Genetic and functional characterization of putative Ras/Raf interaction inhibitors in *C. elegans* and mammalian cells

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ABSTRACT

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(Under the direction of Adrienne D. Cox, PhD)

Molecularly targeted inhibitors are typically screened in cell-based assays for activity and target selectivity. However, early *in vivo* evaluation may improve characterization of specificity and/or detection of toxicity or off-target effects. In this dissertation, I describe use of the nematode *C. elegans* as an *in vivo* model to characterize both on- and off-target activity of novel putative Ras/Raf interaction inhibitors. In mammals, the Ras>Raf>MEK>ERK signaling cascade promotes cellular proliferation. Its aberrant activation is associated with oncogenesis and it is an attractive pharmaceutical target. In *C. elegans*, this pathway is highly conserved and regulates vulval development. Constitutive pathway signaling results in an easily scored Multivulva (Muv) phenotype that provides an accurate *in vivo* readout for activity of inhibitors targeting the pathway. I therefore validated the Muv phenotype for evaluation of the activity and specificity of known and novel pathway inhibitors, beginning with the well-characterized MEK inhibitor U0126. I then characterized its response to small molecule members of the MCP family of putative Ras/Raf interaction inhibitors. Analysis of a *C. elegans* strain expressing activated Ras (worm ortholog, LET-60) showed that MCP110 and MCP116 act downstream of Ras, causing significant dose-dependent reduction of Muv. Analysis of strains

genetically activated downstream of Ras showed that these compounds act upstream of the ETS-like transcription factor (LIN-1) and the MAP kinases, MEK-2 and MPK-1. The best available strain expressing activated Raf (LIN-45AA) was unable to distinguish whether these compounds act at the level of Ras or of Raf, and may not be Ras-independent. I then turned to cell-based assays using NIH 3T3 mouse fibroblasts, and showed for the first time that MCP110 dose-dependently disrupts the physical interaction between Ras and Raf and impairs Ras recruitment of Raf to cellular membranes. I also narrowed the affected protein:protein interaction to that of Ras with the Ras binding domain (RBD) of Raf. Finally, I identified specific NPYR-like off-target effects of MCP compounds in *C. elegans*. Thus, *C. elegans* is a valuable *in vivo* genetic system to characterize on- and off-target activity of inhibitors targeting the Ras>Raf>MEK>ERK pathway and may be useful for other novel therapeutics.

DEDICATION

To my parents and best friends, Amparo and Manuel, who have always been supportive of my career choices, thanks for your great words of encouragement and for always believing I could make all my dreams come true.

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AC	anchor cell
ATP	adenosine triphosphate
BMS	Bristol-Myers Squibb
C-terminus	carboxyl terminus
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FTI	farnesyl transferase inhibitor
GST	gluthatione S-transferase
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
Gf	gain-of-function
GPCR	G-protein coupled receptor
GTP	guanosine-5'-triphosphate
HA	hemagglutinin
H-Ras	Harvey sarcoma virus rat sarcoma protein
HDM2	human protein double minute 2
HOP	Hsp organization protein
HTS	high-throughput screen
IB	immunoblot
L4	larval stage 4

Lf	loss-of-function
MAPK	mitogen-activated protein kinase
MCP	Morphochem products
MOA	mechanism of action
Muv	multivulva phenotype
NPYR	neuropeptide Y receptor
p-ERK	phosphorylated ERK
PI3K	phosphatidylinositol 3-kinase
PM	plasma membrane
PPI	protein-protein interaction
RalGEF	Ral guanine nucleotide exchange factor
RBD	Ras-binding domain
RCC	renal cell carcinoma
S.A.	soft agar
SAR	structure-activity relationship
VEGF	vascular endothelial growth factor receptor
VPC	vulval precursor cell
Vul	vulvaless
YFP	yellow fluorescent protein

CHAPTER I

INTRODUCTION

Molecularly targeted therapeutics

Increased understanding of the molecular events underlying human diseases, coupled with the development of novel techniques and equipment to increase throughput for identification of inhibitors of critical molecular drivers of disease, have allowed rapid growth in the area of molecularly targeted therapeutics. Each year more drug screening approaches are utilized and hundreds of thousands of chemical entities undergo preclinical and clinical evaluation with the goal of becoming successful drug treatments. The drug discovery process is long and costly, where more battles are lost than won, and where the increasing amount of knowledge in each disease only becomes more complex than the day before.

In this chapter I aim to offer a brief summary of the drug discovery process for treatment of Ras-dependent cancers, the elements involved in it and some examples of validated targets and some therapeutics against them. I will then describe the background underlying the search for targeted therapeutics aimed at blocking the interaction of the small GTPase Ras with one of its key effectors, the serine/threonine kinase Raf. Together, these will serve as an introduction to my dissertation research that is described in subsequent chapters.

Target identification and validation

Identifying appropriate anti-cancer targets is an ongoing quest for many researchers seeking to develop novel therapeutics for cancer treatment. Over decades, a variety of genetic lesions have been identified as the driving forces for the development of cancers (Ali and Sjoblom, 2009; Liu, 2008), and as a consequence they have become the focus for the development of molecularly targeted therapeutics. Nowadays, the availability of genomic and proteomic databases have provided endless sources to identify novel genes in a more rapid and efficient way than before. The elucidation of these genetic lesions, which can include the activation of oncogenes or the silencing of tumor suppressor genes, are important not only for the discovery of novel therapeutics but also for the early detection of disease (Midorikawa *et al.*, 2009). However finding genes associated with cancer is just the first step. For example, not all genes associated with cancer are ideal candidates for the subject of a high-throughput screen. First, the gene's disease relevance to the disease must be validated, followed by analysis of its molecular properties that would allow a given target of interest to be targeted by a chemical entity.

A key question for target selection is if the target is sufficient and/or necessary for the onset and progression of a particular malignancy. For example, in a given sample of tumor tissue it is possible to detect many genetic lesions. However, only a subset of those genes would be sufficient and/or necessary for the onset or maintenance of a tumor (i.e., would be "driver" mutations rather than "passenger" mutations). Another important aspect of target selection is to

understand it in the context of cellular signaling networks and its biological role in the progression of a disease. The latter is of great importance because we want to target specific genes or signaling networks, without interfering with other pathways that may be essential for proper cell function. Target selection and validation nowadays often follows the "omics" methods of gene transcription profiling ('transcriptomics'), protein expression profiling ('proteomics'), metabolic pathways ('metabolomics'), protein glycosylation ('glycosilomics'), protein–protein interactions ('interactomics') and systems biology *in silico* (Anders and Vielhauer, 2007). In combination with classical preclinical evaluation methods examining proteins individually, many potentially relevant targets have been discovered, validated and subjected to drug screens. However, a target is only truly validated when inhibitors of it reach the clinic (Colombo and Moll, 2008), and more candidate inhibitors fail than succeed.

Over the years various approaches have been used for the selection of targets for drug development. Some targets surface as a result of a group's independent research in the function of specific genes, proteins and/or pathways. Others have been discovered as the result of data mining of the now enriched genomic databases and genome profiling results. Additional strategies that have also proved to be successful for the identification of therapeutic targets for example in hepatocellular carcinoma, and that can be also applicable to various other malignancies, include: high throughput genetic analysis, comprehensive expression analysis, gene copy number analysis, promoter arrays and tiling arrays (Midorikawa *et al.*, 2009). One new method of choice is gene silencing by interference by RNA

(RNAi). RNAi takes advantage of a naturally occurring mechanism by which cells regulate the expression of genes at the post-transcriptional level, and introduces a new era in loss-of-function experiments, allowing for the rapid measurement of the phenotype observed upon target expression abrogation (Colombo and Moll, 2008). RNAi results in the knockdown or silencing of a gene by the binding of short-double stranded RNAs molecules, also known as small-interfering (siRNAs), to their complementary mRNA, which leads to mRNA degradation.

Currently, RNAi is the most widely used method for the specific and rapid inhibition of gene expression and its high efficiency, strong potency, fast turnaround of results and its potential to be developed into HTS have made it the method of choice for both target identification and validation. Another advantage of using RNAi is that it can be used at different stages of the drug discovery process starting at HTS for initial target identification, in *in vitro* biological assays for in-depth analysis of gene function *in vitro* to *in vivo* models for the analysis of gene function in animals (Lavery and King, 2003) .

Lately, RNAi screens have proved to be a popular approach to confirm known genetic lesions as well to identify novel genetically altered targets in patient samples. A recently reported screen of primary leukemia cells from 30 patients, by RNAi, identified targets that are critical to survival of the malignant cells. Most of these targets were known and widespread, like activating mutations in K-RAS, N-Ras and JAK2, FLT1, CSF1R and PDGFR. Less expected were ROR1, EPHA4/5 and LMTK3. Novel somatic mutations in the thrombopoietin receptor were also identified (Tyner *et al.*, 2009). However, an important caveat with such a widespread use of

RNAi is that because it prevents expression of the entire protein, it does not mimic very well the consequences of drug action, that do not block expression but do impair specific functional aspects. Thus, RNAi has the same downside as described below for gene knockouts, in that it changes stoichiometry in ways that pharmacological agents do not. This can be very important for multi-functional proteins, and especially for scaffolding proteins, as is relevant in Chapter III.

For many years, oncogenic Ras has been considered an attractive target for anti-cancer therapeutics based on its status as the most frequently mutated oncogene in human cancers (Bos, 1989). It was additionally important to validate its ability to regulate cancer-related biological readouts like anchorage-independent growth or tumor growth in xenograft models, as will be discussed below. Recently, RNAi has been used to validate which downstream effectors are needed by Ras, and this helped confirm a role for the serine/threonine kinase, Raf. There was also a need to validate Raf itself, as a critical downstream effector of Ras (see figure 1. 5 and 2.1), as a potential target for anti-cancer therapeutics. Since Raf phosphorylates MEK on serines 218 and 222, a MEK mutant in which these serines are substituted by alanines (MEK218A/222A) cannot be activated by Raf (Arboleda *et al.*, 2001). This MEK dominant negative mutant should block the Raf/MEK/ERK pathway, either by sequestering Raf or by sequestering ERK and blocking their wild-type activity (Lyons *et al.*, 2001; Zheng and Guan, 1994). Their observations showed the inability of cells harboring oncogenic Ras, but also expressing dominant negative MEK mutants, to support anchorage-independent growth, thus correlating with the reduction in MEK activity. Moreover they were also able to detect that the MEK

dominant negative mutant was able to block tumor progression in mice (Lyons *et al.*, 2001). These results were part of the evidence used to validate Raf as a therapeutic target and thus further strengthened the need to pursue drug discovery programs to find inhibitors of Raf.

Proper validation is critical for the drug discovery process, since understanding the role of the putative target will become key in the design of the drug screen, the selection of the screen endpoints and perhaps can aid in the understanding of the mechanism of action of chemical compounds that are potentially interacting with the selected target.

Small molecule compound screens

The purpose of a drug screen is to find small molecules that could serve as a lead compound for the development of a drug. Most commonly, small molecule libraries are browsed using high throughput screens (HTS). HTS are meant to monitor thousands of molecules per day, and the success of the screening platform strongly relies on the selection of the appropriate small molecule compound library in combination with robust assays to measure the selectivity and potency of the compounds being screened against the target of interest. Besides selecting the most appropriate system for the screen, it is also very important to take into consideration the class of compounds in a chemical library. The decision can be made based on the drug-like characteristics of the library, information that can be assessed from records of previous computational modeling analysis of the molecules or simply by the type of active lead candidates selected in the past from the same library. For example, for gene families such as kinases (Stahura *et al.*, 1999) and G-protein-

coupled receptors (Balakin *et al.*, 2002; Stahura *et al.*, 1999), there are hundreds of known active compounds that can be used as starting points for a ligand-based design. It is also important to take into consideration the physicochemical properties that are necessary to increase the likelihood of oral bioavailability as they have been formalized into Lipinski's "rule-of-five" (Lipinski *et al.*, 2001). Bioavailability is one of the most important pharmacokinetic properties of drugs, because compounds with good bioavailability will be able to circulate throughout the body and retain activity.

In vitro biochemical assays possess an advantage over cell-based or *in vivo* assays because there are no concerns about the cell permeability or potency of compounds. Another advantage of *in vitro* assays is that they can be performed at higher drug concentrations, which can help in the identification of novel chemical classes (Walters and Namchuk, 2003). On the other hand, cell-based systems can be employed to complement *in vitro* data, providing greater confidence in compound activity in an intact biological system (Horrocks *et al.*, 2003). An advantage provided by cell-based assays is that cell lines can be engineered to exogenously express or over-express a target of interest, thus making the screening platform more sensitive or powerful to select active compounds against the desired target. Several readouts are available for high-throughput cell-based assays. Among these are: i) reporter assays that link the promoter of the gene target to an appropriate reporter gene and ii) fluorescently modified antibodies in combination with high-throughput microscopy, that help monitor changes in particular aspects of cell morphology or function (Horrocks *et al.*, 2003) and proliferation, cytotoxicity, secretion, translocation, redistribution, protein expression and enzyme activity among others (Johnston,

2002). The use of these techniques has allowed the miniaturization of many cell-based assays and as a consequence increased the throughput of the screens.

Before performing an HTS, there are many important aspects to take into consideration, such as selecting the appropriate cell model, selecting the appropriate control to reduce the selection of false-positives and false-negatives, being able to produce sufficient cells for the HTS, the optimization for the assay for the drug screen and being able to capture a reproducible signal from the assay. All of the aspects mentioned before, are critical for the success of a cell-based HTS. Overall, cell-based assays can provide significant information on the nature of the pharmacological activity of a compound. Once the primary screen yields candidate compounds (hits), they undergo further testing to test their activity and target specificity to fewer active hits (leads) that can be further optimized and validated in preclinical models before advancing into clinical evaluation.

Ras proteins: anticancer targets regulated by GTP/GDP cycling and membrane localization

Ras (Rat sarcoma) is the founding member of a large, evolutionarily conserved superfamily of small GTPases. Ras family members are subdivided into 5 major branches, based on sequence and functional similarities: Ras, Rho, Rab, Ran and Arf (Wennerberg *et al.*, 2005) (Figure 1.1). Ras proteins have been studied for the past 30 years and they are now known to participate in a variety of important biological functions such as regulation of gene expression, cell survival, actin organization and cell cycle progression (Figure 1.2).

Ras is synthesized in the cytosol and post-translationally modified to be recruited to the plasma membrane and endomembranes (Chiu *et al.*, 2002; Hancock *et al.*, 1990). At the plasma membrane, Ras cycles between inactive (GDP-bound) and active (GTP-bound) states (Figure 1.3A). Guanine nucleotide exchange factors (GEFs) positively regulate Ras proteins by stimulating the exchange of GDP for GTP, whereas GTPase activating proteins (GAPs) negatively control Ras family members by catalyzing the hydrolysis of GTP, causing them to become GDP-bound and inactive (Bernards and Settleman, 2004; Boguski and McCormick, 1993; Giglione *et al.*, 1997). The structural differences between GDP- and GTP-bound states of Ras are localized to two regions of the protein, switch I (Ras residues 32-38) and switch II (residues 59-67), with the GTP conformation having increased affinity for downstream effectors (Figure 1.3B) (Mitin *et al.*, 2005). Ras effectors bind through the “effector domain” flanking residues 25-45 (Figure1.4) (Vojtek and Der, 1998) (White *et al.*, 1995) and in fact, one requirement for a given protein to be designated as a Ras effector is that it interacts preferentially with GTP-bound, active Ras compared to GDP-bound, inactive Ras. Direct disruption of the Ras/Raf interaction by specific protein:protein interaction inhibitors may therefore be the result of inhibitor binding to the Ras effector domain.

