells examined. pNW40, pNW41 and pNW42 were injected at 10 ng/µl as the blmp-1 endogenous promoter is much stronger than pes-10Δ minimal promoter. Note that the large round fluorescent nuclei in C, D and E are gut nuclei. Whereas the smaller fluorescent nuclei observed in A, B and E are lateral hypodermal cell nuclei. Negative numbers denote the number of base pairs upstream of the start codon.
Figure 3.35: Sequence conservation in 5'UTR of *blmp-1* across species of *Caenorhabditis* and *Pristionchus pacificus* (top) as created by the UCSC Genome Browser [196].

Figure 3.36: Transcription sites predicted by Cis-BP [190] using the 8-mer E-scores option were overlapped with these alignments, and the conserved regions that contain transcription factor binding sites were annotated in red rectangles
A. in the region 2(-1895..1696) and B. region 10(-295..0).
Figure 3.37: Expression dynamics of egl-17 and pax-3 in the vulval cells. Expression patterns of egl-17 is derived from data previously published in [43,57,193].

Table 3.1: List of miRNA binding sites in C. elegans blmp-1 3'UTR as predicted by miRWIP [156].

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* blmp-1 3'UTR has multiple let-7 family miRNA sites since let-7, miR-241 and miR-84 are let-7 family miRNAs.
Table 3.2: Vulval cell lineages of *let-60(n1046)* and *blmp-1(tm548);let-60(n1046)*

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</tbody>
</table>

* indicates animals with potentially an extra cell division after the three cell cycles are completed, as denoted by Lx2 where the vulval cells seem to have divide once more after the three rounds of vulval cell division are completed.

Average vulval induction of *let-60(n1046)* is 3.48 and *blmp-1(tm548);let-60(n1046)* is 4.39. Divisions of VPC granddaughters are represented by S, L, T and N (See Section 1.2.2 and Figure 1.1). S = When cell P3.pa (anterior daughter of P3.p) fuses with the syncytium and does not undergo further cell divisions. L = longitudinal cell division. T = Transverse cell division. N = no cell division.
Table 3.3: Phenotypes of \( blmp-1(tm548) \) worms targeted for 6 \( synMuv \) genes by RNAi, and \( lin-15A(n767) \) and \( lin-15B(n744) \) worms fed with \( blmp-1(RNAi) \).

\( blmp-1 \) is therefore not a \( synMuv \) gene because \( blmp-1 \) does not have synthetic Muv phenotype with known \( synMuv \) genes.

<table>
<thead>
<tr>
<th>RNAi</th>
<th>mutant</th>
<th>( lin-15A(n767) )</th>
<th>( lin-15B(n744) )</th>
<th>( blmp-1(tm548) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( smo-1 ) (( synMuvA ))</td>
<td>not Muv</td>
<td>Muv</td>
<td>not Muv</td>
<td></td>
</tr>
<tr>
<td>( trr-1 ) (( synMuvC ))</td>
<td>Muv</td>
<td>Muv</td>
<td>not Muv</td>
<td></td>
</tr>
<tr>
<td>( lin-8 ) (( synMuvA ))</td>
<td>not Muv</td>
<td>Muv</td>
<td>not Muv</td>
<td></td>
</tr>
<tr>
<td>( blmp-1 )</td>
<td>not Muv</td>
<td>Egl, cuticle defects</td>
<td>not Muv</td>
<td></td>
</tr>
<tr>
<td>( lin-35 ) (( synMuvB ))</td>
<td>Muv</td>
<td>not Muv</td>
<td>not Muv</td>
<td></td>
</tr>
<tr>
<td>( lin-53 ) (( synMuvB ))</td>
<td>Muv, Let</td>
<td>not Muv</td>
<td>not Muv</td>
<td></td>
</tr>
<tr>
<td>( mys-1 ) (( synMuvC ))</td>
<td>Muv</td>
<td>Muv</td>
<td>not Muv</td>
<td></td>
</tr>
</tbody>
</table>

All worms treated with \( blmp-1 \) RNAi exhibited all other characteristic superficial phenotypes of \( blmp-1 \), i.e. Unc, Dpy, darker color, and Pvl.

\( n > 200 \) for each genotype examined.

Table 3.4: Baseline \( %Muv \) of Muv mutants at different temperatures.

<table>
<thead>
<tr>
<th>strain</th>
<th>median</th>
<th>min</th>
<th>max</th>
<th>SEM</th>
<th>temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>( lin-1(e1275) )</td>
<td>27.19</td>
<td>0</td>
<td>66.67</td>
<td>5.833</td>
<td>16</td>
</tr>
<tr>
<td>( let-60(n1046) )</td>
<td>25.29</td>
<td>14.31</td>
<td>51.16</td>
<td>3.4</td>
<td>16</td>
</tr>
<tr>
<td>( lin-31(n301) )</td>
<td>69.05</td>
<td>48.72</td>
<td>88.23</td>
<td>3.62</td>
<td>16</td>
</tr>
<tr>
<td>( lin-15(n765) )</td>
<td>75.77</td>
<td>47.61</td>
<td>100</td>
<td>4.72</td>
<td>16</td>
</tr>
<tr>
<td>( let-23(sa62) )</td>
<td>54.16</td>
<td>0</td>
<td>80</td>
<td>8.2</td>
<td>16</td>
</tr>
<tr>
<td>( lin-1(e1275) )</td>
<td>59.09</td>
<td>0</td>
<td>90</td>
<td>5.93</td>
<td>20</td>
</tr>
<tr>
<td>( let-60(n1046) )</td>
<td>52.73</td>
<td>35.68</td>
<td>80</td>
<td>3.51</td>
<td>20</td>
</tr>
<tr>
<td>( lin-3(n301) )</td>
<td>66.67</td>
<td>46.42</td>
<td>97.37</td>
<td>4.36</td>
<td>20</td>
</tr>
<tr>
<td>( let-23(sa62) )</td>
<td>75.86</td>
<td>0</td>
<td>100</td>
<td>7.47</td>
<td>20</td>
</tr>
<tr>
<td>( lin-15(n765) )</td>
<td>100</td>
<td>97.62</td>
<td>100</td>
<td>0.13</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3.5: Vulval induction phenotypes of \( blmp-1 \) compound mutants.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Average vulval induction ± SEM</th>
<th>WT vulva</th>
<th>Vul</th>
<th>Pvl*</th>
<th>number of worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.00 ± 0.00</td>
<td>All</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>( blmp-1(tm548) )</td>
<td>3.00 ± 0.00</td>
<td>All</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>( lin-35(sy96) )</td>
<td>2.19 ± 0.31</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>( lin-35(sy96);blmp-1(tm548) )</td>
<td>1.62 ± 0.32</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>( let-60(n2021) )</td>
<td>1.77 ± 0.33</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>( let-60(n2021);blmp-1(tm548) )</td>
<td>2.07 ± 0.17</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>( lin-3(e1417) )</td>
<td>0.61 ± 0.15</td>
<td>1</td>
<td>11</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>( lin-3(e1417);blmp-1(tm548) )</td>
<td>1.65 ± 0.18</td>
<td>1</td>
<td>6</td>
<td>21</td>
<td>28</td>
</tr>
</tbody>
</table>

*Pvl worms as indicated in this table are worms that have reduced vulval induction, so they have less vulval induction than wildtype, but more vulval induction than Vul worms.