Ras proteins are synthesized in the cytosol where they are inactive, but immediately after synthesis they become lipid-modified and targeted to the plasma membrane. At the plasma membrane, GEF proteins are localized in response to extracellular stimuli, which in turn activate Ras to become GTP-bound and interact with effectors to transmit its signals. Oncogenically mutated Ras is chronically GTP-

bound and independent of upstream signals, but still requires correct localization to the plasma membrane for biological activity, presumably so it can promote activation of its downstream effectors such as Raf.

Ras association with the plasma membrane is mediated by a series of steps involving the modification of the carboxyl termini (CAAX motif) of all Ras proteins, where C= cysteine, A= aliphatic amino acid and X= terminal amino acid. This sequence drives the recognition of the Ras protein by the enzyme farnesyl transferase (FTase), which adds a 15-carbon (C_{15}) farnesyl isoprenoid lipid to the cysteine of the CAAX motif (Reiss *et al.*, 1990) to start the targeting to the plasma membrane. This step is followed by proteolytic cleavage of the -AAX residues by the endopeptidase, Rce1 (Ras and factor converting enzyme) (Boyartchuk *et al.*, 1997; Kim *et al.*, 1999; Otto *et al.*, 1999), and carboxyl methylation of the now terminal cysteine, by isoprenylcysteine carboxyl methyltransferase (Icmt) (Dai *et al.*, 1998; Hrycyna *et al.*, 1991). In addition to farnesylation, a second signal is need to target Ras to the plasma membrane. The three Ras isoforms that are ubiquitously expressed in the cell: H-Ras, K-Ras and N-Ras and they share sequence homology between amino acids 1-165, but they have distinct regulatory sequences for their efficient positioning in the plasma membrane (Apolloni *et al.*, 2000; Choy *et al.*, 1999; Hancock, 2003; Hancock *et al.*, 1990). It is noteworthy that these post-translational modifications are not only necessary for Ras localization to the plasma membrane and promotion of its activity but are also they are necessary to propagate the signals of oncogenic Ras in cancer cells. As will be discussed below, given the important role of enzymes mediating Ras plasma membrane localization in the

activation of Ras, they soon were considered attractive targets to generate novel anti-cancer therapeutics against Ras-driven malignancies. The lipid modification status also may influence the interactions of Ras with Raf by dictating the specific membrane domains for Ras and Raf localization (Fischer *et al.*, 2007; Thapar *et al.*, 2004). Finally, targeting Raf to the plasma membrane by the addition of the Ras C-terminal domain, including both CAAX and immediate upstream sequences, is sufficient to cause constitutive activation of Raf signaling (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), indicating the importance of the Ras/Raf interaction for Raf activation.

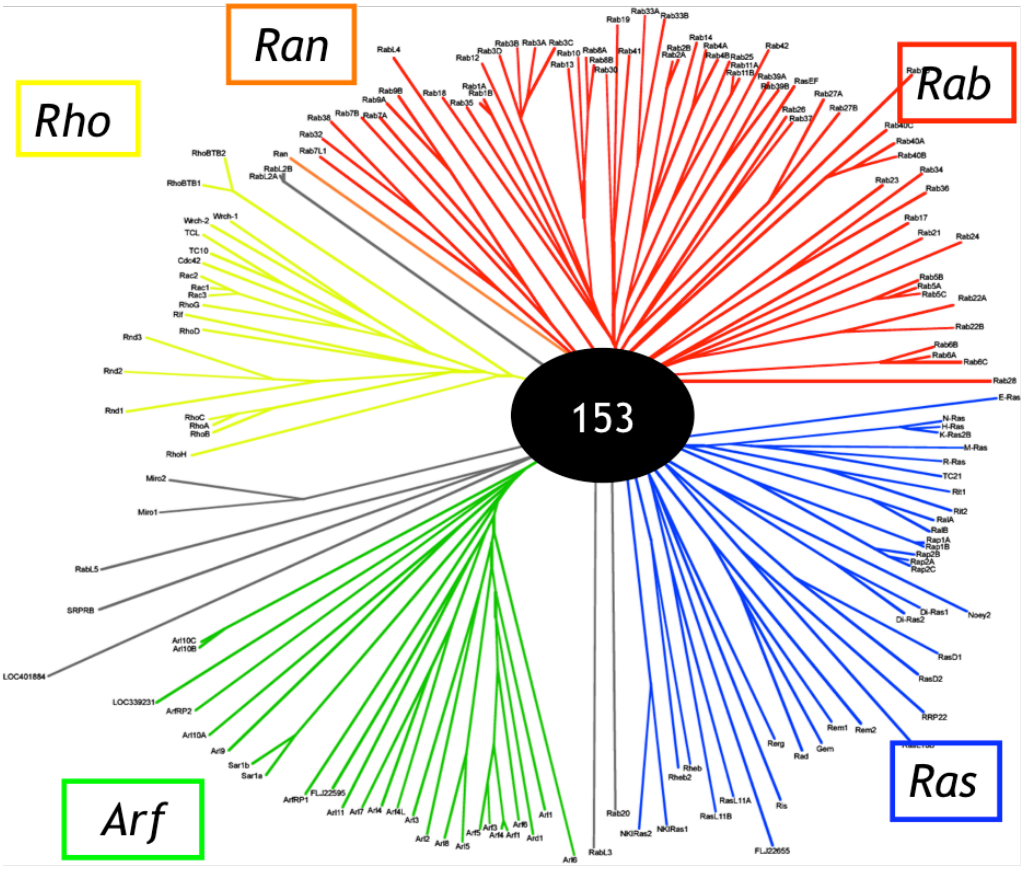

Figure 1.1 Ras belongs to a superfamiy of small GTPases. All five branches: Ras, Arf, Rho, Ran and Rab share biochemical mechanisms and functional similarities. Adapted from: Wennerberg et al. (2005) J Cell Science 117:1301

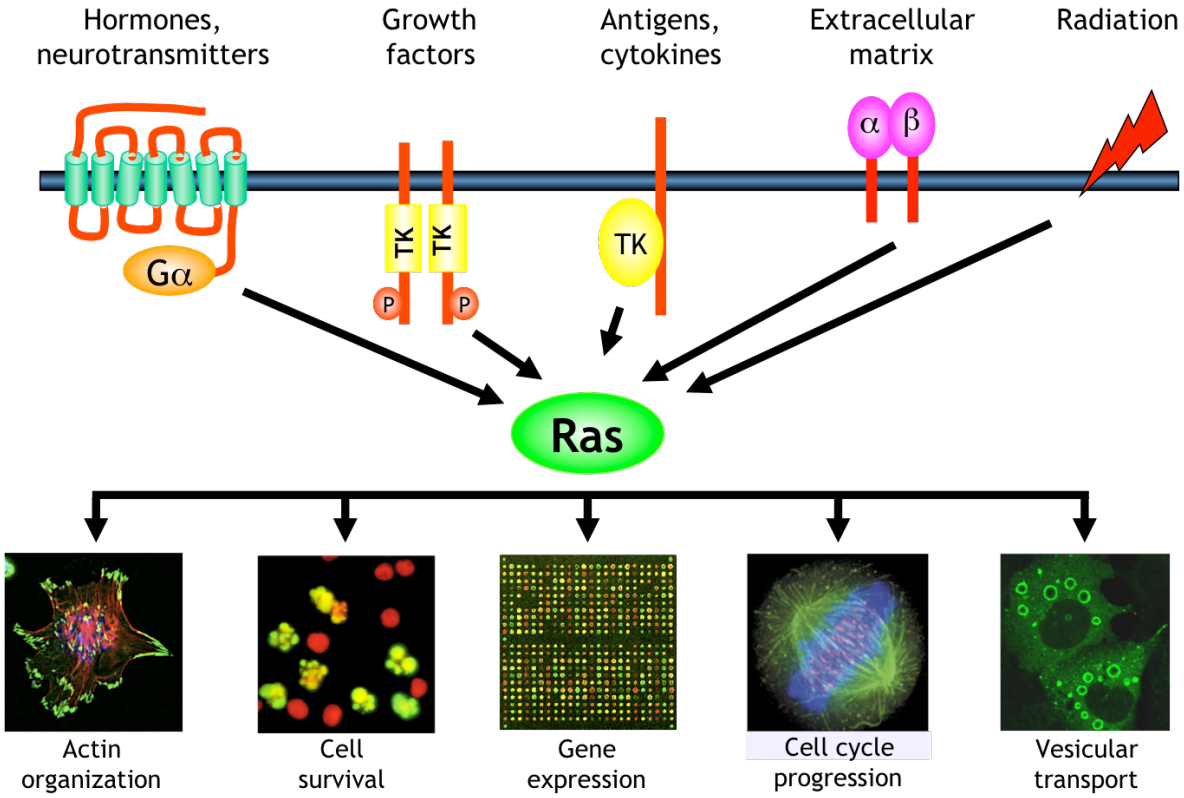

Figure 1.2 Ras acts as a signaling node
Ras transduces activating signals from upstream extracellular cues into the cell. In turn Ras acts as a signaling node to activate downstream effectors that regulate a wide variety of biological functions.

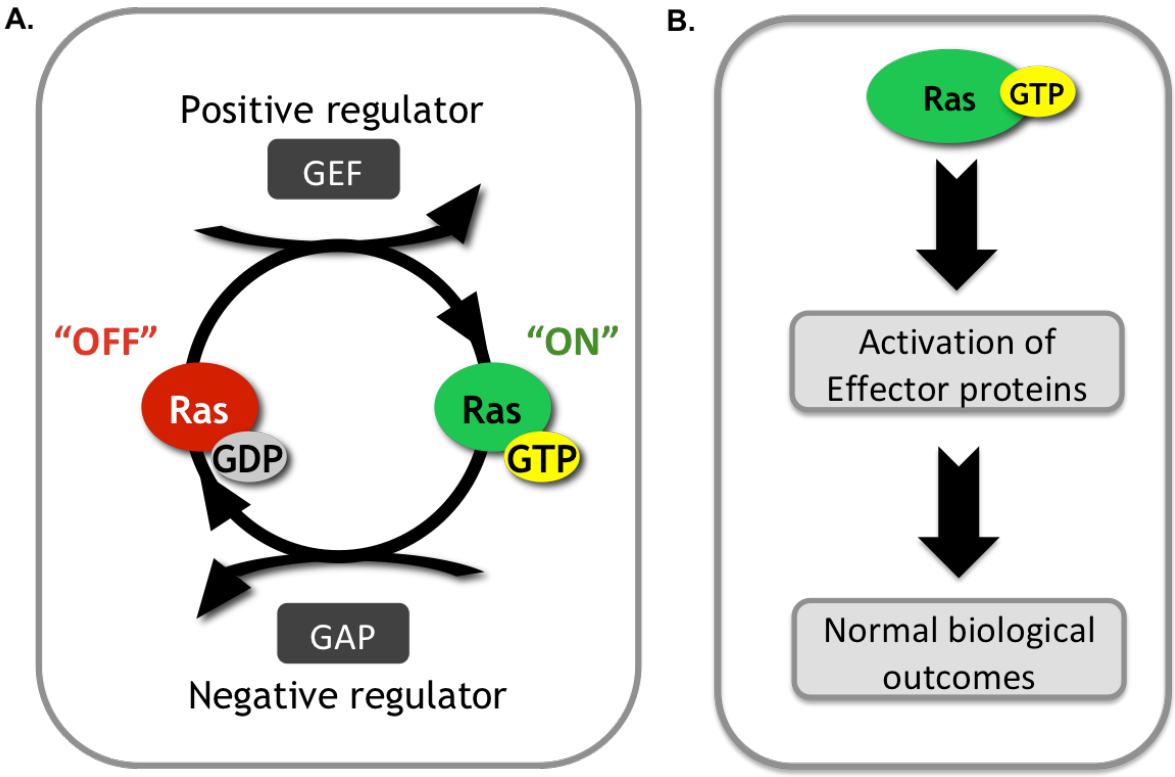

Figure 1.3 Ras cycles between an "ON" and "OFF" state
A) Regulation of Ras: Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP for GTP (Ras-GTP = active conformation), whereas GTPase activating proteins (GAPs) catalyze the hydrolysis of GTP (Ras-GDP = inactive conformation). B) Ras binding to GTP causes a conformational change in the effector binding domain that facilitate its interaction with and activation of downstream effectors to then regulate a variety of biological outcomes.

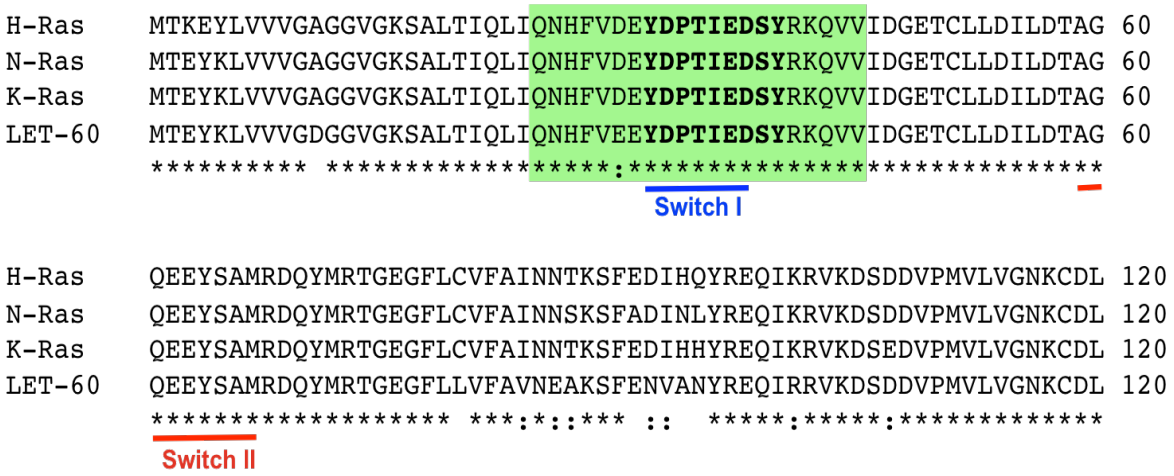

Figure 1.4. Ras protein effector domains
The effector domain, including core residues 32-40 (bold fonts) and flanking residues 25-45 (green box), is also known as switch I, because it switches conformation depending on whether Ras is in the active, GTP-bound state or the inactive, GDP-bound state. When GTP-bound, the effector loop is more solvent-exposed and therefore more available for interactions with effectors such as the cytoplasmic Raf serine/threonine kinases, which bind to Ras through their Ras binding domains (RBDs). The effector domain sequences of mammalian Ras proteins are closely conserved with that of the *C. elegans* Ras ortholog, LET-60. Sequence alignments were performed in ClustalW2 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html), were aligned residues marked (*) are identical, (:)conserved substitutions and (.)semi-conserved substitutions.

Ras is genetically altered in cancer

Genetic lesions in Ras are present in about 30% of all human cancers, with higher incidence in pancreas (90%), colon (50%), thyroid (50%), lung (30%) and melanoma (25%) (Bos, 1989) (Malumbres and Barbacid, 2003). Point mutations in residues 12, 13 or 61, turn Ras proteins into active oncogenes; mutations at these positions makes Ras insensitive to negative regulation by GAP proteins, therefore leaving the protein constitutively activated (Bos, 1989) (see Fig 1.6). The biological role of oncogenic Ras was initially measured by its ability to transform established rodent fibroblast cell lines leading to their growth in soft agar and tumorigenesis in nude mice (Clark *et al.*, 1995). Aberrant Ras activity leads to the de-regulation of numerous processes in the cell, such as cell proliferation, cell survival, apoptosis, migration, cell adhesion that in turn can contribute to cell transformation, invasion and metastasis (Campbell and Der, 2004).

As previously stated, Ras acts as a signaling node that transmits many upstream signals into many downstream signals (Figure 1.2). Ras activity can also be mis-regulated by some of its upstream regulators, and not only by mutations in Ras itself. One mechanism by which this happens is overexpression of the epidermal growth factor receptor (EGFR), for example in colorectal, pancreatic, lung and non-small lung cell cancer (Roberts and Der, 2007). On the other hand, downstream of Ras there are multiple effectors that contribute to transducing diverse biological consequences caused by oncogenic Ras, but for the purposes of this dissertation I will focus my discussion on the deregulation of the

Ras>Raf>MEK>ERK signaling pathway, initiated by signals from oncogenic Ras or Raf, and their biological consequences in cancer.

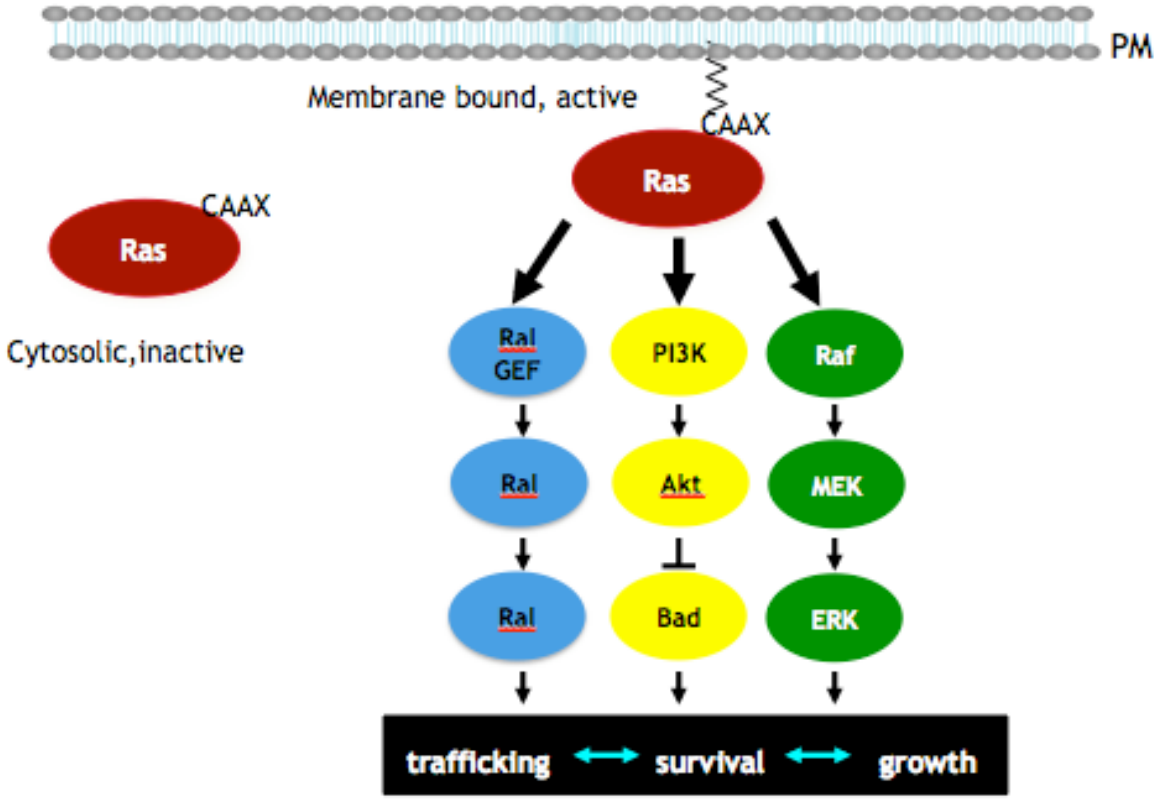

Figure 1.5. Simplified overview of Ras effector signaling pathways. Of the many Ras effectors (>20 to date), the best studied are the phosphatidylinositol 3-kinases (PI3-kinases), Ral guanine nucleotide exchange factors (RalGEFs) and the serine/threonine Raf kinases (A-Raf, B-Raf and Raf-1), whose combined signaling networks contribute to biological outcomes in the cell like trafficking, survival and growth among others.

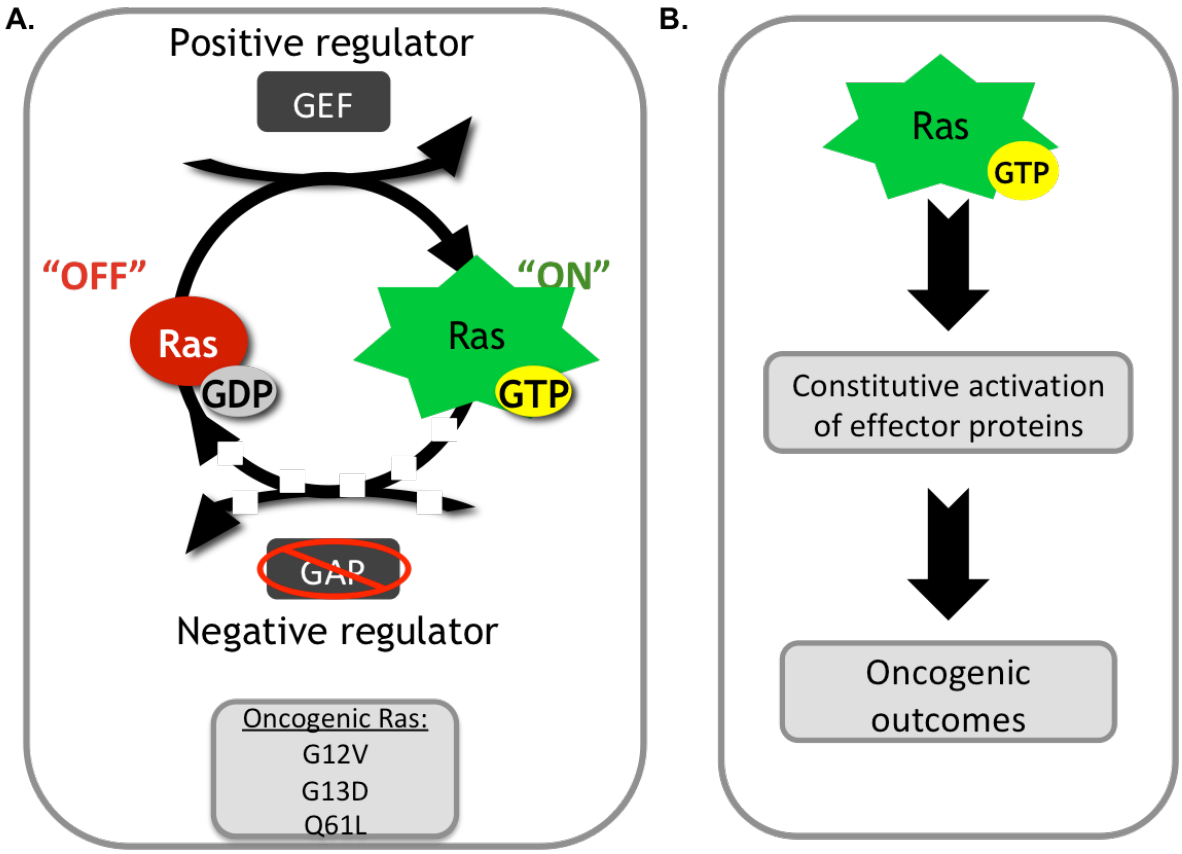

Figure 1.6 Oncogenic Ras regulation and biological effects. A) Point mutations in residues G12V, G13E or Q61L render Ras insensitive to GAP negative regulation and as a result oncogenic Ras (green starburst shape), remains constitutively activated. B) Constitutive activation of many Ras effectors leads to oncogenic outcomes.

Role of the Ras>Raf>MEK>ERK pathway and its activation in cancer

Ras has many downstream effectors that contribute to amplifying Ras signaling for normal function and disease-related events. Among the best studied downstream effectors of Ras that also contribute to its transforming activity are the phosphatidylinositol 3-kinases (PI3-kinases), Ral guanine nucleotide exchange factors (RalGEFs) and the serine/threonine Raf kinases (Raf-1, A-Raf and B-Raf) (Figure 1.5). Here I will focus on the Ras>Raf>MEK>ERK MAPK pathway and its role in cancer.

Classic activation of the MAPK cascade occurs following ligand binding to a receptor tyrosine kinase such as EGFR at the cell surface, but additional receptors such as integrins, serpentine receptors, heterotrimeric G-proteins and cytokine receptors can also activate this signaling cascade (Friday and Adjei, 2008). In an oncogenic context, overexpression or mutation of members of the EGFR family is a driving factor for numerous cancers including pancreatic, lung, head and neck squamous cell cancer, colorectal and glioblastoma (Khazak *et al.*, 2007). Such alterations are a source for the deregulation of Ras activation and therefore de-regulation of its effector pathways, thus adding another layer to the complex signaling network that can contribute to the onset of cancer and to its progression and maintenance.

Ras activation is the first step in the activation of the Ras>Raf>MEK>MAPK cascade, followed by Raf (A-Raf, B-Raf, or Raf-1) activation, which is recruited to the cell membrane through binding to Ras. Activation of Raf involves several de-phosphorylation and phosphorylation steps. Once recruited to the plasma

membrane by interaction with active Ras, Raf is activated by phosphorylation of residues in its kinase domain to unfold the protein from its inactive conformation and make the kinase catalytic activity available to be activated and to interact with downstream effectors (Figure 1.7). Activation signals from EGFR, then subsequently Ras and Raf leads to the activation of the mitogen-activated protein kinase-kinases (MAPKK), MEK1 and MEK2. MEK1/2 then phosphorylate and activate the mitogen-activated protein kinases (MAPKs), ERK1/p44 and ERK2/p42 (Hamad *et al.*, 2002; Kyriakis *et al.*, 1992). In turn ERK has multiple targets including transcription factors Elk-1 and Ets1/2, ribosomal protein kinase p90RSK1, MNK1/2, etc., whose cellular functions are to regulate cell proliferation, survival and mitosis by regulating transcription and translation respectively (Friday and Adjei, 2008). This pathway plays a major role in both normal and aberrant EGFR signaling by transducing signals through Ras and Raf. However, some components of the pathway can be mutationally activated and therefore render the pathway active independent of EGFR or other upstream inputs.

Additional studies of the Ras>Raf>MEK>ERK pathway have revealed that Ras is not the only protein found mutated in this pathway, since the serine/threonine kinase Raf also is found to be mutationally activated. A single base substitution, from valine (V) to glutamic acid (E) in codon 600 (V600E), was identified in the catalytic domain of B-Raf (Davies *et al.*, 2002). B-Raf has been found to be mutated in many types of cancer and is commonly found in tumors such as melanoma (~66%), colorectal cancer (~15%), thyroid cancer (~40%) and gliomas (11%), (Davies *et al.*, 2002; Garnett and Marais, 2004; Young *et al.*, 2009). However, oncogenically

mutated Ras or Raf are generally mutually exclusive among cancer patients (Davies *et al.*, 2002), suggesting that they have overlapping functions. Additional studies confirmed that expression of B-Raf(V600E) in NIH3T3 cells and murine melanocytes stimulates constitutive ERK signaling, induces proliferation and transformation, and allows these cells to grow as tumors in nude mice (Davies *et al.*, 2002; Wellbrock *et al.*, 2004), therefore supporting B-Raf V600E) as an mutationally activated oncogene. Once the oncogenic potential of B-Raf was validated by its ability to induce transformation and tumor formation in mice, it was then examined to test its potential as a target for anti-cancer therapeutics. Soon after, studies with RNA interference and a multikinase pharmacological inhibitor demonstrated that depleting oncogenic B-Raf in cancer cells reduced ERK activity, inhibited proliferation, and induced apoptosis (Karasarides *et al.*, 2004), ultimately showing the potential of B-Raf as a therapeutic target for tumors harboring B-Raf mutations.

Early studies aimed at characterizing the Ras>Raf>MEK>ERK signaling cascade showed that constitutive activation of MAPK is sufficient and necessary for cell differentiation and proliferation of PC12 and NIH3T3 cells, respectively (Cowley *et al.*, 1994). Soon after altered MEK proteins were implicated in carcinogenesis. First, engineered mutants with alterations in the key regulatory serine residues showed their ability to transform NIH 3T3 cells (Mansour *et al.*, 1994). More recently, gain-of-function mutations have been found in MEK1 in ovarian (Estep *et al.*, 2007) and lung adenocarcinomas (Marks *et al.*, 2008). Functional characterization of the mutant *MEK1(K57N)* found in lung adenocarcinomas showed that cells transfected with this mutant displayed increased levels of ERK

phosphorylation that was sensitive to inhibition by the MEK1 inhibitor AZD6244 (Marks *et al.*, 2008; Yeh *et al.*, 2007). Altogether, this showed increasing evidence of additional clinically relevant targets besides Ras for the inhibition of the Ras>Raf>MEK>ERK pathway for anti-cancer therapeutics.

The high incidence of oncogenic Ras and Raf mutations and the dependency on Raf to transduce many of the signals caused by Ras activation, as well as the finding of altered MEK and increased MAPK signaling in some but not all types of cancer with upstream activation of the pathway has led to many studies attempting to target these proteins (Wagner and Nebreda, 2009). For this dissertation, I will focus on discussing inhibitors of Ras or Raf activity and inhibitors of Ras/Raf interactions.

Inhibitors of the Ras>Raf>MEK>ERK pathway

Ras inhibitors

Given that signaling caused by oncogenic Ras quickly became attractive for the design of anti-cancer therapeutics, is not surprising that the first efforts to inhibit the Ras signaling pathway focused on targeting Ras activation. Efforts to target the active GTP-bound form of Ras would be optimal but have failed, possibly because of the very high (picomolar) affinity of GTP for the GTPase (Wittinghofer and Herrmann, 1995). One study (Fischbach and Settleman, 2003) showed that it was possible to target mutationally active Ras-GTP with nucleoside diphosphate kinase *in vitro*, but this approach has also not led to any clinically useful entities. Therefore

other attempts to target Ras have focused on its subcellular localization or its downstream targets, particularly kinase targets that are thought to be “druggable”.

As described above, Ras activation requires its association with the plasma membrane, and its interaction with positive modulators (GEFs) to become activated to transmit downstream signals (Der and Cox, 1991). One of the first steps required for translocation of Ras to the plasma membrane involves the addition of a farnesyl lipid to the CAAX motif of Ras by the enzyme farnesyltransferase (FTase). By helping wild type Ras to be targeted to the correct cellular localization, this lipid modification is known to be necessary for Ras transforming activity (Hancock *et al.*, 1990; Kato *et al.*, 1992), making FTase an attractive target to prevent oncogenic Ras activity (Kohl, 1999; Lerner *et al.*, 1997). Because Ras modification by FTase is necessary for its activation, one approach to inhibit the pathway was to target FTase, with the expectation of preventing active Ras from localizing to the plasma membrane and subsequently blocking aberrant activation of downstream effectors. Several farnesyltransferase inhibitors (FTIs) have been shown to inhibit Ras farnesylation in cell cultures and reverse the transformed phenotype caused by oncogenic Ras *in vitro* and in xenograft models (James *et al.*, 1993; Kohl, 1999; Kohl *et al.*, 1993). Given the positive results in preclinical models, some of these FTIs reached clinical trials. Further evaluation of FTIs, showed they could block the farnesylation of all Ras isoforms in *in vitro* enzyme assays in which only Ras and FTase were present, but failed to effectively block the isoprenoid modification and membrane association of K-Ras and N-Ras in cells (James *et al.*, 1995; Lerner *et al.*, 1997). Soon after it was understood that in the presence of FTIs in the cell, N-

and K-Ras, but not H-Ras undergo alternate prenylation by the enzyme geranyl geranyltransferase I (GGTase I) (James *et al.*, 1995; Lerner *et al.*, 1997; Zhang *et al.*, 1997) . Further evaluation of FTIs showed that they are not truly anti-Ras drugs since they had preclinical antitumor activity against a wide array of tumors, not all harboring Ras mutations, and their activity did not require inhibiting Ras farnesylation (Cox and Der, 2002).