#p<0.05, one-way t-test.
Table 3.6: Moderate positives identified from the RNAi screening for suppressors of blmp-1(tm548); let-60(n1046) Muv phenotype.

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Gene public name</th>
<th>Overlap with suppressor screen in let-60(n1046) (Chapter 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1007.1</td>
<td>ceh-17</td>
<td></td>
</tr>
<tr>
<td>E03H4.13</td>
<td>nhr-89</td>
<td></td>
</tr>
<tr>
<td>F23F1.1</td>
<td>nfy-c-1</td>
<td>yes</td>
</tr>
<tr>
<td>Y17G7A.1</td>
<td>hmg-12</td>
<td></td>
</tr>
<tr>
<td>ZK131.2</td>
<td>his-73</td>
<td></td>
</tr>
<tr>
<td>F54C1.3</td>
<td>mes-3</td>
<td></td>
</tr>
<tr>
<td>F02E9.9</td>
<td>dpt-1</td>
<td></td>
</tr>
<tr>
<td>T23D8.8</td>
<td>cfi-1</td>
<td></td>
</tr>
<tr>
<td>K10C3.6</td>
<td>nhr-49</td>
<td></td>
</tr>
<tr>
<td>C45E1.1</td>
<td>nhr-64</td>
<td></td>
</tr>
<tr>
<td>C32F10.2</td>
<td>lin-35</td>
<td>yes</td>
</tr>
<tr>
<td>Y48A6C.5</td>
<td>pha-1</td>
<td>yes</td>
</tr>
<tr>
<td>ZK993.a</td>
<td>ceh-45</td>
<td></td>
</tr>
<tr>
<td>C32F10.7</td>
<td>nhr-2</td>
<td></td>
</tr>
<tr>
<td>Y51H1A.5</td>
<td>hda-6</td>
<td></td>
</tr>
<tr>
<td>F45C12.3</td>
<td>F45C12.3</td>
<td></td>
</tr>
<tr>
<td>F23H11.1</td>
<td>bra-2</td>
<td></td>
</tr>
<tr>
<td>C50A2.2</td>
<td>cec-2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7: Cis-BP [190]-predicted transcription factor binding sites that overlap with highly conserved regions of blmp-1 promoter in the regions #2(-1895..1696) and #10(-295..1) as indicated in Figure 3.36.

<table>
<thead>
<tr>
<th>Region #</th>
<th>Transcription factor families</th>
<th>Included members</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Forkhead</td>
<td>fkh-6, fkh-10, unc-130, let-381, pha-4, lin-31*, fkh-2, fkh-7, daf-16</td>
</tr>
<tr>
<td>A2</td>
<td>Homeodomain</td>
<td>ceh-18, lin-4,</td>
</tr>
<tr>
<td>A3</td>
<td>Z2H2 Zinc Finger</td>
<td>ztf-2, ztf-28</td>
</tr>
<tr>
<td>A5</td>
<td>E2F</td>
<td>efl-2, ces-2</td>
</tr>
<tr>
<td>A6</td>
<td>MADF</td>
<td>C01G12.1</td>
</tr>
<tr>
<td>B single conserved region of 24 bp in region 10 (-295..0) with transcription factor binding sites</td>
<td>E2F</td>
<td>efl-2*</td>
</tr>
<tr>
<td></td>
<td>At hook</td>
<td>athp-1, hmg-12, Y116A8C.22</td>
</tr>
<tr>
<td></td>
<td>TBP</td>
<td>thp-1</td>
</tr>
<tr>
<td></td>
<td>POU</td>
<td>ceh-6</td>
</tr>
<tr>
<td></td>
<td>Nuclear receptor</td>
<td>nhr-177</td>
</tr>
</tbody>
</table>

* genes that have been implicated in vulval development.
Chapter 4
Summary, Discussion and Future Directions

4.1 Summary

The main goals of this thesis were, first, to identify genes that cooperate with activated Ras using RNAi screening in *C. elegans*; and second, to understand how such cooperations occur between activated Ras and *blmp-1*. In Chapter 2, I described a quantitative RNAi screen which identified 22 genetic suppressors and 24 enhancers of activated Ras phenotypes (Table 2.2 and 2.3). Two of these genetic suppressors (*akt-1* and *pha-1*) and four enhancers (*blmp-1*, *sma-4*, *pdhk-2* and *cki-2*) were confirmed by crossing mutant alleles with *let-60(n1046)*.

*blmp-1(RNAi)* strongly enhanced the *let-60(n1046)* Muv phenotype, and the phenotype was recapitulated with a double mutant *blmp-1(tm548);let-60(n1046)*. Blimp1 has been implicated in cancers, and mutation in the *C. elegans* *blmp-1* alone does not result in Muv phenotype (Table 2.3). Therefore, I decided to further characterize *blmp-1* (Chapter 3).

*blmp-1* encodes the direct *C. elegans* ortholog of the mammalian B-Lymphocyte-Induced Maturation Protein 1 (Blimp1), a protein which is conserved between worms and mammals. With a SET domain and 5 C2H2 Zinc Fingers, Blimp1 generally functions as a transcriptional repressor. While it has pleiotropic functions and is dynamically expressed in all model systems in which it is characterized, Blimp1 is best characterized for its roles in B cell terminal differentiation into antibody-secreting plasma cells. Loss of Blimp1 function is a typical driver mutation in activated-B cell diffuse large B cell lymphoma, suggesting that Blimp1 is a tumor suppressor gene [14].

While loss of *blmp-1* alone does not lead to Muv phenotypes, I found that *blmp-1(tm548)* worms have slightly increased Ras activation (Figure 3.8 and 3.9). Interestingly, *blmp-1* transcript levels appear to be downregulated downstream of Ras activation upon vulval induction, which is consistent with the role of *blmp-1* as an attenuator of Ras signals (Figure 3.11). This downregulation of *blmp-1* levels appeared dependent on vulval induction because mutants with abolished vulval induction did not have this downregulation (Figure 3.12). Forced expression of *blmp-1* in the vulva did not result in vulvaless phenotype, but consistently
decreased vulval induction phenotype resulting in a protruding vulva (Pvl) phenotype (Figure 3.13 and 3.14A). In addition, this forced expression of *blmp-1* in the vulva reduced vulval induction but did not suppress the Muv phenotype of *let-60(n1046)* worms (Figure 3.14B). Therefore, *blmp-1* may function late in vulval development.

In order to understand the mechanism of *blmp-1* downregulation upon vulval induction, I tested two different hypotheses. *blmp-1* may be regulated by miRNA via its 3’UTR or by transcriptionally via *blmp-1* upstream promoter. Since mammalian Blimp1 is a target of the *let-7* family miRNAs via its 3’UTR, *C. elegans* *blmp-1* is a predicted miRNA target and several *let-7* family miRNAs have complementary expression patterns of *blmp-1*, I tested whether *C. elegans* *blmp-1* is a target of *let-7* family miRNAs. Notably, targeting *alg-2* argonaute by RNAi attenuated *blmp-1* downregulation so my findings remain inconclusive as to whether *blmp-1* is a *let-7* family miRNA target via its 3’UTR in *C. elegans* (Figure 3.28-3.29).