The failure of FTIs to be effective anti-Ras inhibitors led many researchers to try other approaches to inhibit Ras signaling to the Ras>Raf>MEK>ERK pathway, among them to target downstream effectors of Ras like Raf and perhaps Ras/Raf interactions, as will be described in Chapter III. However, there are ongoing studies of other inhibitors of Ras membrane localization that suggest this may be an effective way of targeting Ras for cancer treatment. In particular, the "Ras inhibitor" salirasib (also called FTS, for farnesyl-S-thiosalicylic acid (Elad *et al.*, 1999; Gana-Weisz *et al.*, 1997; Haklai *et al.*, 1998; Marciano *et al.*, 1995)) has been reported to disrupt the interaction of H-Ras with its chaperone galectin-1 (Paz *et al.*, 2001), itself an oncogene, and of K-Ras with its chaperone galectin-3 (Elad-Sfadia *et al.*, 2004). This is thought to occur because FTS mimics the farnesyl group of Ras (Blum *et al.*, 2008). The consequences of these disruptions include dislodging Ras from the plasma membrane and also decreased signaling to phospho-ERK as well as reversal of transformed growth *in vitro* and *in vivo* (Rotblat *et al.*, 2008). An impressive list of Ras-driven tumors has been shown to be susceptible to salirasib (Blum *et al.*, 2008), and the results of two clinical trials of salirasib reported last summer (ASCO abstracts 2009) also suggest the possibility that this means of

interfering with Ras membrane targeting and biological functions could someday become a useful cancer treatment.

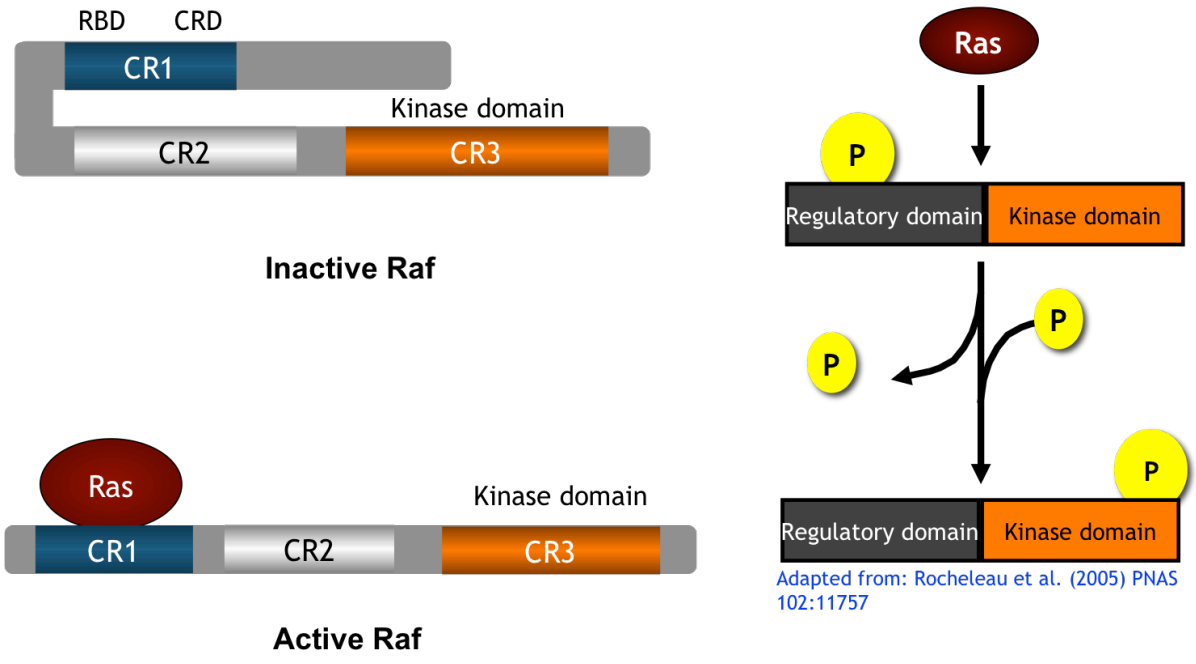

Figure 1.7 Raf activation is a multistep process
Upon interaction with Ras, Raf undergoes conformational changes and a series of phosphorylation and dephosphorylation steps to render Raf in an open conformation that is able to interact with and phosphorylate its downstream target, MEK.

Raf inhibitors

It is well documented that the Ras>Raf>MEK>ERK signaling pathway includes more than one oncogene, since the serine/threonine kinase B-Raf also is found to be mutationally activated in a smaller set of cancer types like malignant melanoma, thyroid cancer and colorectal cancer among others (Davies *et al.*, 2002; Garnett and Marais, 2004). It is noteworthy that the presence of oncogenic Ras or Raf are generally mutually exclusive thus making Raf an additional attractive and relevant target for anti-cancer therapeutics of this pathway.

One putative Raf kinase inhibitor, BAY43-9006 (sorafenib) (Lee and McCubrey, 2003; Lyons *et al.*, 2001), which had promising pre-clinical results and bioavailability, advanced to clinical trials. Soon, after additional characterization of BAY 43-9006, it was uncovered that this compound had additional activity towards the pro-angiogenic vascular endothelial growth factor receptor (VEGFR)-2 and -3, and additional receptor tyrosine kinases also involved in tumorigenesis (Wilhelm *et al.*, 2006; Wilhelm *et al.*, 2004). In a phase I clinical trial involving BAY 43-9006, the partial response of patients with renal cell carcinoma (RCC), whose tumors have high VEGFR expression, led to additional testing of this inhibitor in this selected group of patients. Assessment of BAY 43-9006 in RCC patients eventually led to its approval for the treatment of patients with advanced RCC (Khazak *et al.*, 2007; Wilhelm *et al.*, 2006). Despite the unexpected activity of BAY 4309006 against other kinases, the observations made through the evaluation of this compound suggested that angiogenesis via targeting VEGFR may be the major therapeutic activity of the drug in these tumors, and that BAY 43-9006 also interrupts proliferative signaling

arising upstream of Ras (Khazak *et al.*, 2007). Given the additional activity of BAY-43-9006, it was soon known that in order to target Raf activity other inhibitors needed to be isolated and evaluated for better specificity against Raf.

Additional Raf kinase inhibitors have successfully gone through preclinical and/or clinical evaluation. Some examples of these are RAF-265 and PLX-4032. Briefly, PLX-4032 is an orally bioavailable kinase inhibitor developed by Plexxikon and Roche. Characterization of PLX-4032 has shown potent inhibition of wild-type and mutant B-Raf V600E and also showed significant tumor growth delay, including tumor regression without evidence of toxicity (Tsai *et al.*, 2008). PLX-4032 has entered Phase I clinical trials for melanoma patients in which occurrence of B-Raf V600E has been confirmed (Khazak *et al.*, 2007). One additional Raf kinase inhibitor is Raf-265, a compound originally developed by Chiron and Novartis. Raf-265 is a novel, orally active, small molecule with potent inhibitory activity against all wild-type Raf kinases, as well as B-Raf oncogenic mutant kinases. Like sorafenib, it also inhibits vascular endothelial growth factor receptor type 2 (VEGFR-2). Raf-265 is currently in pre-clinical evaluation, and is currently under evaluation in Phase I clinical trial in patients with metastatic melanoma (Khazak *et al.*, 2007) and [http://clinicaltrials.gov/ct2/show/NCT00304525?term=raf-265&rank=1].

Evaluation of the selectivity of Raf-targeted kinases can be complex since some of the efficacy of these compounds may arise from activity against non-Raf kinases (Khazak *et al.*, 2007). Therefore it still under debate if it is better to improve existing Raf-kinase inhibitors that will improve either their efficacy or their specificity. Regardless of the challenge imposed by the specificity of kinase activity inhibitors,

these drugs represent one class of inhibitor presently available. Other approaches are also available to target protein activity by altering the expression of the target, rather than its activity. One class of drugs that can target protein expression are includes anti-sense oligonucleotides.

Antisense therapy consists of the design and use of an oligonucleotide which is complementary to the messenger RNS (mRNA) of the gene of interest's messenger RNA (mRNA). This oligonucleotide prevents expression by binding to the mRNA and thus inactivating it, thereby preventing its proper translation. Therefore another approach to inhibit Raf is by using anti-sense oligonucleotides to inhibit itsexpression. Some examples are the ISIS 5132 and LErafAON. ISIS 5132 is a phosphorothioate DNA oligonucleotide that was designed to hybridize to the 3' untranslated region of c-Raf-1 mRNA (Monia *et al.*, 1996). The success of ISIS 5132 in its preclinical evaluation allowed the advancement of ISIS 5132 to clinical trials. However, the lack of patient response in phase II clinical trials caused the withdrawal of this compound as a therapeutic agent (Cripps *et al.*, 2002; Oza *et al.*, 2003). Different from ISIS 5132, LErafAON is liposome-entrapped derivative of a 15-mer antisense oligodeoxyribonucleotides, directed against the translation initiation site of c-Raf (Gokhale *et al.*, 2002). Preclinical evaluation led to the advancement of this compound to Phase I clinical trials and the partial response and stabilization of disease in some patients were enough to conclude that LErafAON is well tolerated at low doses. It then underwent clinical evaluation after modification of the liposomal formulation (Khazak *et al.*, 2007). Currently, inhibition through anti-sense therapy looks possible but still far-off, and its further evaluation in clinical trials will be

required to prove its efficacy as a therapeutic agent.

MEK inhibitors

In addition to Ras or Raf inhibitors, other drug discovery efforts have yielded inhibitors targeting a different element of the Ras>Raf>MEK>ERK pathway, including the MAPKKs, MEK1/2. U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), is a potent and selective inhibitor of MEK1/2 kinase activity that has been used extensively in cell-based studies (Campbell *et al.*, 2006; Davies *et al.*, 2002; Duncia *et al.*, 1998). Despite the high specificity and usefulness of U0126 for cell-based assays, pharmacological limitations didn't allow it to proceed to clinical trials. Nowadays U0126 is widely used as a tool for examining the MEK-ERK pathway in research laboratories (Cox and Der, 2002).

On the other hand, there are several MEK inhibitors that have reached clinical evaluation. In contrast to the majority of protein kinase inhibitors which are competitive ATP inhibitors, MEK inhibitors are non-ATP competitive inhibitors, which may account for their highly selective properties (Roberts and Der, 2007). For example, the MEK1/2 inhibitor CI-1040 reached phase II clinical evaluation but its development was stopped due to insufficient efficacy (Rinehart *et al.*, 2004). However, structural analysis of a CI-1040 derivative (PD0325901) in complex with MEK1 and MEK2 showed that inhibitor binding did not perturb ATP binding, and instead, bound to a novel allosteric binding pocket adjacent to the ATP binding site (Ohren *et al.*, 2004). As a consequence the inhibition of the kinase activity of MEK1 and MEK2 are likely the result of the stabilization of a catalytically-inactive conformation (Ohren *et al.*, 2004). This recognition of MEK sequences that are not

shared with other protein kinases, and their association with an inactive conformation, may account for MEK inhibitor target selectivity (Roberts and Der, 2007), thus this target selectivity may be exploited in the future for the development and isolation of novel MEK inhibitors.

A second generation MEK1/2 inhibitor, AZD6244 (ARRY-142886), is an orally bioavailable benzimidazole derivative known to potently inhibit MEK1/2 *in vitro* and in cell-based assays (Yeh *et al.*, 2007). The promising results from its preclinical evaluation at inhibiting cellular growth and inducing apoptosis, along with its efficacy against tumor growth in *in vivo* xenograft models (Yeh *et al.*, 2007), allowed AZD6244 to progress to complete its Phase I clinical trials and to advance to Phase II clinical evaluation (Adjei *et al.*, 2008).

Protein-protein interaction inhibitors and MCP Ras/Raf inhibitors

So far I have discussed some of approaches and advances towards characterizing and testing inhibitors of the Ras>Raf>MEK>ERK pathway. The typical screening strategies have mostly been designed to identify compounds targeting the enzymatic activity (i.e., the kinase activity) of the target proteins. Although some of these inhibitors seem to have promising antitumor activity, they generally showed poor selectivity towards their target, most likely due to the high conservation of this domain among proteins (Bain *et al.*, 2007).

Another way to target signaling pathways is to prevent required protein-protein interactions. Identifying protein-protein interaction inhibitors can be a complex process since it is difficult to establish the specific interface at which the inhibitor may be acting. Additionally, protein interaction surfaces don't provide a highly

tractable binding site for drug design (Arkin and Wells, 2004). Despite these challenges, there are ongoing efforts to use traditional screening methods along with designing better strategies like using computer-generated systems to assess the druggability of protein-protein interactions to isolate novel protein-protein interaction (PPI) inhibitors (Sugaya and Ikeda, 2009). Such efforts have led to the identification of several PPI inhibitors of which only a few have reached clinical evaluation. A few examples of protein-protein interactions for which inhibitors have been isolated are: i) inhibitors of the cytokine Interleukin-2 (IL-2) with the α chain of IL-2 receptor (IL-2Rα), ii) inhibitors of the family of B-cell lymphoma2 (Bcl-2)/Bcl-X_L with the pro-apoptotic molecule BAK, iii) inhibitors of the human protein double minute 2 (HDM2) interaction with the tumor suppressor p53, and iv) inhibitors of the ATP-dependent chaperone protein HSP90 with the cochaperone Hsp organization protein (HOP) among others (Arkin and Wells, 2004; Arkin and Whitty, 2009; Wells and McClendon, 2007). The identification of these PPI inhibitors represents proof of principle that the use of traditional methods, potentially in combination with new methods, will soon drive the field towards greater success at identifying PPIs. As more protein-protein interaction inhibitors are isolated and evaluated, the knowledge about this class of inhibitors and their mechanism of action will enrich the drug discovery field and will serve as a starting point for future drug design strategies.

Given the relevance of both Ras and Raf as targets for anti-cancer therapeutics for the onset and development of cancer, there was interest in developing protein-protein interaction inhibitors targeting the Ras/Raf interaction. Therefore, a novel family of Ras/Raf inhibitors of the MCP (Morphochem, Inc.) family

was identified by using a yeast two-hybrid system as a platform for a primary screen of small molecule compounds library to probe for inhibitors capable of disrupting the interaction between Ras and Raf (Kato-Stankiewicz *et al.*, 2002; Khazak *et al.*, 2005; Lu *et al.*, 2004). This screen was designed using C-terminally truncated H-Ras as a bait and full-length Raf-1 as the prey. H-Ras was favored over N-Ras or K-Ras due to its better expression profile in the yeast (Khazak *et al.*, 2005). Among the compounds selected by this screen were MCP1, which was further chemically improved to become MCP110 (Figure 1.8) (Lu *et al.*, 2004); and a poorly active analog MCP122 (Kato-Stankiewicz *et al.*, 2002). The early characterization of these compounds was performed *in vitro*, using cell-based assays to measure the ability of the MCP compounds to inhibit Ras-Raf interactions and their subsequent biological functions (Kato-Stankiewicz *et al.*, 2002). This study revealed the ability of MCP compounds to inhibit Ras-mediated cell proliferation, anchorage-independent growth and downstream effectors of Ras signaling in cell-based systems and xenografts (Campbell *et al.*, 2007; Kato-Stankiewicz *et al.*, 2002; Skobeleva *et al.*, 2007). However, the mechanism of action of these putative Ras/Raf interaction inhibitors is not well understood. More recent evidence also indicates that MCP compounds have activity towards melanoma cell lines in which B-Raf is mutated (Hao *et al.*, 2007), thereby bringing into question their specific site of action.

Characterizing the mechanism of action of protein-protein interaction inhibitors is a challenging task. In this dissertation we present the nematode *Caenorhabditis elegans* vulva development as tractable genetic model for the evaluation of MCP activity *in vivo,* in combination with biochemical assays and cell-based assays, with

the goal to further understand the mechanism of action of MCP compounds as is described further in Chapter III of this dissertation.

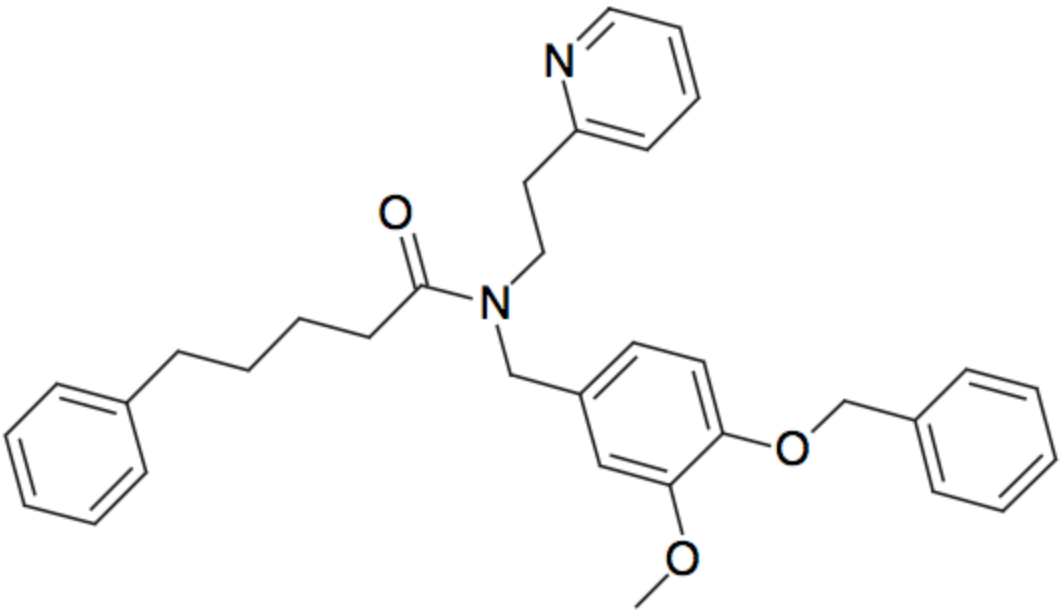

Figure 1.8 Chemical structure of MCP110 (Khazak *et al., 2007)* This polar compound is well-suited to interact with the flat interfaces of membrane-bound Ras and Raf.

C. elegans as a model for drug discovery

Caenorhabditis elegans was originally described as a powerful research model by Sydney Brenner (Brenner, 1974). As a model system, this nematode offers many advantages such as transparent internal structures that are easily detected using a dissecting microscope, a short life cycle of 3 days that allows for rapid phenotypic analysis and simple maintenance of laboratory strains. *C. elegans* reproduces asexually, and hermaphrodites can produce approximately 300 progeny in one life cycle, resulting in robust sample sizes for the design of high throughput studies. To date, there has been extensive research and characterization of *C. elegans* at the molecular, developmental and behavioral level. Given this extensive characterization, *C. elegans* is now often used to study a large variety of biological processes including apoptosis, cell signaling, cell cycle, cell polarity, gene regulation, metabolism, aging and sex determination (Kaletta and Hengartner, 2006).

In terms of pharmacological studies, *C. elegans* possesses many advantages as a model organism. For example, it is easy to design simple dosing schemes and drug studies in the context of a whole organism. In addition, because the worm is transparent, fluorescent probes can be engineered to easily monitor protein expression in living animals. Because of the worm's small size (1mm), most assays can be carried out in microtiter plates either on agar or in liquid using more than one hundred animals in a single well of a 96-well plate (Kaletta and Hengartner, 2006). Most importantly, *C. elegans* possesses signaling processes that are evolutionarily

conserved among species, which allows the opportunity to exploit this model organism for the better understanding of complex signaling pathways in mammals.

Another potential advantage of *C. elegans* versus *in vitro* or cellular models is that instead of using a target of interest in an isolated system it can be instead studied in the context of a whole organism. (Kaletta and Hengartner, 2006). Performing whole organism studies adds perspective in understanding the bioavailability of the compound, although it can be problematic if the particular compound of interest is poorly penetrant through the worm cuticle.

In addition to the high degree of conservation of proteins and signaling pathways whose functions are well understood, the existence of fewer orthologs also simplifies the study of single pathways in the worm. For example, in mammals there are three Ras homologs (H-, K- and N-Ras), whose varied activation mechanisms and activity in cell-dependent contexts add significant complexity to the interpretation of experimental results. In contrast, the worm possesses only one functional Ras ortholog (LET-60), which simplifies the characterization and function of this protein in the worm. Moreover, it also simplifies the study of Ras-induced signaling for genetic or pharmacological studies (Hara and Han, 1995; Reiner *et al.*, 2008), as will be further discussed in Chapters II and III of this dissertation. It is also relevant to mention that *C. elegans* phenotypes are not expected to correlate with human pathology but this is not required in order to obtain relevant pathway information; in other words, one can study pathways relevant for human cancer although worms do not get cancer. However, even existing mammalian models are often not reliably predictive of drug action in humans; thus the use of non-mammalian models early in

the process are cost-effective and should deliver fast answers to a discovery problem (Kaletta and Hengartner, 2006).

One critical aspect of the drug discovery process is early analysis of bioavailability, related toxicity and potential off-target effects. These are compound qualities that are poorly assessed in mammalian cell-based assays and often are not detected until lead compounds are being evaluated at the very late preclinical stage and unfortunately often not even until evaluating expensive clinical trials are undertaken. For this reason, another advantage offered by the use of the worm for drug discovery is that if specific drug effects are detected, it means that the drug is bioavailable, the drug has reached the target tissue and most likely has been able to target the protein activity, which are necessary features of whole-animal pharmacology in mammals (Kaletta and Hengartner, 2006).

So far I have discussed the general advantages of using *C. elegans* as a model for drug discovery; however I have yet to discuss one of the major advantages of using this system, which is the power to combine genetic screens with pharmacology. For many years *C. elegans* has been used for genetic high throughput screens as the standard to uncover gene function. The idea of using a similar platform, but for high throughput drug screens with *C. elegans* seems like a workable idea. The key elements are to develop an assay using a specific readout that would be sensitive and selective to the action of small molecules targeting the protein of interest. For this purpose, the target of interest should be conserved in the worm and its function should be well understood. Based on the previous information, transgenic worm strains with sensitized genetic backgrounds could be easily

generated to create a robust readout that would fit the purpose of a HTS. The success of a screen of this nature relies in the selection of a proper readout and also in the composition (type of chemical entities) of the compound library being screened. An example of the validation and future prospects for a drug screen using *C. elegans*, will be discussed in the fourth chapter of this dissertation, where a novel platform was generated and validated for the screen of novel Rac or Rac pathway inhibitors.

In classical or forward genetic screens, the genome of an organism is randomly mutagenized to generate large numbers of mutants, which are screened for a desirable phenotype or trait, such as alteration in growth, appearance or behavior. Mutants with the desired phenotype are collected and used to identify and characterize genes involved in the process of interest (Jones *et al.*, 2005; Kaletta and Hengartner, 2006; Zheng and Chan, 2002). This classical genetic screen method can be modified to suit pharmacology studies. The resulting approach is termed chemical genetics. This approach takes advantage of the traditional “forward genetics approach” to screen for mutants that are resistant or hypersensitive to the effect of a small molecule compound, resulting from mutations that alter the key target or a pathway-related element (Jones *et al.*, 2005; Kaletta and Hengartner, 2006) (Alaoui-Ismaili *et al.*, 2002). With this type of screen the worm becomes useful for the identification of the target of compounds of interest. An example of the success of this approach was reported in Ranhanathan et al, where worms harboring loss-of-function mutations in the *C. elegans* serotonin reuptake transporter (SERT) MOD-5 were resistant to the action of the serotonin reuptake inhibitor

fluoxetine, as shown by changes in specific behavior patterns, identifying MOD-5 as a candidate target of fluoxetine. These *in vivo* studies contributed to increase our knowledge about the mechanism of action of fluoxetine and also offered the opportunity to find novel targets by further characterization in the worm model (Ranganathan *et al.*, 2001).

So far I have discussed that the worm can provide information that can be beneficial for further characterization and understanding of the molecular targets of a given compound. Besides the expected results in a drug screen, the use of *C. elegans* could also reveal unexpected off-target effects by displaying changes in behavior, phenotype or defects in development. Given the excellent characterization of *C. elegans* in various biological aspects, it would be possible to revisit databases or the literature to establish new correlations between the off-target effect and a particular gene. In work published by Lackner et al., *C. elegans* was presented as a valuable model to genetically identify the target of unusual pro-apoptotic FTIs and thereby unveil their true mechanism of action (MOA) (Lackner *et al.*, 2005). During the development of a next generation FTI, BMS-214662, at Bristol-Myers Squibb, it was observed that this FTI possessed proapoptotic activity that did not correlate with the mutational status of Ras or the activity against farnesyl transferase or geranylgeranyl transferases (Rose *et al.*, 2001). As a consequence, activity of this BMS-214662, was thought to be the result of off-target activity. Given the lack of positive results in mammalian cells and the well-characterized apoptosis mechanisms in the worm, *C. elegans* was then chosen to as the model of choice to further characterize of this compound. First it was found that BMS-214662 also had

pro-apoptotic activity in the worm, thus adding evidence that the real target of these inhibitors may be also conserved across species. The use of forward genetic screens and RNAi analysis then identified candidate genes that phenocopied the effects of the pro-apoptotic FTIs. Among these genes were proteins involved in vesicular trafficking and the prenyltransferase RabGGTII, which is responsible for the geranylgeranylation of the Rab family of small GTPases (Figure 1.1) (Lackner *et al.*, 2005). Further genetic analysis in *C. elegans* identified RabGGTII as the target responsible for FTI-driven apoptosis in worms and further testing confirmed the target in mammalian cells. This study provided evidence supporting the worm as a powerful genetic tool for the *in vivo* characterization of pharmacological inhibitors (Lackner *et al.*, 2005).

Led by these examples, there is now strong evidence that supports *C. elegans* as an alternative model for pharmacology studies. It is noteworthy that *C. elegans* does not replace mammalian cell culture models or other higher animal models; nevertheless, it provides the advantage of a living organism due to its simplicity and well-characterized development and behavior. The extensive characterization of this model also increases the chances to uncover potential off-target effects earlier in the drug discovery process, which can reveal critical information in the activity of the drug and the potential existence of additional targets. Like on-target effects, any off-target effects observed in the worms may not translate directly into humans in terms of phenotypic endpoints, but can still provide valuable clues to the pathway or protein being targeted.

In summary, there is considerable information derived largely from proof-of principle experiments showing the potential of *C. elegans* as a model for drug discovery. More importantly a successful platform would need to be selected along with the appropriate readout to develop and validate the corresponding assay. *C. elegans*, like any other preclinical model, has its advantages and disadvantages, but the availability of tools for its study, the ease with which new transgenic animals are created, the low cost of maintenance plus the valuable databases available for the better understanding of the model, have collectively made *C. elegans* a highly useful model for drug discovery. In work presented in this dissertation, I have used *C. elegans* to characterize the actions of small molecule MCP compounds targeting the Ras-Raf interaction.

CHAPTER II

USE OF *CAENORHABDITIS ELEGANS* TO EVALUATE INHIBITORS OF RAS FUNCTION *IN VIVO*

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Abstract

The human RAS genes constitute the most frequently mutated oncogenes in human cancers, and the critical role of aberrant Ras protein function in oncogenesis is well established. Consequently, considerable effort has been devoted to the development of anti-Ras inhibitors for cancer treatment. An important facet of molecularly targeted cancer drug discovery is the validation of a target-based mechanism of action, as well as the identification of potential off-target effects. This chapter describes the use of the nematode worm *Caenorhabditis elegans* for simple, inexpensive pharmacogenetic analysis of candidate molecularly targeted inhibitors of mutationally activated Ras, with a focus on the Ras>Raf>MEK>ERK mitogen-activated protein kinase pathway. This protein kinase cascade is well conserved from worms to humans and is well established as a critical player in the signaling events leading to vulval formation in *C. elegans*. Excess activity results in the development of a multivulva (Muv) phenotype, whose inhibition by test compounds can be characterized genetically as to the specific step of the pathway that is blocked. In addition, off-target activities can also be identified and characterized further using different strains of mutant worms. This chapter presents proof-of-principle analyses using the well-characterized MEK inhibitor U0126 to block the Muv phenotype caused by the constitutively activated Ras homolog *C. elegans* LET-60. It also provides a detailed description of protocols and reagents that will enable researchers to analyze on- and off-target effects of other candidate anti-Ras inhibitors using this system.

Introduction

The nematode worm *Caenorhabditis elegans* is an organism commonly used by researchers wishing to model human biology. The simple lifestyle and body plan of *C. elegans* allow many facets of its development and behavior to be perturbed genetically (Brenner, 1974). Coupled with the extensive evolutionary and functional conservation of signaling proteins, the tractable genetics of *C. elegans* allow the creation of many valuable genetic tools for manipulating signaling pathways. These tools can be isolated in screens or created transgenically, and the resulting phenotypes can be assayed relatively easily. Perhaps the best studied of such pathways in *C. elegans* is the epidermal growth factor receptor (EGFR) receptor tyrosine kinase pathway that activates Ras, which subsequently activates the Raf>MEK>ERK mitogen-activated protein kinase (MAPK) cascade (Figure. 2.1). This EGFR-stimulated Ras pathway is very well conserved in evolution, with all components found in *C. elegans* and *Drosophila* as well as in all vertebrate species. In *C. elegans*, this pathway controls the development of the vulva, an epithelial aperture through which fertilized eggs are laid.

Mutations perturbing vulval development are easy to identify (Figure. 2.2). Defective vulval induction results in a Vulvaless (Vul) phenotype, and embryos hatch inside the animal. Excessive vulval induction results in the development of nonfunctional ectopic pseudovulvae, resulting in the Multivulva (Muv) phenotype. The molecular pathways that regulate these developmental events are highly conserved amongst all metazoans (Moghal and Sternberg, 2003). By screening for these phenotypes and modifiers of these phenotypes, mutations in over 50 genes that govern vulval development have been identified. These studies have

demonstrated that the worm LET-23>LET-60>LIN-45>MEK-2>MPK-1 MAPK cascade, which is highly conserved with the homologous EGFR>Ras>Raf>MEK>ERK MAPK pathway in mammals, is a critical signaling pathway that governs cell fate decisions needed for proper vulval formation.