To test whether *blmp-1* is downregulated by transcriptional modulation, I first compared the expression patterns of an upstream DNA sequence (2095 bp) in the transcriptional, versus fosmid-based reporter. I found that the transcriptional reporter signal is downregulated post-vulval induction similarly to that for the fosmid-based reporter. Therefore, the promoter region 2095 bp upstream of the start codon which is common to both the transcriptional and the fosmid reporters should contain sequence elements that are necessary and sufficient for this vulval expression pattern. To identify such sequence elements, I dissected the 2095 bp *blmp-1* promoter region for its transcriptional elements that may be required for the downregulation. I found that tissue-specific expression of *blmp-1* is controlled by distinct regions within the *blmp-1* promoter (Figure 3.33). In addition, isolating the 50 bp minimal region required for vulval and hypodermal cell lineage abolished the uniform downregulation of *blmp-1* downstream of vulval induction (Figure 3.34D).

Since BLMP-1 is a transcriptional repressor, it is possible that it functions to repress downstream targets that cooperate with activated Ras in ectopic vulval induction. Therefore, these downstream targets may be revealed as genetic suppressors of *blmp-1(tm548);let-60(n1046)* phenotypes among a large series of 711 transcriptional/chromatin regulators. An RNAi screen for suppressors of *blmp-1(tm548);let-60(n1046)* identified 18 positive RNAi
clones (Table 3.5). Interestingly, several positives from this screen were also identified in my initial suppressor screen for let-60(n1046) Muv phenotypes, suggesting that genes that are required for ectopic pseudovulval induction or any process required for vulval development may be identified in such suppressor screens.

*pax*-3 was identified as the strongest suppressor with nearly all worms appeared non-Muv. However, since *pax*-3 worms lack vulval tissue, the gene may be required at an early stage of vulval development. In addition, expression of *pax*-3 is also upregulated in specific vulval cells after cell divisions are completed, indicating that *pax*-3 may also function late in vulval development, such as the specification of VPC great-granddaughter cell fate or vulval morphogenesis.

4.2 The power of the quantitative RNAi screen in identifying new genetic modifiers of *let-60(n1046)* Muv phenotype

I carried out my initial RNAi screen to identify genetic modifiers of *let-60(n1046)* Muv phenotypes through two different RNAi libraries: one was the library of 215 chromatin remodeling factors and another is the 193 orthologs of cancer-associated genes as defined by the Tumor Sequencing Project [12].

While the screen could, in theory, be performed to identify either suppressors or enhancers, I found that the enhancement could be more readily scored, such that the enhancers were easier to identify than suppressors. This could be due to many reasons. Firstly, many genetic suppressors of vulval induction have essential functions, so few or no worms would survive to adulthood and only weaker knockdowns or escapers would be seen. Secondly, other essential genes may lead to spurious positives or negatives because the majority of the knocked down worms did not survive to adulthood. To address this latter problem, I tested and re-tested my positives at least twice, with five biological replicates for each positive RNAi clone. In addition, reduction in the exposure time by starting the RNAi feeding at later stages significantly increased the recovery of true positives that are essential genes. These approaches reduce the possibility of identifying false positives due to bottleneck effects caused by reduced viability. Furthermore, I found that several positives identified as suppressors of *let-60(n1046)* Muv phenotypes were also identified as suppressors of *blmp-1(tm548);let-60(n1046)* Muv
phenotypes, suggesting that a suppressor screen in a strain that is ~100% Muv may be more streamlined and more sensitive.

One advantage of forward genetic screening is that it is possible to identify poorly characterized genes and implicate their functions through genetic interactions. Several positives consistently identified from the RNAi screens were not previously characterized and even standard genetic mutants were not available. With the advent of direct gene manipulation tools like CRISPR technology, it will be possible to mutate the endogenous copies of these genes in order to further confirm their cooperation with activated Ras.

4.3 Genetic interactions between existing known Ras interactors in human tumors and in C. elegans

4.3.1 Ras and PI3K signaling

Ras signaling in human cancers often signal through MAPK, PI3K-Akt or Ral-GDS pathways and components of these pathways have been shown to cooperate with Ras in tumorigenesis [30]. In C. elegans, MAPK signaling and Ral-GDS have been shown to function downstream of Ras [20,197], while crosstalks between PI3K-Akt and Ras signaling have been established [108]. In addition, many pathway components are highly conserved between worms and mammals. Results from the RNAi screen for modifiers of let-60(n1046) Muv phenotype establish that genetic perturbations within the PI3K-Akt pathway genetically interact with activated Ras in a similar manner as do their homologs. I found that akt-1(RNAi) suppressed the Muv phenotype, and akt-1(mg306);let-60(n1046) worms were only 13% Muv compared to let-60(n1046) (~60 – 80% Muv). In agreement, starvation during L2 – L3 suppresses the Muv phenotype in let-60(n1046) [198]. Nakdimon (2012) found that PTEN daf-18 reduction of function mutation significantly enhanced the Muv phenotype of let-60(n1046) [108]. Interestingly, while I found that akt-1(lf) strongly suppressed let-60(n1046) Muv phenotypes, it only partially suppressed the Muv phenotype of let-60(n1046);daf-18(lf) [108]. Further investigation would be needed to further understand the genetic cooperation between Akt kinases and other PI3K pathway components.
4.3.2 synMuv genes and synMuv suppressors

synMuv genes encode nuclear proteins that function redundantly to inhibit ectopic vulval induction. Many synMuv genes encode chromatin remodeling and transcription factors such as lin-35 Rb, hda-1 HDAC and efl-1 E2F, while others encode nuclear proteins of unknown functions [58]. A single mutation of a synMuv gene does not result in a Muv phenotype, but certain combinations of synMuv gene mutations do. Several synMuv genes are orthologs of known cancer genes, such as lin-35 Rb, efl-1 E2F, dpl-1 DP. In addition, some synMuv genes, i.e. egl-27 and egr-1 which encodes MTA1 orthologs are known to be enhancers of Muv phenotypes [63,96]. Therefore, many of these genes were included in my screen through the chromatin factors library.

Several synMuv suppressors were also identified as suppressors by Cui et al (2006) [8], while many others were not. The suppressors that were identified included htz-1, dpy-30 and isw-1. This could mean that these synMuv suppressors may be required for ectopic pseudovulval induction.

Since htz-1 is a histone variant, it is possible that it has roles more globally or more dependent on chromosomal context than other genes which may function in vulval signaling pathways. One example is that, in yeast, the SWR1 complex mediates gene silencing by incorporating Htz1p near telomeric regions [199]. It is also possible that some genes that suppress ectopic vulval induction are among genes that are near telomeric regions, which get de-repressed when htz-1 is mutated.

Similar location-dependent effects can also be observed with dosage compensation genes and synMuv suppressors. For example, mes-6 is a dosage compensation gene, and is only found to suppress Muv phenotype of synMuv double mutants when both mutations are on the X chromosome (lin-15AB) but enhancement of the Muv phenotype when a hypomorphic allele is present on the X chromosome (such as lin-8(n111);lin-15(n374)) [8]. In the RNAi screen, I found several dosage compensation genes that modify let-60(n1046) Muv phenotypes, including dpy-30 as a suppressor, and mes-6 as an enhancer. While mammals use X-inactivation for sex-specific dosage compensation, which is different from the widespread
transcriptional repression used by nematodes, it is possible that chromosome- or motif-specific widespread transcriptional repression play roles in cancer.