Studies in model systems like *C. elegans* and *Drosophila* have been instrumental in identifying previously unknown components of these pathways, or determining the functional role of known pathway components. For example, the human Raf scaffolding protein Ksr was originally identified in mutant screens in *C. elegans* and *Drosophila* (Kornfeld *et al.*, 1995; Sundaram and Han, 1995; Therrien *et al.*, 1995), and the functional relationship of the SEM-5/Grb2 adaptor protein to other pathway components was originally determined in *C. elegans* (Clark *et al.*, 1992). Thus, regulation of vulval cell fate in *C. elegans* is a useful differential biological readout of EGFR>Ras>Raf>MEK>ERK pathway activity, such as the R7 photoreceptor in the *Drosophila* eye or like cell proliferation in human epithelia.

Because the signaling module itself, from the EGFR ligands (*e.g.,* EGF) to the Ets-like transcription factors, is highly conserved and similarly regulated in both worms and humans, it is likely to have the same pharmacological targets and be modified by the same pharmacological treatments in both worms and humans. For

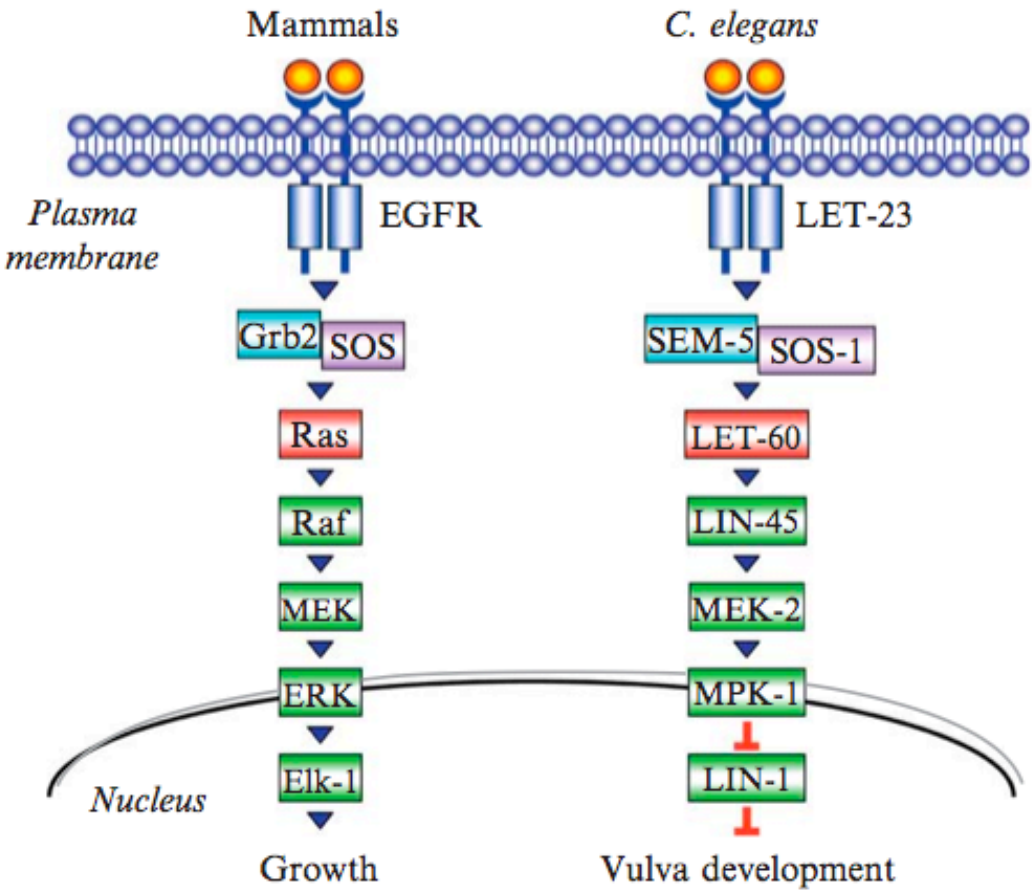

Figure 2.1. A conserved Ras>Raf>ERK MAPK pathway specifies growth in mammalian cells and vulval cell fate in *C. elegans*. In the worm, an EGFR-ligand-like signal, LIN-3 (released from the anchor cell in the gonad; see Fig. 30.2), binds to the LET- 23 receptor tyrosine kinase, which becomes activated and binds the adaptor/exchange factor complex SEM-5/SOS-1 (Grb- 2/SOS) to activate the Ras small GTPase LET- 60. Activated Ras then triggers the equivalent of the Raf>MEK>ERK MAPK cascade of kinases. ERK/MPK-1 enters the nucleus to phosphorylate and activate or inactivate, respectively, Ets family transcription factors such as LIN-1, which negatively regulates the induction of vulval precursor cells.

example, many human small GTPases, such as the LET-60 orthologs K-Ras, H-Ras and N-Ras, require post-translational modification by farnesylation at their C-termini to target the proteins to membranes and to promote correct biological activity. First generation FTase inhibitors (FTIs) manumycin and gliotoxin were capable of blocking excessive LET-60 signaling in worm vulval induction (Clark *et al.*, 1995), although subsequent studies found these and other FTIs to be ineffective against the Ras isoforms most commonly mutated in human cancers (Rowinsky, 2006).

In addition, the degree of functional conservation of *C. elegans* pathways is such that off-target drug effects can also be conserved across species and identified in *C. elegans*. When a newer generation FTI, BMS-214662, was unexpectedly found to have off-target pro-apoptotic activity (Rose *et al.*, 2001), many attempts to identify the mechanism for this property failed in mammalian cells. However, the pro-apoptotic function was conserved in *C. elegans*. Genetic screens then identified genes whose loss of function caused the same phenotype as the p53-independent, caspase-dependent germline apoptosis induced by a panel of pro-apoptotic FTIs (Lackner *et al.*, 2005). These genes control endosome-lysosome and autophagosome-lysosome docking and fusion, and the conserved Rab prenylation enzyme, Rab geranylgeranyltransferase (RabGGTase/GGTase II), was shown to be the key FTI target responsible for induction of apoptosis. Target identification was then confirmed in mammalian cells. This study illustrates the utility of using *C. elegans* to identify novel pharmacological targets of known drugs. Several conserved pharmacological targets in the nervous system have been identified in *C. elegans.* For example, in *C. elegans* nicotinic acetylcholine agonists activate

acetylcholine receptors (Lewis *et al.*, 1980) and GABA-A receptor agonists activate GABA-A receptors (McIntire *et al.*, 1993). Furthermore, tricyclic (Horvitz *et al.*, 1982) and SSRI (Weinshenker *et al.*, 1995) antidepressants promote serotonin signaling, and putative molecular targets of off-target effects of fluoxetine were identified using *C. elegans* genetics (Choy *et al.*, 1999). Together, these observations suggest that *C. elegans* is an excellent system for studying both intended and off-target effects of pharmacological agents.

Because of the potential for detailed genetic manipulation of conserved pathways, the use of *C. elegans* allows the researcher to determine at which level in the pathway a drug acts *in vivo*. For example, the mutant allele *let-60(n1046*gf*)* introduces a gain-of-function (gf) G13E mutation into the worm Ras protein, LET-60, thereby inactivating its intrinsic and GAP-stimulated GTP hydrolysis activity and trapping the protein in its active GTP-bound form. Hyperinduction of vulval tissues causes a Muv phenotype that is easily scored by counting the ectopic ventral tissue protrusions on the normally smooth surface of the animal (Figure. 2.2). The strong Muv phenotype caused by *let-60(n1046*gf*)* can be suppressed genetically or pharmacologically by compromising pathway function either at or downstream of *let-60*, whereas perturbation of upstream pathway function has little effect (Beitel *et al.*, 1990). Similar types of manipulation can be performed at most levels of the pathway, either by use of isolated strains with *in situ* mutations or by generation of transgenic strains in which the pathway is manipulated in *trans*. In Table I we have listed activated pathway reagents that cause a Muv phenotype.

Lacking this extensive genetic toolkit for analyzing EGFR>Ras>Raf>MEK>ERK MAPK signaling, other model systems have their own advantages and disadvantages. Human cell culture can be readily manipulated genetically, and has the advantage of excellent biochemical readouts, but it is an *ex vivo* system, and many cancer cell lines have extensive genetic abnormalities that are distinct from those found in the original tumors. Mice have the advantage of being an *in vivo* and mammalian system, but they are expensive, lack the reagents capable of altering each pathway component, and extensive manipulation of this pathway would result in lethality; further, despite their widespread use in preclinical anti-tumor efficacy studies, they are surprisingly nonpredictive of anti-tumor and off-target normal cell toxicity in the cancer patient (Sharpless and Depinho, 2006). Zebrafish are relatively inexpensive and have the benefit of complex organ systems, but they are also subject to some of the *caveats* of the mouse system. For our purposes, although no system is perfect, *C. elegans* is particularly useful because these animals are inexpensive, the genetic tools are currently available and simple to work with, and one can rapidly assay different inhibitors.

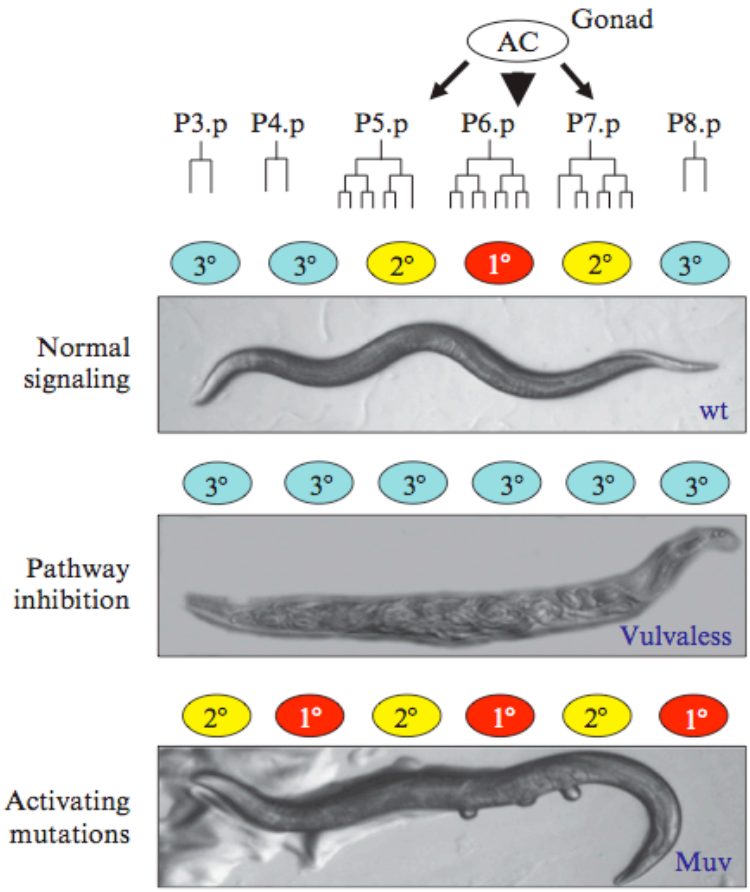

Figure 2.2. Normal or aberrant vulval formation in C. elegans is determined by the exposure of vulval precursor cells to graded levels of activation of the Ras>Raf> MEK>ERK MAPK signaling pathway. Under ‘‘normal signaling’’ conditions (top worm photograph), the anchor cell (AC) of the gonad releases signals (e.g., LIN-3 ligand) to activate the Ras pathway in adjacent VPCs (P6.p, P7.p, etc.). In wild-type animals, VPCs adjacent to the AC receive the strongest signal. These cells assume 1° and 2° fates and together develop into a functional vulva. The more distal VPCs are normally exposed to lower levels of signaling inputs from the Ras pathway. These cells remain uninduced and therefore assume a 3° fate and fuse to the hypodermal syncytium rather than contributing to vulval formation. Under conditions of ‘‘pathway inhibition’’ (middle worm photograph), whether because of loss -of-function mutations or drug treatments, blockade of critical elements of the pathway leads to the absence of inductive signals. All VPCs therefore remain undifferentiated and assume a 3 cell fate that results in a failure to develop a functional vulva (vulvaless phenotype) and hence the inability to lay eggs. As a consequence, the mature eggs hatch inside the parent, causing the ‘‘bag-of-worms’’ phenotype shown. Conversely, constitutive signaling (‘‘activating mutations,’’ bottom worm photograph) leads to hyperinduction of all VPCs, which adopt only 1 and 2 fates, resulting in the formation of both a single functional vulva and additional protruding nonfunctional vulva-like structures called pseudovulvae. Worms with such pseudovulvae are described as exhibiting a multivulva (Muv) phenotype.

2. Experimental protocol

The experimental protocol described in this section has been developed to study both on-target and off-target effects of pharmacological inhibitors of Ras function in the EGFR>Ras>Raf>MEK>ERK MAPK pathway, in which inhibition of the Muv phenotype serves as a readout for inhibitor activity. The following section illustrates the use of this protocol by describing a proof-of-principle experiment to inhibit the pathway downstream of Ras. We used the well-characterized MEK inhibitor U0126 (Duncia *et al.*, 1998) to block the Muv phenotype induced by the constitutively activated worm Ras homolog, LET-60. This protocol can be adapted to analyze other pharmacologic inhibitors and other signaling pathways.

2.1. Overview of experimental procedure

As shown in the schematic overview (Figure. 3), each experiment involves pouring the fresh agar plates on which the experiment will be performed, adding inhibitor or vehicle to the agar, growing a lawn of bacteria on the agar to provide food for the worms, growing the worms in the presence or absence of the inhibitor, and finally scoring the phenotype of the worms under each condition. Each experiment therefore generally takes 5 to 8 days to complete, depending on the developmental stage to be scored and on any growth delay induced by the inhibitors tested. For consistency and accuracy, it is critical to follow the recommended time course every time an experiment is run.

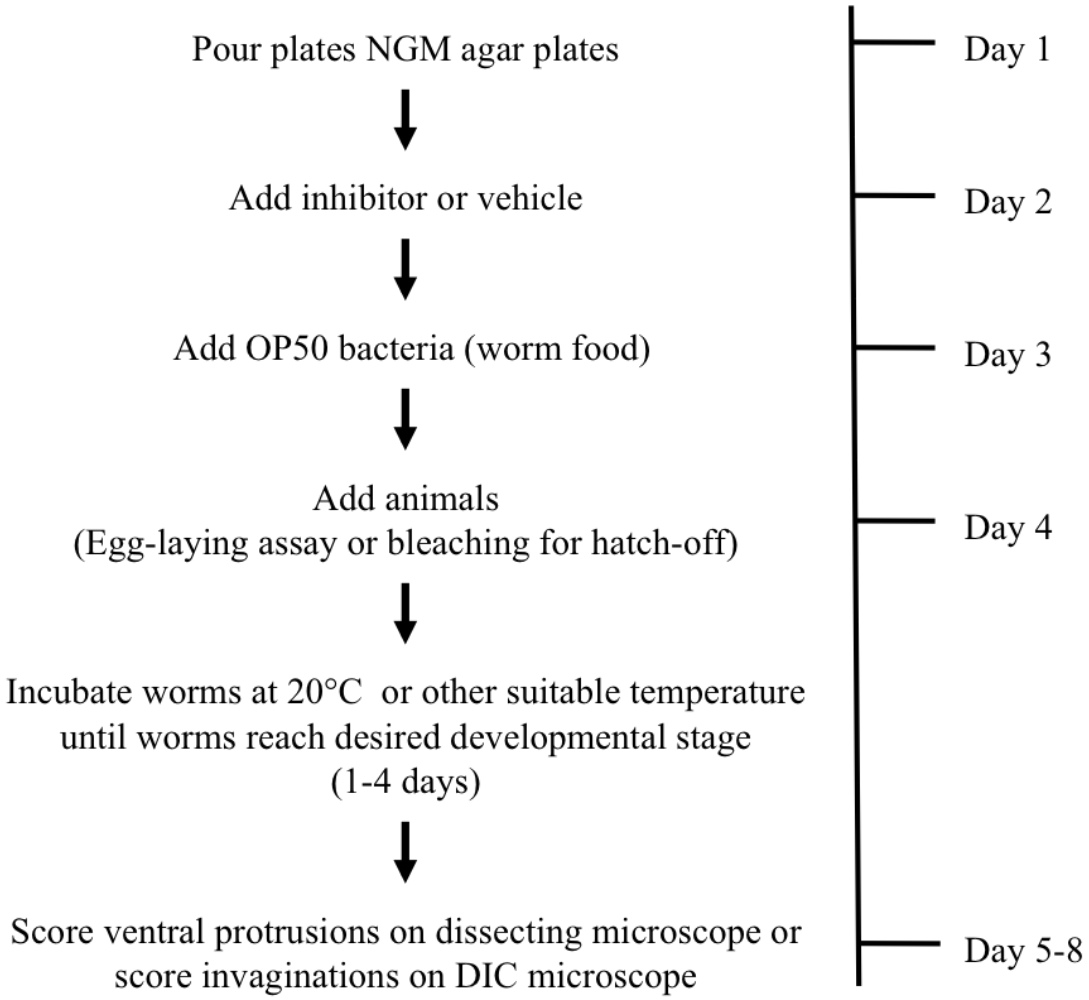

Figure 2.3. Schematic overview and time line for a typical experiment testing inhibitors of the Muv phenotype in robust or sickly animals. Details are provided in the text.

2.2 Preparation of materials and reagents

Instructions were adapted from the following sources:

- Alkaline buffer and bleaching for synchronized L1 larvae (Bianchi and Driscoll, 2006)
- NGM agar, M9 buffer, OP50 strain of *E. coli* (Brenner, 1974)
- Mounting worms on agar pads and DIC microscopy (Shaham, 2006)
- General *C. elegans* culturing techniques (Stiernagle, 2006)

2.2.1 2% Neutral Growth Medium (NGM) agar (1 liter)

- 20 g Bacto agar
- 3 g NaCl
- 2.5 g Bacto peptone
- 1 ml of 5 mg/ml (dissolved in 100% ethanol) cholesterol
- 975 ml dH_2O

Autoclave for 60 minutes. When cooled to approximately 50˚C, add sterile:

- 1 ml of 1M $MgSO_4$
- 1 ml of 1 M $CaCl_2$
- 25 ml of 1 M KPO_4, pH6.0*

*To make 1 M KPO_4, pH 6.0, first prepare and autoclave solutions of 1 M KH_2PO_4 (monobasic) and 1 M K_2HPO_4 (dibasic). Adjust the KH_2PO_4 pH to 6.0 by gradually adding 1 M K_2HPO_4.

This agar is used to pour plates. Make only enough 2% NGM agar for use in pouring plates that day. If excess agar remains after pouring plates for the inhibitor experiment, it can be used to pour stock plates to maintain worm strains. Store the other solutions (salts, buffers) at room temperature in 250-ml glass bottles, each containing 100 ml.

2.2.2 3% NGM agar (1 liter)

This agar is used for mounting worms for microscopy. To prepare the 3% NGM agar, follow the same recipe as for 2% agar above, but increase the Bacto agar to 30 g per liter. Aliquot 2 to 3 ml into sterile disposable glass tubes. Seal the opening of each tube with Parafilm and store at room temperature until needed. Each aliquot is sufficient to make enough agar pads to mount 10-15 slides (see later), so it is probably unnecessary to make more than 250 ml of 3% NGM agar at any given time.

2.2.3 1X M9 buffer (minimal salts) (1L)

- 5.8 g Na_2HPO_4
- 3.0 g KH_2PO_4
- 0.5 g NaCl
- 1.0 g NH_4Cl
- Add dH_2O to adjust the final volume to 1 liter and autoclave (no longer than 30 m) before use.

Sterile 1X M9 buffer is commonly used for the handling of worms in many different protocols. Here it is used to dilute the test drugs and to wash the worms after the bleaching protocol.

2.2.4 Inhibitor / drug dilutions

Inhibitors are generally made up and stored as stock solutions of 10, 20 or 50 mM in dimethyl sulfoxide (DMSO) or ethanol solvent, and each of these solvents can be used as vehicle-only controls as appropriate. All working dilutions of stock solutions of inhibitors (whether containing control or test inhibitor, or vehicle only) should be prepared at the time of use in sterile 1X M9 buffer, which is compatible with worm development and can be distributed evenly in the agar plates. Working dilutions of each inhibitor should be planned such that the desired final concentration can be achieved by distributing 150 μl of inhibitor over 5 ml of agar. The rationale for choosing a range of working concentrations is described below in the proof-of-principle experiment. It is important to note that the sensitivity of different worm strains to a given inhibitor may vary considerably for reasons that may or may not be related to the gene of interest for the pathway being tested. Therefore each strain must be subjected to control dose-finding experiments and not assumed to be equally sensitive to the same inhibitor dose as another strain. Furthermore, a consistent method for obtaining progeny (synchronous egg-laying versus bleaching parental worms) should be used for each strain, since the method may alter the results of some experiments.

2.2.5 Alkaline buffer / bleaching solution (5 ml)

- 1.25 ml 1M NaOH
- 1 ml commercial hypochlorite bleach
- 2.75 ml ddH_2O

2.2.6 Worm strains

Table I lists the properties of various worm strains suitable for evaluating Ras function, particularly with respect to the EGFR>Ras>Raf>MEK>ERK MAPK pathway. We note that independent strains containing *in situ* mutations or transgenes are not necessarily otherwise genetically uniform, so appropriate controls should be performed for each strain. For each strain, it is also particularly important to note the basal distribution of worms expected to display the Muv phenotype.

Standard worm husbandry steps are taken to maintain stock worm strains. For each generation, two or three adult worms are transferred to standard 60-mm dishes containing 12 ml 2% NGM agar and spotted with approximately 250 µl OP50 bacteria (see later). It is also important to pursue best practices to avoid genetic drift of strains. Strains with a growth disadvantage are particularly prone to acquiring modifiers. *C. elegans* strains survive two to three months on their NGM plates if Parafilmed after starvation. All strains should be kept as Parafilmed stocks, and active cultures can be renewed as necessary from the Parafilmed plates. *C. elegans* strains can also be frozen, and active cultures should be periodically renewed from frozen reserves (Stiernagle, 2006).

3. Experimental procedure

Investigators new to *C. elegans* are highly recommended to also consult the references cited in Bianchi and Driscoll (2006), Shaham (2006), Stiernagle (2006) for excellent visual aids and basic worm handling tips.

3.1 Neutral growth medium agar plate preparation

NGM agar plates must be prepared fresh for each experiment, since they tend to dehydrate over time, which affects the final drug concentration. Therefore, pouring fresh plates for each experiment ensures consistent volume and drug concentration and decreases inter-experiment variability. To further improve consistency and minimize dehydration, we recommend the use of six-well tissue culture plates, in which each well is the equivalent of a single 35 mm tissue culture dish, and filling the wells about half full with agar. On Day One, prepare and pour NGM agar (5 ml per well) into only the four outer wells of the 6-well tissue culture plates, leaving the two center wells empty. This approach further avoids variability due to uneven dehydration across the plates, and ensures that all wells will continue to contain an equal volume of agar throughout a given experiment. Freshly poured plates should be stored in a tightly covered container at room temperature; plastic food storage containers do nicely. To improve the even dissemination of inhibitors into the agar, wait approximately 24 h before the next step.

Table I. Mutant strains of *C. elegans* available for characterization of Ras/ERK MAPK signaling pathway inhibitors

Worm gene	Ref.	Mammalian homolog	Type of mutation*	Allele	Strain‡	Basal Muv § %	Notes
lin-3	Hill and Sternberg (1992)	EGF	Transgenic over-expression	*syIs1*	PS1123	90	Integrated transgene containing wild-type *lin-3* genomic DNA. Confers a dominant multivulva (Muv) phenotype.
lin-15	Ferguson and Horvitz (1985); Huang *et al.*,(1994)	None known	lf	*n765*	MT 8189	77	Temperature sensitive mutant: animals are Muv at 25°C, wild type at 15˚C. LIN-3/EGF ectopically expressed in mutant.
let-23	Katz *et al.,* 1996)	EGFR	gf (C359Y)	*sa62*	PS1524	89	Activating mutation in the extracellular domain of the LET-23 receptor; Muv phenotype is ligand-independent.
let-23	Moghal and Sternberg (2003)	EGFR	gf (C359Y, G270E)	*sa62, sy621*	PS 4064†	100	Double extracellular domain mutations confer a stronger ligand-independent Muv phenotype.

Worm gene	Ref.	Mammalian homolog	Type of mutation*	Allele	Strain ‡	Basal Muv § %	Notes
sos-1	Modzelewska *et al.,* (2007)	Sos	gf (G322R)	*sy262, let-23(sy1)*	ND91 ††	68	Muv phenotype due to *sy262* is visible only in the sensitized *let-23(sy1)* background.
let-60	Ferguson and Horvitz (1985)	Ras	gf (G13E)	*n1046*	MT 2124	57-90§§	Mutation predicted to disrupt intrinsic GTPase activity, resulting in constitutive activation (similar to G12V but predicted to be weaker).
lin-45 (AA)	Chong *et al.,* (2001); Rocheleau *et al.,* (2001)	Raf	TG overexp. gf (S312A, S435A)	*kuIs57*	MH 2209	91	Integrated transgene driving full-length *lin-45(AA)* with mutational loss of the Akt negative regulatory phosphorylation serines.
lin-45 (TM)	Sieburth *et al.,* (1998); Dickson *et al.,* (1992)	Raf	Conditional TG overexp. gf	*kuIs17*	UP1154	13	Integrated transgene in which *hsp16-41* heat-shock promoter drives expression of *Drosophila* Raf kinase domain (411 C-terminal amino acids) fused to the Torso transmembrane domain. Activation is independent of Ras.

Worm gene	Ref.	Mammalian homolog	Type of mutation*	Allele	Strain‡	Basal Muv § %	Notes
lin-45 (ED)	(Chong *et al.,* (2001); Rocheleau *et al.,*(2005)	Raf	Conditional TG overexp. gf (T626E, S629D)	*csEx72*	UP1226	37	Non-integrated transgene (unstable) with *hsp16-41* heat-shock promoter driving *lin-45(ED)* containing T626E, S629D phosphomimetic activating mutations.
mek-2 + *mpk-1*	Lackner and Kim (1998)	MEK/ ERK	Conditional overexp. gf	*gaIs36* or *gaIs37*	SD418✦ or SD470✦	Not avail.	Integrated transgene with both *Drosophila* MEK [*Dsor1* gf mutation] and *C. elegans* ERK [*mpk-1* gf mutation (D324N)]. Both constructs are driven by the *hsp16-41* heat-shock promoter. Muv phenotype is observed only when animals are grown at 25°C, but not at 15-20°C.
lin-1	Beitel *et al.,* (1995)	Ets-related transcription factor	lf	*sy254*	MT 7567	90-100	Loss of function of *lin-1* confers Muv phenotype.

Worm gene	Ref.	Mammalian homolog	Type of mutation*	Allele	Strain‡	Basal Muv § %	Notes
lin-31	Hill and Sternberg (1992); Miller *et al.,* (1993); Tan *et al.,* (1998)	Winged helix transcription factors	gf	*n301*	MT3031	~70	Identified in a general screen for Muv or Vul enhancement. Loss of function of *lin-31* confers Muv phenotype. Healthier than *lin-1(sy254)*.

* overexp, overexpression; gf, gain of function; lf, loss of function.

‡ Most strain names were identified using the public domain *C. elegans* database Wormbase (http://www.wormbase.org/). This database also provides basic information on each strain, as well as links to literature in which the strain of interest has been cited. Not all the mutant strains mentioned here were publicly available at the time of preparation of this document. We obtained marked strain numbers from the original laboratory sources as follows: † Paul W. Sternberg, †† Nadeem Moghal, ✦ Stuart K. Kim.

§ The penetrance of the multivulva (Muv) phenotype among mutant strains of the Ras/ERK MAPK pathway varies. This variation may be due to the type of genetic lesion and/or to the role of the mutated gene in Ras/ERK MAPK signaling that regulates worm vulval development.

§§For this strain, we routinely observe 85-90% Muv worms when they are grown from fresh cultures.

3.2 Inhibitor addition to agar plates

Add inhibitor 24 h after pouring the agar (i.e., on day 2). Each plate is generally devoted to a single concentration of a given inhibitor, with one well containing the DMSO or ethanol vehicle control and the three remaining wells containing inhibitor, all at the same concentration in order to generate triplicate data points. Each inhibitor concentration should be tested in its own plate. It is strongly recommended to use the same layout for all plates in the experiment, both for consistency and to decrease the chances of error. Add 150 μl of the working dilution of inhibitor or vehicle, freshly prepared in sterile 1X M9 buffer as indicated in earlier, drop wise to the agar surface of the appropriate well. Immediately swirl and then rock the plate in perpendicular directions to ensure uniform drug distribution. Apply the drug at a sufficient concentration that will result in the desired final concentration, once the drug has been absorbed into the entire 5 ml volume of agar. Return the plates to their container and allow the inhibitors to absorb into the agar for 24 h.

3.3 Seeding NGM plates with bacterial food for the worms

It is best to use OP50, a laboratory strain of *E. coli,* to feed the worms for these experiments (Brenner, 1974; Stiernagle, 2006). Grow OP50 to stationary phase in LB medium without antibiotics. It is not necessary to use fresh OP50 cultures, but the same culture should be used for all experimental plates. On Day Three, spot *E. coli* OP50 bacteria onto the plate to provide food for the worms by adding 80 μl of bacteria to the center of each well. Swirl to distribute. This lawn of OP50 will be sufficient to feed the animals for the duration of the experiment. Store plates as before, in tightly covered containers at room temperature.

3.4 Adding animals to the bacterial lawn

Add animals to the plates 24 h after spotting the bacteria. On Day Four, begin either the egg-laying assay or the bleaching for hatch-off protocol as described later. Healthy strains grown at 20˚C will form L4 larvae between 48 and 60 h post-egg laying or bleaching, and adults thereafter (Brenner, 1974). The timing for sickly, slower growing strains will need to be adjusted accordingly.

3.5 Egg-laying assay to obtain semi-synchronized populations

In one generation, the worms from a single stock 60-mm dish will grow to produce sufficient progeny to test one experimental condition, that is, 4 wells of a 6-well plate. To start the egg-laying assay, transfer 12 to 15 adult hermaphrodite worms to each well. More parents can be added for less fecund strains. Place the plates in a sealed plastic container at 20˚C (or a viable temperature appropriate for the strain). Allow them to lay eggs for 3 h. Then, remove all the parents and return plates to the incubator until the progeny hatch and reach the desired developmental stage. This can be as long as 4 days (*e.g.*, for *lin-1(null)* worms that will be scored as L4 larvae) or as short as 60 h (*e.g.*, *let-60(*gf*)* worms that will be scored as adults). It is not necessary to begin with exactly the same number of adult animals in each well because the eventual worm progeny will be scored according to the percentages of all the resulting worms that display a given phenotype, not according to absolute numbers.

3.6 Bleaching worms for hatch-off to obtain a synchronized population of L1 larvae

This protocol is used to obtain eggs from an asynchronous culture of adult hermaphrodite worms that will hatch to produce a culture of synchronized L1 larvae. It is generally used when a mutant worm strain lays eggs poorly, such as strains harboring null mutations in the Ets family transcription factor *lin-1*, or any strain with a Vulvaless phenotype.