4.3.3 Paradoxical roles and results for lin-35 in the RNAi screen

The *C. elegans* RB1 ortholog *lin-35* is a synMuv B gene with pleiotropic functions, including roles in cell cycle progression, organogenesis and RNAi [7,116,200]. The human Rb gene is a known tumor suppressor gene that cooperates with Ras, as loss of Rb in the presence of activated Ras can lead to tumorigenesis [201-203]. Therefore, it was expected that *lin-35(RNAi)* could enhance the Muv phenotype of *let-60(n1046)*. However, I consistently found that *lin-35(RNAi)* strongly suppressed the *let-60(n1046)* Muv phenotype and *blmp-1(tm548);let-60(n1046)* (Table 2.4 and Table 3.6). The null allele *lin-35(n745)* also suppressed the Muv phenotype of *let-60(n1046)*, albeit less strongly than *lin-35(RNAi)* (compare Figure 2.13A with 2.14).

Curiously, Cui et al (2006) observed that *lin-3* levels are reduced in *lin-35* mutants [8], which may explain why *lin-35* perturbation suppresses the *let-60(n1046)* Muv phenotype. However, this does not explain the paradoxical finding that *lin-35(RNAi)* produced much stronger suppressive effect than did *lin-35(n745)* null mutant.

While it is possible that the discrepancy between the RNAi results and the null mutant could be due to off-target effects of the RNAi expression vectors, I suspect that this is due to a different mechanism, which may reflect both deactivation of *lin-35* and interference of the RNAi machinery. *lin-35* deficient worms are hypersensitive to RNAi [112]. In addition, *lin-35* mutations display synthetic genetic interactions with mutants of the RNAi/miRNA pathway with loss of *lin-35* leading to an increase in intestinal number cell divisions, and synthetic lethal phenotype with the *alg-1* null mutation [204]. *lin-35* is required for repression of endogenous siRNA targets in the worms as well as RNAi-induced post-transcriptional gene silencing [205]. Therefore, it is possible that the strong suppression of *let-60(n1046)* Muv phenotypes by *lin-35(RNAi)* is due to the disruption of both *lin-35* functions and global changes involving small RNAs or RNAi machinery. While this may be considered an unfortunate artifactual result, it may be a hypothesis worth testing because the genetic suppression of *let-60(n1046)* Muv
caused by the disruption of lin-35 together with the activation of the RNAi machinery is likely to be interesting and biologically significant.

4.3.4 Genetic circuitry in pharyngeal development

In *C. elegans*, pharyngeal organ development is a well-coordinated organogenesis process. David Fay’s group found that *ubc-18* and the retinoblastoma ortholog *lin-35* function redundantly to negatively regulate SUP-35 protein levels, which in turns negatively regulate *pha-1* [206]. Therefore, *ubc-18;lin-35* double mutants phenocopy *pha-1* single mutant in terms of pharyngeal development defects. In addition, several other *ubc-18;synMuv B* double mutants phenocopy *pha-1* pharyngeal defect phenotypes [116]. The authors proposed that the pharyngeal development roles of *lin-35* and these other *synMuv* genes are due to non cell cycle-related functions of *lin-35*. Consistent with this, only *synMuv* B genes that had functions unrelated to cell cycle regulation produced the pharyngeal defect phenotypes, while other *synMuv* B genes did not. [116].

In my initial RNAi screening, I consistently identified *pha-1* and the RB1 ortholog *lin-35* as strong suppressors of *let-60(n1046) Muv* phenotype. I was able to confirm that *lin-35(n745)* weakly suppressed the Muv phenotype, but *pha-1(e2123ts)* did so only marginally at permissive temperature (16°C). Possibly, *pha-1(RNAi)* suppression of *let-60* Muv phenotype was biologically significant, but *pha-1(e2123ts)* may not have had sufficiently strong perturbation of *pha-1* function elicit the strong suppression phenotype at permissive temperature. Subsequently, I also tested whether *ubc-18* modifies Muv phenotype, and indeed found that *ubc-18(ku354)* significantly suppressed the Muv phenotype (Figure 2.15). Since *lin-35* and *ubc-18* both suppress Muv phenotype, and *pha-1(RNAi)* suppresses the Muv phenotype, it is possible that similar genetic circuitry involving *lin-35, pha-1* and *ubc-18* also positively regulates vulval development.

Although *pha-1* contains sequence motifs similar to a leucine zipper motif, it actually encodes a largely cytoplasmic protein [115,207]. *pha-1* is an essential gene whose loss of function result in defective pharyngeal development [114], although I observed no overt lethality RNAi phenotype. Mosaic analyses indicate that it has pleiotropic functions. It is required for embryonic viability, may be required for the intestinal cells as well as in the gonad. In the same
mosaic analysis study, Kuzmanov et al (2014) also found that some \textit{pha-1} mosaic animals have Egl phenotype, suggesting that \textit{pha-1} may be required for wildtype vulval development or the development of egg laying muscles [115]. Additional characterizations would be required to confirm the roles of \textit{pha-1} and the presence of \textit{pha-1/ubc-18/synMuv B} genetic circuitry in vulval development.

4.4 \textit{blmp-1} as an enhancer of \textit{let-60(n1046)} Muv phenotype

\textit{blmp-1} was identified in an RNAi screen for modifier of the \textit{let-60(n1046)} Muv phenotype, consistent with its function as a cell-fate specifier and a tumor suppressor gene. Interestingly, while mammalian Blimp1 has been established as a tumor suppressor in ABC-DLBCL, it was never characterized in the context of activated Ras. I examined several possibilities in which \textit{blmp-1} may cooperate with gain of function Ras, including these following six possibilities which may not be mutually exclusive of each other.

4.4.1: \textit{blmp-1} may be an attenuator of ras signaling

In wildtype worms, a promoter that expresses downstream of Ras-MAPK activation, \textit{egl-17}, is expressed in P5.p – P7.p during 1-cell stage and subsequently becomes largely restricted to P6.p stage after vulval induction and during 2-cell stage. \textit{gap-1} and several other genes that negatively regulate Ras signals led to increase in the percentage of worms that ectopically express \textit{egl-17} reporter in either P5.p or P7.p daughters during 2-cell stage [43]. In order to test whether \textit{blmp-1} has a similar property, I found that \textit{blmp-1} worms had significantly increased percentage of worms that ectopically expressed \textit{egl-17} reporter relative to wildtype worms, but to a lesser extent than do \textit{gap-1} deficient worms. In addition, I found that \textit{blmp-1(tm548);gap-1(ga133)} had a very low (<1%) penetrant Muv phenotype. This suggests that \textit{blmp-1} may be a negative attenuator of Ras signaling, but to a much lesser degree than the ones described by Yoo and colleagues [43]. In addition, since \textit{blmp-1} is a transcription factor and most other genes described by Yoo \textit{et al} encoded cytoplasmic proteins, it is possible that \textit{blmp-1} may transcriptionally regulate them.
4.4.2: *blmp-1* is not a *synMuv* gene

Andersen and Horvitz (2007) previously tested whether *blmp-1* was a *synMuv* gene, and found it was not [164]. Interestingly, I found that a small percentage of *blmp-1(tm548); lin-15B(n744)* worms have ventral protrusions (Figure 3.10). Since *blmp-1* worms have cuticular defects and the ventral protrusions are much subtler than the pseudovulval protrusions of *Muv* worms, it is possible that this phenotype is due to cuticular defects rather than an ectopic vulval induction. Therefore, *blmp-1* is not a *synMuv* gene, but *blmp-1* may genetically interacts with *lin-15B* in cuticular development.

4.4.3: *blmp-1* as a potential miRNA target downstream of vulval induction

Several lines of evidence suggest that *blmp-1* may be a miRNA target, and that miRNA may mediate the downregulation of BLMP-1 protein levels downstream of vulval induction. First, *blmp-1* 3’UTR is abnormally large (~1 kb), placing it among the top 1% of *C. elegans* 3’UTR, suggesting that its 3’UTR may have important regulatory sequences. Secondly, *blmp-1* 3’UTR contains multiple *let-7* miRNA sites from a study that used both computational and biochemical predictions [156]. Thirdly, some *let-7* family miRNAs have complementary expression patterns to *blmp-1* in the vulval cells (Figure 3.29A and B). Fourthly, *blmp-1* is a heterochronic gene and mammalian Blimp1 has been shown to be a miRNA target [155]. Lastly, *blmp-1* worms have defective alae, where normal development requires miRNA functions during development [157].

However, I found that both the transcriptional reporter and fosmid reporter of *blmp-1* have similar downregulation of GFP reporter signals in induced VPC daughters (P5.p – P7.p), suggesting that the most significant downregulation of *blmp-1* happens through transcriptional regulation. This, however, does not rule out the possibility that *blmp-1* is a miRNA target in other tissues, such as in the hypodermal seam cells or the intestinal cells, or other stages. Additional direct assays to test whether *blmp-1* is downregulated by miRNA, such as one using a sensitive heterologous reporter, would be required to determine whether *blmp-1* is a miRNA target in specific tissues of interest.
4.4.4: blmp-1 may be a heterochronic gene that is regulated through cell cycle regulation in the hypodermal lineage and the vulva

In a multicellular organism, the temporal coordination of cell division and cell fate decisions are critical to ensure that each organ develops at the right pace relative to the rest of the animal. The C. elegans vulva, for example, must coordinate with somatic gonad development and match stages with the rest of the body. Other examples include the timing for seam cell terminal differentiation and gonad migration.

blmp-1 worms have incomplete alae formation or retarded seam cell terminal differentiation, and an epistatic analysis placed blmp-1 late in the heterochronic circuit [157]. Because the vulva structurally hangs on the alae to maintain structure, incomplete alae formation, rather than the morphogenesis defects of vulval cells, may be the cause of Pvl phenotype in blmp-1 mutants.

Cyclin-CKI controls vulval cell division and these genes are controlled by miRNAs, which allows for temporal coordination of development between the vulva and the rest of the body. Cyclin mutants have reduced vulval cell division as G1 takes much longer than for wildtype worms [208]. In addition, cye-1(ku256);let-60(n1046) worms still have ectopic pseudovulval induction but reduced number of cells in all vulva and pseudovulvae [208]. Whereas, cki-1(RNAi) worms not only develop extra VPCs due to precocious cell division, the worms also have extra rounds of cell divisions [209]. In addition, genes that control post-translational modification of these cyclin and CKI genes, such as the E3 ubiquitin ligase dre-1 and cul-1, also lead to similar extra rounds of vulval cell induction phenotypes as CKI mutants.

BLMP-1 is a degradation target of dre-1. Loss of blmp-1 suppresses several dre-1 mutant phenotypes and blmp-1 mutants display opposite heterochronic phenotypes to dre-1 [157]. While the blmp-1 single mutant worms do not have heterochronic vulval cell division phenotype, I observed that 3 out of 16 blmp-1(tm548);let-60(n1046) worms, an additional round of cell division can occur even after morphogenesis (Figure 3.15). In addition, I consistently observed more ectopic pseudovulval cells in blmp-1(tm548);let-60(n1046) worms.
It is possible that \textit{blmp-1(tm548)} worms have slowed vulval cell cycle progression since the mutant strain does grow slowly.

To determine whether \textit{blmp-1(tm548)} or \textit{blmp-1(tm548);let-60(n1046)} worms have defective cell cycle regulation, additional experiments using cell cycle markers such as \textit{mrn-1::gfp} or quantification of nuclear DNA would be necessary. In addition, tissue-specific reconstitution of \textit{blmp-1} function in the null mutant may illuminate the contribution of seam cells to the Pvl phenotype and perhaps the non-cell autonomous functions of \textit{blmp-1} in vulval development. The cooperation between \textit{blmp-1} and Ras signaling in heterochrony and cell-cycle regulation remain to be elucidated.

In mammals, cyclin dependent kinase inhibitors expression is tightly tied to terminal cell fate commitment, with upregulation once terminal cell fate is decided [210]. Since \textit{blmp-1} has conserved roles as a terminal cell fate differentiation transcription factor, the cooperation between \textit{blmp-1} and cyclin-dependent kinase inhibitors are highly plausible. However, additional studies are required to determine the precise mechanisms and manner of interactions.

\textbf{4.4.5: \textit{blmp-1} as a transcriptional regulation target downstream of Ras-MAPK signaling pathway}

The RTK-Ras-MAPK signaling pathway typically results in changes in gene expression due to MAPK phosphorylation of downstream transcription factors. In \textit{C. elegans}, the heterodimeric transcription factors LIN-31 Winged Helix and LIN-1 ETS are phosphorylated by MAPK MPK-1 downstream of Ras signaling. This phosphorylation dissociates the LIN-31/LIN-1 dimer, which relieves its inhibition of vulval induction [41].

In wildtype or \textit{let-60(n1046)} worms, \textit{blmp-1} reporter signals are downregulated in a manner that is inversely correlated to the strength of vulval induction (Figure 3.11 and 3.12A-B). However, in \textit{lin-31(n301)} and \textit{lin-1(e1275)} worms, \textit{blmp-1} expression is low in all VPC daughters (Figure 3.12C and D), suggesting that correct expression of \textit{blmp-1} may require \textit{lin-31} and \textit{lin-1}.

To identify transcription factors that regulate \textit{blmp-1} transcription, I used CisBP to search for cognate sites within conserved promoter subregions that I showed to be sufficient and
necessary for wildtype \textit{blmp-1} expression in the vulva [190] (Figure 3.32-3.34). Interestingly, this search identified a cognate site for Forkhead-family transcription factor in 2\textsuperscript{nd} 200 bp subregion of the 2.1 kB promoter, which includes LIN-31. In addition, several cognate sites of other transcription factors that have been implicated roles in vulval development were also identified (Table 3.7). To confirm the roles of these transcription factors as transcriptional regulators of \textit{blmp-1}, additional experiments including direct binding assays and mutations of these cognate sites will be required.

### 4.4.6: BLMP-1 may transcriptionally regulate putative proto-oncogene orthologs or growth promoting genes

Blimp1 functions as a transcriptional repressor that functions by recruiting other chromatin remodeling factors such as HDAC1 and G9a, among others. In addition, in B-cell terminal differentiation, Blimp1 transcriptionally represses protooncogenes including Pax5, E2F, Myc and CIITA [179]. Therefore, \textit{blmp-1} may cooperate with Ras by limiting expression of downstream Ras-cooperating proto-oncogenes.

To determine which of these were key targets in vulval development, I carried out an RNAi screen for suppressors of \textit{blmp-1(tm548);let-60(n1046)} phenotype. I identified \textit{pax-3} as a strong suppressor in the screen, as I found that nearly all \textit{blmp-1(tm548);let-60(n1046);pax-3(RNAi)} worms were non-Muv. In addition, modENCODE ChIP-Seq identified \textit{blmp-1} binding peak in the \textit{pax-3} promoter. However, analyses of \textit{pax-3} reporters in the \textit{blmp-1} background did not show significant differences in vulval-specific \textit{pax-3} expression levels, suggesting that \textit{blmp-1} is unlikely to directly regulate \textit{pax-3} expression in the vulva. It is possible, however, that \textit{pax-3} or other Paired-box transcription factors function to regulate \textit{blmp-1} expression.

\textit{pax-3} encodes a transcription factor in the paired-box (Paired and Homeobox or Pax) family [211-213]. Generally, in humans, Pax genes are proto-oncogenes [211,212]. In many vertebrates, Pax3 is expressed in the neural tube, and is important for CNS, heart development and skeletal muscle development [211]. Mice mutated for Pax3 has Splotch phenotype, due to melanocyte developmental defects [211]. In humans, Pax3 mutations are implicated in Waardenberg syndrome, which is characterized by pigmentation defects and deafness due to neural crest defects [214,215]. Generally, Pax genes are proto-oncogenes. Chromosomal
translocations which increase transcriptional enhancer activity of Pax3, such as t(2;13) resulting in Pax3-FKHR fusion proteins, have been reported in cases of alveolar rhabdomyosarcoma [216,217]. In addition, increased Pax3 expression has been implicated in melanoma [218,219]. However, *pax-3* was not previously characterized in the context of activated Ras, and it remains a relatively poorly characterized gene in *C. elegans*.

### 4.5 The roles of *pax-3* in vulval development and as putative Ras-cooperating proto-oncogene

In an RNAi screen to map out genetic interaction network in *C. elegans*, Byrne et al (2007) found that *pax-3* genetically interacted with several genes that function in vulval development including RTK *let-23*, beta-catenin *bar-1*, GRB2 *sem-5* and GEF *sos-1* [183]. *pax-3* is an essential gene in *C. elegans* and to date no viable hypomorphic mutant of *pax-3* has been characterized. However, other existing evidences also suggest that *pax-3* is very likely to have roles in vulval development. In *Pristionchus pacificus*, *Ppa-pax-3* is involved in the formation of vulval equivalent group as it regulates cell survival of central Pn.p cells and cell death of posterior epidermal cells. In addition, *Ppa-pax-3* is a direct target of *Ppa-lin-39* [182]. In *C. elegans*, *lin-39* mutants are generation-vulvaless, because all the VPCs fuse with the hypodermis. In *P. pacificus*, *Ppa-pax-3* as well as *Ppa-lin-39* are both generation vulvaless.

*C. elegans pax-3(RNAi)* worms generally have body morphology defects, uncoordinated movements and egg laying defects, emphasizing that *pax-3* has pleiotropic phenotypes and is generally required for viability. Homozygosed *pax-3(tm1771)* worms typically die as embryos or during L1 stage. Therefore, tissue-specific and time-specific perturbation of *pax-3* functions or a mosaic analysis would be essential to understand the roles of *pax-3* in any post-embryonic organogenesis process such as vulval development. In addition, with the advent of CRISPR as a targeted genomic editing tool [118], it may be possible to create hypomorphic viable mutants of *pax-3*. 

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4.6 *pax-3* may function at more than one step in vulval development

In *C. elegans*, PAX-3 regulates the specification of cells that would develop to become VPCs [194], which is consistent with my observation that *pax-3* worms did not completely develop vulval tissue but still have a ventral protrusion. In addition, *pax-3* expression patterns during vulval development suggest that *pax-3* may also play roles in the vulval development.

Based on my analysis of fosmid-based reporter expression, during 1-cell fate *pax-3* is expressed in P4.p – P8.p, and is most highly expressed in P6.p, with less in P5.p/P7.p and even less in P4.p/P8.p (Figure 3.24A). After the first cell division, expression is only detectable in P6.p daughters and granddaughters before upregulating again. During the L4 stage, after three rounds of VPC cell division, *pax-3* is expressed at high levels in vulC and vulD, and less so in vulE and vulF (Figure 3.24D and 3.25C). Therefore, *pax-3* may be upregulated downstream of vulval induction, and it is possible that this upregulation is an important step of vulval cell fate determination.

This dynamical expression pattern is remarkably similar, although not identical, to genes that also function late in vulval development like *egl-17* (Figure 3.37). Possibly, the modest differences in observed expression patterns of *egl-17* reporters and *pax-3* fosmid-based reporters may reflect differences in protein turnover or post-translational modifications between that of GFP and full-length *pax-3* tagged with GFP, while both genes share similar transcriptional regulation. *egl-17* is expressed in all induced VPC cells with highest level in P6.p at 1-cell stage, before upregulation only in P6.p cell and its descendants and shutting off in all other VPCs in subsequent stages [35,57]. During L4 stage, *egl-17* expression is again upregulated in vulC and vulD cells but is turned off in other cells. Since *egl-17* expression is regulated by a network of transcription factors including *lin-11*, *cog-1*, *lin-29* and *egl-38* [193], I suspect that *pax-3* may also be regulated by these transcription factors.

Since the *pax-3* promoter sequence (defined as the 3 kb upstream region from the start codon) was not sufficient to drive reporter expression, I tested whether intronic sequences of *pax-3*
promoter were sufficient to restore expression together with the promoter sequence (Figure 3.22). From this investigation, I found that the 5th intron of pax-3 contains sequence elements which may be responsible for expression in vulC and vulD cells (Figure 3.22, fragment A), whereas, other introns of pax-3 appears to contain sequence element which may be responsible for pax-3 expression in vulE and vulF cells during L4 stage (Figure 3.22, fragment B). A phylogenetic footprinting analysis together with direct DNA binding assays may reveal transcription factors that regulate pax-3 expression in the vulva. In addition, specific perturbation of such DNA sequence elements in the introns may make it possible to generate viable hypomorphic alleles of pax-3 which only have vulval development defects. As with several other examples, perhaps multiple levels of added complexity of transcriptional regulation allows this one gene to be used multiple times during development, and in different cell types.

4.7 Overall significance

Tumorigenesis is a process where genetic interactions occur between various mutations that accumulate in the tumor genome. Distinguishing between the key driver and passenger mutations, as well as the mechanisms of interactions among them, are critical to determining prognosis and successful treatments for cancer. In this thesis, I addressed these problems by employing in vivo RNAi screening in an activated Ras mutant of C. elegans. From the screen, I identified several new enhancers and suppressors of activated Ras phenotype.

Of particular interest is the transcription factor blmp-1, which encodes the ortholog of the mammalian Blimp1 gene. Blimp1 regulates B-lymphocyte terminal differentiation into antibody-secreting cells, which is consistent with the role of blmp-1 in regulating terminal differentiation of vulval cell fates [179]. I made the first connection between blmp-1 and Ras signaling for the first hallmark of cancer, and established that blmp-1 is a Ras-cooperating tumor suppressor gene. In addition, blmp-1 appears to be downregulated downstream of vulval induction which involves Ras signaling pathway. Just as loss of Blimp1 alone in activated B cells would not be sufficient to drive full-blown tumorigenesis, loss of blmp-1 alone is not sufficient to cause Muv phenotypes. Interestingly, overexpression of blmp-1 in the vulva results in reduced vulval induction both in the vulva and in ectopic pseudovulvae, but did not suppress
the Muv phenotype. This suggests that \( blmp-1 \) is downregulated downstream of Ras signaling and would appear to play a role late in vulval development. The precise mechanisms in which \( blmp-1 \) cooperates with Ras or is downregulated downstream of Ras remain to be elucidated.
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### Supplementary Table 1: List of genes included in RNAi screens

### Supplementary Table 1A: Chromatin factor list

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<td>TCA GAA AAA TTC AAG CCT GTT CAA AAG GAG CCG ACA ACT TTC</td>
</tr>
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<td>nw20</td>
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<td>nw21</td>
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</tr>
<tr>
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<td>CGA GGT TTA TGA ACA AAA ATT ATG ATC CCC ACA AGT CAT AGT CC</td>
</tr>
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<td>nw23</td>
<td>Rev7</td>
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</tr>
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<td>nw24</td>
<td>Fwd7</td>
<td>GTA TCT AAA GCA ATT TGA TGC AAA CGC GAA AGT TGA GCA ACG</td>
</tr>
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<td>nw26</td>
<td>Rev8</td>
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</tr>
<tr>
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<td>GGA GAG GGA AAA GGC GAA GGA AGA ACT CGA AGA ATT G</td>
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<td>TGT CTT TCT TGG CAA AAA TCT TCT TTC CTT CTT CTA CTA GAG</td>
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<td>nw41</td>
<td>10-6 Fwd blmp-1prdel</td>
<td>GTT TTC AAT TTC AGA AAA TAA ATA ATC TCA TGG GTC AAG GAA GTG GG</td>
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<td>nw42</td>
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<td>nw43</td>
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<td>AAG AAC AGG TTC AAG GGG ATG CAA GAG AGT GCG CTA</td>
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<td>nw46</td>
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<td>2-3 fwd blmp-1prdel</td>
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<td>nw48</td>
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<tr>
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<td>nw50</td>
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<td>TCT TCC GAA GAG TGT GAG TTA TCA GAC GAA GTT CAAG AAA GGG ACC GG</td>
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<td>GAA AGT TGT CGG CTC CTT TTC CCG CCT CGC TTC TTC TTT T</td>
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<td>nw52</td>
<td>Oligo 5-1 reverse</td>
<td>AAA AGG AGC GAG GCG GGA AAA GGA GCC GAC AAC TTT C</td>
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<tr>
<td>nw53</td>
<td>Oligo 5-2 forward</td>
<td>TGA AAA AGA CAA ACA GAT CTG ACT TAC TTC TTT TGA AAG TTG CCA</td>
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<tr>
<td>nw54</td>
<td>Oligo 5-2 reverse</td>
<td>TGG CAA CTG CCA AAA AAA GTG AGT CAG ATC TGT TTC TCT TTT TCA</td>
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<td>nw55</td>
<td>Oligo 5-3 forward</td>
<td>CTT TTA ACT TTC TTT TCT CAC CCG TTT GTG CCC CGC GTG AT</td>
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<td>Oligo 5-3 reverse</td>
<td>ATC ACC GCG GGC ACA AAC GGG TGA GAA AAA AAA GTT AAA AG</td>
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<tr>
<td>nw57</td>
<td>Oligo 5-4 forward</td>
<td>GGT TTT TTA TAC AAG CTC TGG AAT TGA ACA AGC TTG AAT TTT TCT GA</td>
</tr>
<tr>
<td>nw58</td>
<td>Oligo 5-4 Reverse</td>
<td>TCA GAA AAA TTC AAG CTT GTT CAA TTA CAG AGC TTG TAT AAA AAA CC</td>
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<td>dpy-7F-Hind3</td>
<td>ACA GAC AAG CTT GAA CAA TCT ATT TGT AAT CTC ATT C</td>
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<tr>
<td>nw60</td>
<td>dpy-7R-BamHI</td>
<td>ACA CAC AGG ATC CGA GTT TTG ACA AAA AAT ATA AAT TAA A</td>
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<td>nw61</td>
<td>blmp-1pr-sub200_2A</td>
<td>GAC GAT CCT CTC CTC GAA TC</td>
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<tr>
<td>nw62</td>
<td>blmp-1pr-sub200_2B</td>
<td>AAC AGT GCC AGC AAA ACC TTC TTC CGA AGA GTG TGA GGT</td>
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<td>nw63</td>
<td>blmp-1pr-sub200_5A</td>
<td>GAA AGT TGT CGG CTC CTT TT</td>
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<td>nw64</td>
<td>blmp-1pr-sub200_5B</td>
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<td>nw65</td>
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<td>ACT TTG GCG GAT CTC TAC AGG CCC GAT ATG CAA GAA CAG GTT CAA GGG GAA AGG TTT TGC TGC CAC TGT T</td>
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<tr>
<td>nw66</td>
<td>blmp-1pr-sub300-10_stitchF</td>
<td>AAG GTT TTG CTG GCA CTG TTT TTG TTC TCT TCT TAA CTC TGC</td>
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<td>nw67</td>
<td>5th50bp(2del1)blmp-1+10</td>
<td>ACT TTG GCG GAT CTC TAC AGG CCC GAT ATG CAA GAA CAG GTT CAA GGG GAA AGG TTT TGC TCT TCA CTC TGC</td>
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<td>nw68</td>
<td>unc-54_3'UTR_D</td>
<td>AAG GGC CCG TAC GGC CGA CTA GTA GG</td>
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<td>nw69</td>
<td>unc-54_3'UTR_D*</td>
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<td>nw70</td>
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<td>CTG GAT CTG GTC AAC AGC AA</td>
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<td>nw71</td>
<td>3'FosGenotype_blmp-1R</td>
<td>AAT GGG AGG ATG GAG AAA GG</td>
</tr>
<tr>
<td>nw72</td>
<td>pax-3(tm1771)F</td>
<td>AGA GCT CAT CAA GCA TCA CA</td>
</tr>
<tr>
<td>nw73</td>
<td>pax-3(tm1771)R</td>
<td>TTT GCT TTC GCT AGG CTT AT</td>
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Supplementary Table 3: Plasmids generated in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>pNW1</td>
<td>The ORFeome plasmid was verified for correct sequence, and point mutations were eliminated using restriction cloning. BLMP-1 cDNA was then amplified with oligos nw1 and nw2 and subcloned into pPD95.75 in frame with GFP using HindIII and KpnI.</td>
</tr>
<tr>
<td>pNW2</td>
<td>blmp-1 promoter (2 kb upstream of start codon) and the first 703 bp of blmp-1 CDS was amplified with oligos nw3 + nw4 from fosmid WRM0635aE10 and subcloned into pUC19 with XbaI.</td>
</tr>
<tr>
<td>pNW3</td>
<td>Pblmp::GFP::unc-54 3' UTR blmp-1 promoter and the first 35 bp of blmp-1 ORF was subcloned with endogenous XbaI and NgoMIV sites from pNW2 into pPD95.77 (digested with XbaI and SmaI) resulting in the first 35 bp of BLMP-1 expressing in-frame with GFP.</td>
</tr>
<tr>
<td>pNW4</td>
<td>Pblmp::BLMP-1::GFP::unc-54 3' UTR blmp-1 promoter was subcloned with XbaI and NgoMIV from pNW2 into pNW1.</td>
</tr>
<tr>
<td>pNW5</td>
<td>pNW3 with 2xNLS added, or Pblmp::GFP2xNLS::unc-54 3' UTR. A fragment of GFP from pPD135.83 was subcloned into pNW3 with XbaI and NgoMIV sites from pNW2 into pPD95.77.</td>
</tr>
<tr>
<td>pNW6</td>
<td>Pblmp::BLMP-1::GFP2xNLS::blmp-1 3' UTR blmp-1 3' UTR was subcloned into pNW5 using EagI and EcoRI.</td>
</tr>
<tr>
<td>pNW7</td>
<td>pUC19-lin-31pr ~2 kb sequence upstream of lin-31 start codon was amplified off genomic DNA using oligos nw5 and nw6, adding HindIII sites and subcloned into pUC19.</td>
</tr>
<tr>
<td>pNW8</td>
<td>Lin-31::GFP::unc-54 3' UTR 2 kb promoter from pNW7 was subcloned into pPD95.75 using HindIII.</td>
</tr>
<tr>
<td>pNW9</td>
<td>Lin-31::BLMP-1::GFP::unc-54 3' UTR 2 kb lin-31 promoter from pNW7 was subcloned into pNW1 using HindIII.</td>
</tr>
<tr>
<td>pNW10</td>
<td>L4440-pax-3_B</td>
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<tr>
<td>pNW11</td>
<td>pNW5 deleted for the first 200 bp (-1896..-2095) Two overlapping PCR fragments M13Rev-27 (nw10) + nw9 and nw11 + nw12 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with InvitrogenTM TOPO TA cloning before subcloning back into pNW5.</td>
</tr>
<tr>
<td>pNW12</td>
<td>Two overlapping PCR fragments M13Rev-27 (nw10) + nw9 and nw11 + nw12 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with InvitrogenTM TOPO TA cloning before subcloning back into pNW5.</td>
</tr>
<tr>
<td>pNW13</td>
<td>pNW5 deleted for the second 200 bp (-1696..-1895) Two overlapping PCR fragments M13Rev-27 (nw10) + nw13 and nw11 + nw14 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with InvitrogenTM TOPO TA cloning before subcloning back into pNW5.</td>
</tr>
<tr>
<td>pNW14</td>
<td>pNW5 deleted for the 3rd 200 bp (-1496..-1695) Two overlapping PCR fragments M13Rev-27 (nw10) + nw15 and nw11 + nw16 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with InvitrogenTM TOPO TA cloning before subcloning back into pNW5.</td>
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<tr>
<td>pNW15</td>
<td>pNW5 deleted for the 4th 200 bp (-1296..-1495) Two overlapping PCR fragments M13Rev-27 (nw10) + nw17 and nw11 + nw18 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with InvitrogenTM TOPO TA cloning before subcloning back into pNW5.</td>
</tr>
<tr>
<td>pNW16</td>
<td>pNW5 deleted for the 5th 200 bp (-1096..-1295) Two overlapping PCR fragments M13Rev-27 (nw10) + nw19 and nw11 + nw20 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with InvitrogenTM TOPO TA cloning before subcloning back into pNW5.</td>
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| pNW17 | pNW5 deleted for the 6th 200 bp (-896..-1095)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw21 and nw11 + nw22 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW18 | pNW5 deleted for the 7th 200 bp (-696..-895)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw23 and nw11 + nw24 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW19 | pNW5 deleted for the 8th 200 bp (-496..-695)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw25 and nw11 + nw26 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW20 | pNW5 deleted for the 9th 200 bp (-296..-495)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw27 and nw11 + nw28 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW21 | pNW5 deleted for the 10th interval of 295 bp just upstream of blmp-1 start codon (-1..-295)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw29 and nw11 + nw30 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 295 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW22 | pNW5 deleted for the 5th 50 bp (-1846..-1895)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw44 and nw11 + nw43 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 50 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW23 | pNW5 deleted for the 6th 50 bp (-1796..-1845)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw46 and nw11 + nw45 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 50 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW24 | pNW5 deleted for the first 7th 50 bp (-1746..-1795)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw48 and nw11 + nw47 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 50 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW25 | pNW5 deleted for the 8th 50 bp (-1696..-1745)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw50 and nw11 + nw49 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 200 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW26 | pNW5 deleted for the 17th 50 bp (-1246..-1295)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw52 and nw11 + nw51 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 50 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
| pNW27 | pNW5 deleted for the 18th 50 bp (-1196..-1245)  
Two overlapping PCR fragments M13Rev-27 (nw10) + nw54 and nw11 + nw53 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 50 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5. |
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<td>Two overlapping PCR fragments M13Rev-27 (nw10) + nw56 and nw11 + nw55 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 50 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5.</td>
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<td>Two overlapping PCR fragments M13Rev-27 (nw10) + nw36 and nw11 + nw35 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 50 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5.</td>
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<td>Two overlapping PCR fragments M13Rev-27 (nw10) + nw42 and nw11 + nw41 were amplified in separate reactions, and then subsequently used as templates together with oligos nw10 + nw11 to delete the 50 bp. The fragments were then captured with Invitrogen™ TOPO TA cloning before subcloning back into pNW5.</td>
</tr>
</tbody>
</table>

| pNW36 | pPD95.75-dsRed was made by swapping GFP with dsRed from pPRRF162 using KpnI and EcoRI. 200 bp region containing hypodermal-specific enhancer of dpy-7 promoter [208] was amplified with oligos nw59 + nw60 and subcloned into pPD95.75-dsRed with HindIII and BamHI. |

| pNW37 | 2nd 200 bp region of blmp-1 was amplified from pNW5 with nw61 + nw62. PCR product was captured with TOPO TA cloning. Clone with the forward orientation was selected and subcloned into pPD122.53 with XbaI and HindIII. |

| pNW38 | 5th 200 bp region of blmp-1 promoter was amplified with oligos nw63 + nw64. PCR product was captured into pCR2.1 with TOPO TA cloning. Clone with the reverse orientation was then selected and subcloned with SpeI and SphI into pPD122.53 digested with SphI and XbaI. |

<p>| pNW39 | 5th 50 bp region of blmp-1 promoter was attached to pes-10 promoter::gfp::nls::unc-54 3'UTR using oligo nw65 + nw68 and plasmid pPD122.53 as template. Fragments were then captured into pCR2.1 with Invitrogen™ TOPO TA cloning kit. |</p>
<table>
<thead>
<tr>
<th>pNW40</th>
<th>Last 295 bp region of <em>blmp-1</em> promoter, with gfp and unc-54 3’UTR was amplified with oligos nw66 + nw69 using pNW5 as template and captured into pCR2.1 with InvitrogenTM TOPO TA cloning.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNW41</td>
<td>Last 295 bp region of <em>blmp-1</em> promoter amplified with 5th 50 bp sequence of <em>blmp-1</em> promoter using oligos nw67 + nw68 and pNW5 as template. PCR product was captured into pCR2.1 with InvitrogenTM TOPO TA cloning kit.</td>
</tr>
<tr>
<td>pNW42</td>
<td>Last 295 bp region of <em>blmp-1</em> promoter, with gfp2xNLS and unc-54 3’UTR was amplified from pNW5 using oligos nw66 + nw68. The 2nd 200 bp region of <em>blmp-1</em> promoter was amplified from pNW5 using oligos nw61 + nw62. These two PCR products were stitched together using overlapping PCR with oligos nw61 + nw69. The correct band of PCR product was captured into pCR2.1 with InvitrogenTM TOPO TA cloning kit.</td>
</tr>
</tbody>
</table>