- Grow worms as usual on a standard 60-mm stock 2% NGM plate seeded with OP50 bacteria, until the plate is overgrown with adult worms. Unlike the egg-laying assay, to obtain enough worms from the bleaching protocol, it is necessary to start with 2 overgrown 60 mm plates of stock worms per 4 wells of each 6 well plate.
- Add 2 ml of sterile 1X M9 solution to the plate. Use a sterile Pasteur pipette to transfer the worm suspension to a 15 ml conical tube.
- Spin 30 s in a clinical centrifuge (~1000 RPM) and remove the supernatant.
- Add 5 ml of freshly made alkaline buffer (bleach solution). This kills all adult animals, but leaves eggs (protected by shells) alive, thus producing a culture of animals that are all within a ~12 h developmental time window. It is important to make the bleach solution fresh for each experiment in order to assure good lysis of the adult worms.
- Incubate at room temperature for 3 min, with occasional gentle agitation.
- Quickly repeat the centrifugation step and remove the supernatant.

- Add 5 ml of 1X M9 buffer (this step MUST be completed within 5 min of the addition of alkaline buffer) to resuspend the eggs. Mix, spin, and remove supernatant.
- Repeat this wash step twice by adding 5 ml of 1X M9 buffer each time.
- At the final wash step, leave ~50 µl of 1X M9 buffer in the tube and resuspend the eggs in it.
- Use a Pasteur pipette to distribute a few drops of the suspension containing the eggs (and adult worm carcasses) to each well containing inhibitor or vehicle. Similar to the egg-laying assay above, it is not necessary to distribute exactly the same number of eggs into each well, because the data are collected as percentages of all worms in the well displaying a given phenotype, rather than absolute numbers of worms in the well.
- Place the plates, again in a sealed plastic container, at 20˚C (or a viable temperature appropriate for the strain) and continue as with the egg-laying assay above, until the worms reach the desired developmental stage for scoring.

3.7 Mounting worms for DIC microscopy

This mounting procedure creates a small “pad” of anesthetic-containing agar on a microscope slide, onto which the animals can be placed before a cover slip is applied. It is necessary to do this to immobilize the worms in order to score their

phenotypes by using Differential Interference Contrast (DIC/Nomarski) optics. When ready to score worms:

- Melt one aliquot at a time of 3% NGM agar by placing the glass tube first into a 100˚C block, then partially cool by transferring it to 65˚C. Once an aliquot has been melted, any excess should be discarded.
- Add 1 M sodium azide, which will serve as an immobilizing agent for the worms, to a final concentration of 10 mM. Vortex briefly to mix.
- On both sides of a clean slide place two “spacer” slides, each of which have a single piece of laboratory tape running the length of the slide. The thickness of the labeling tape will determine the thickness of the agar pad. To the center slide add two drops of the melted agar with a Pasteur pipette, avoiding bubbles. Immediately, drop another clean slide crosswise on it. Allow the agar to solidify and then remove the top slide, thereby uncovering the resulting agar pad.
- Add 5 µl 1X M9 buffer to the top of the agar pad.
- With a pick, collect the worms to be scored and gently swish them off into the buffer solution on the pad.
- Immediately and gently place an appropriate coverslip on top of the agar pad. For the Nikon Eclipse DIC microscope we use for these experiments, a No. 1.5 (18 mm square) coverslip is recommended.
- If a long session (> 1 h) is planned, seal the edges of the cover slip with petroleum jelly or VALAP to avoid desiccation.

4. Scoring wild-type versus Muv phenotypes

Accurate quantification of the Muv phenotype of animals treated with vehicle or inhibitor requires understanding how to identify the ventral protrusions or invaginations that represent pseudovulvae, and consistently applying these criteria at the appropriate developmental stage. It is also important to recognize that throughput is inversely related to resolution. Thus, rapid, high throughput scoring is possible at low resolution by using a dissecting microscope, and slow, low throughput scoring is possible at higher resolution by using DIC/Nomarski optics, but it is not possible to achieve both high throughput and high resolution. Therefore, a reasonable sized assay will include 4 or 5 conditions (i.e., four or five plates) for worms that will be scored under a dissecting microscope, or 1 or 2 conditions for worms that will be scored under a DIC microscope.

Criteria for distinguishing Muv animals from wild-type (WT) animals always include the appearance of one or more ventral protrusions in Muv animals, that is, pseudovulvae, or their precursors, that are in addition to the normal WT vulva (Figure. 2 and 4). However, the ease and manner of detecting these pseudovulvae may differ, depending on the strain of worms being tested. Worm strains displaying robust Muv phenotypes at the adult stage of development, for example, *ras*/*let-60(n1046*gf) mutants, can be rapidly and accurately scored under the dissecting microscope, allowing one person to score large samples (hundreds of worms) in a single day. Because the vulva and pseudovulvae are easily observed through the dissecting microscope only at the adult stage, it is critical that all the scoring done simply by counting these protrusions be performed only on adult worms, and thus

the experiment must be timed accordingly. At 20˚C, the time from egg laying to adulthood is approximately 60 hours (Brenner, 1974), though certain sickly strains can take longer, so their developmental timing must be measured empirically.

Data should be collected not only on the numbers of worms that are WT vs Muv, but also on the numbers of protrusions that appear in the Muv worms: Muv +1, Muv+2, Muv+3, etc. (see Figure. 4). This is because some inhibitor treatments may reduce the percentage of worms that are Muv, whereas others simply reduce the numbers of protrusions in Muv worms, and still others do both. This situation is analogous to anchorage-independent growth assays using mammalian cells, in which either colony number or colony size or both can be affected. For an example, see our recent analyses of Raf and MEK inhibitors in human tumor cell lines (Hao *et al.*, 2007). The biological mechanisms underlying these different effects remain unclear, however.

While some mutant strains displaying a Muv phenotype, such as *let-60*(gf), are easily scored on the dissecting microscope, there are other strains of worms that display a Muv phenotype but that cannot be scored by this means. An example of such a strain is the *lin-1(null)* mutant (Table I), mentioned earlier in the context of poor egg-laying that necessitates the use of the bleaching protocol to obtain synchronous populations of L1 larvae. The *lin-1(null)* mutant worms are relatively unhealthy in other ways as well. These worms can undergo significant developmental delays (more than 3 days) and become unsynchronized, which causes the scoring process to be both less efficient and less accurate. In addition, vulval protrusions are poorly distinguished in adult *lin-1(null)* mutant worms.

Therefore, instead of scoring treated *lin-1(null)* worms at the adult stage, they should be scored at the earlier L4 larval stage. Although highly accurate, this procedure is considerably more complex and time-consuming than counting very obvious protrusions under the dissecting microscope.

The numbers of ventral protrusions that will be present at the adult stage (Figure 4, top row) can be measured very accurately at the L4 stage by counting under high resolution DIC microscopy the structures formed at that point by the vulval precursor cells (VPCs) (Figure 4, bottom row) (Sulston and Horvitz, 1977). When worms are scored under DIC, the main (wild type) pre-vulval structure can be differentiated easily from the additional ventral protrusions or invaginations that are seen in adult or L4 worms, respectively, that display a Muv phenotype. VPCs are induced by the Ras>MAPK pathway, and the VPCs that will form the WT vulva first form a “Christmas tree”-like structure. In contrast, those cells inappropriately adopting vulval fates, because of activating mutations or excess activity in this pathway, form a rounded, generally asymmetrical invagination that will eventually become a ventral protrusion or pseudovulva (Figure 4). The high resolution of DIC microscopy thus makes possible easy and accurate identification and distinction of the invaginations that will become either the normal vulva or the pseudovulvae. However, the processes of worm synchronization and mounting that are required prior to quantification at the DIC microscope make this scoring method rate-limiting, due to the low number of worms that can be analyzed at one sitting (<100 worms per experiment). In unhealthy worm strains, loss of synchronous growth is common. Therefore, not all worms present will be at the appropriate developmental stage

suitable for scoring. To achieve the best accuracy, it is always important to count as many worms as are present at the appropriate (in this case, L4) developmental stage.

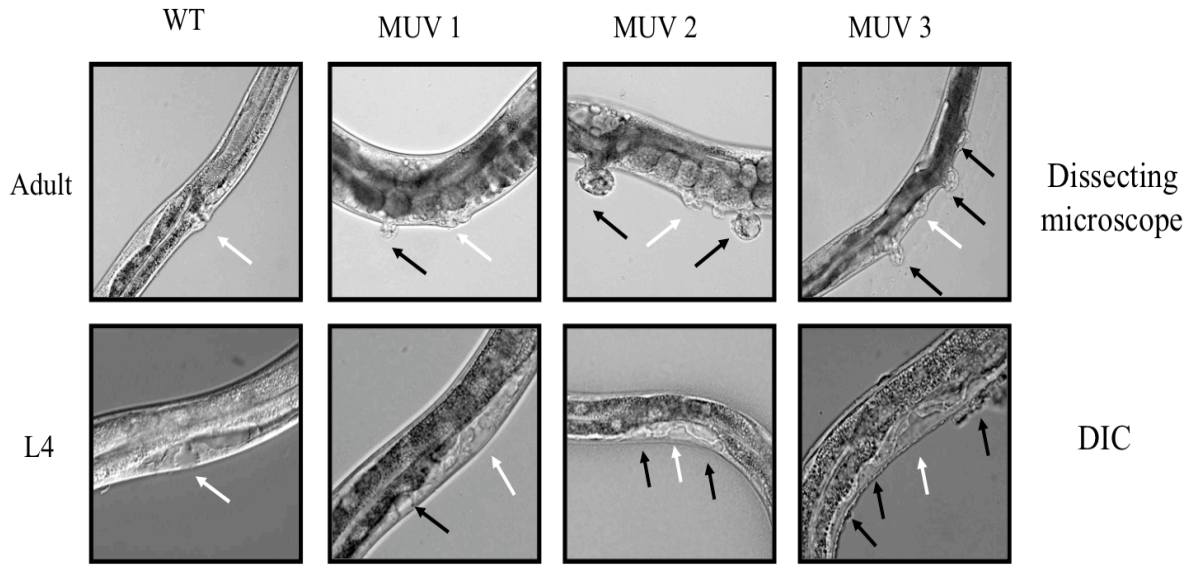

Figure 2.4 Quantification of vulval induction through the Ras>Raf>ERK MAPK pathway at adult and L4 larval stages. In the adult worm (top row), the Muv phenotype of the activated Ras homolog *let-60(n1046gf)* is very robust and is easily identifiable by the appearance of one or more of the excess ventral protrusions called pseudovulvae (black arrows; Muv1, Muv2, Muv3, etc.) that develop in addition to the normal wild-type vulva (white arrows). The Muv phenotype of these worms can be scored easily and rapidly by simply counting the pseudovulvae present at the end of a standard egg-laying assay. In some sickly mutant strains with a Muv phenotype, such as *lin-1(null)* worms, pseudovulvae are hardly distinguishable in adults. These worms must be subjected to the bleaching protocol and the Muv phenotype quantified instead at the L4 larval stage (bottom row), utilizing DIC microscopy. In L4 worms the future wild-type vulva appears as a ‘‘Christmas tree’’ structure (white arrow), whereas future pseudovulvae (black arrows) form characteristic rounded and frequently asymmetric invaginations. All images were obtained using DIC microscopy at 400 x magnification.

5. Proof-of-principle experiment

We show here a proof-of-principle experiment designed to demonstrate the utility of this protocol for evaluating pharmacological inhibitors of activated Ras pathway function. The widely used and well-characterized MEK inhibitor U0126 is a potent and selective small molecule inhibitor of both of the dual specificity kinases MEK1 and MEK2; it blocks MEK-induced phosphorylation and activation of ERK MAPKs both in mammalian cells and in *in vitro* enzymatic assays (Duncia *et al.*, 1998). U0126 should thus inhibit the Muv phenotype of worm strains in which the activating mutation is at or upstream from MEK (in *C. elegans*, MEK-2; see Figure 1 and Table I), but should not affect the Muv phenotype of worms in which the activating mutation is downstream of MEK. We therefore tested the ability of U0126 to inhibit the Muv phenotype of worm strains with mutations affecting either Ras or a downstream (transcription factor) step in the Ras>Raf>MEK>ERK MAPK pathway.

As indicated in the protocol described previously, we diluted both the DMSO vehicle and the U0126 inhibitor to equivalent concentrations in sterile M9 buffer, applied the working dilutions to separate wells containing NGM agar, and added a lawn of OP50 bacteria. We then performed an egg-laying assay in the vehicle- or inhibitor-impregnated agar, using *let-60(n1046gf*) worms expressing an endogenously activated LET-60 mutant protein (G13E). This mutation causes activation of the LIN-45-MEK-2-MPK-1 MAPK pathway and hence we expected it to be sensitive to Muv inhibition by U0126. To make sure that any Muv inhibition seen was mechanism-based and not just due to general VPC toxicity, we also grew L1 larvae following a bleach hatch of *lin-1(null)* worms lacking an Ets-like inhibitory

transcription factor that regulates the induction of VPCs (recall that the Muv phenotype of *lin-1(null)* worms must be evaluated following bleach hatch-off due to poor egg-laying properties). Because the Muv phenotype of these worms is driven instead by alterations at the transcription factor level, which is clearly downstream of MEK-2 (Figure 1 and Table I), we expected it to be insensitive to MEK inhibition.

The IC_{50} of U0126 for MEK1 *in vitro* was reported to be ~0.07 μM, and ~1 μM in COS-7 cells (Duncia *et al.*, 1998). Although it is not clear whether there is a definable relationship between the IC_{50} of U0126 or other inhibitors as identified *in vitro* or in mammalian cell systems and an effective dose in *C. elegans*, this information on U0126 did provide us with a starting point to select doses for assay *in vivo*. Knowing that the presence of their cuticle barrier means that worms do not efficiently take up most small molecules, we chose to test U0126 at 1, 3, 10 and 30 μM. Treatment with U0126 reverted the Muv phenotype of *let-60(*gf*)* worms in a dose-dependent manner (Figure 5). The IC_{50} of U0126 in *this strain* was ~7 μM, and ~30 μM was required to completely block the Ras>MAPK signaling pathway. By contrast, even 30 μM had no effect whatsoever on the Muv phenotype of *lin-1* null animals (Figure 5, top). These results demonstrate that the Muv inhibition by U0126 was selective, and are consistent with the mechanism of U0126 acting as a MEK inhibitor. In additional experiments (not shown) using worm strains expressing other activating mutations, U0126 also acted as expected, by completely blocking the Muv phenotype induced by the activated Raf ortholog, LIN-45(AA), but not by the ERK MAPK ortholog, MPK-1. Although U1026 is a highly specific non-ATP-competitive inhibitor of MEK activation of ERK (Ahn *et al.*, 2001; Davies *et al.*, 2000), it has poor

pharmacologic properties, and newer generation MEK inhibitors are now under clinical evaluation (Roberts and Der, 2007). If their anti-MEK activities are also mechanism-based, we would expect similar results, albeit perhaps at a shifted dose range. However, off-target effects may not be shared with U0126, and may be identifiable by these assays as was seen with the unexpectedly pro-apoptotic FTIs (Lackner *et al.*, 2005).

The same type of experiments as we have described here can be applied to previously characterized or novel inhibitors of Ras function, or to inhibitors of other pathways, by utilizing appropriate strains of worms. Consult Table I for additional information about available worm strains with activating mutations in signaling components that function both upstream and downstream of Ras. These strains provide more focused systems to study inhibitors that target the Ras signaling network at distinct nodal points.

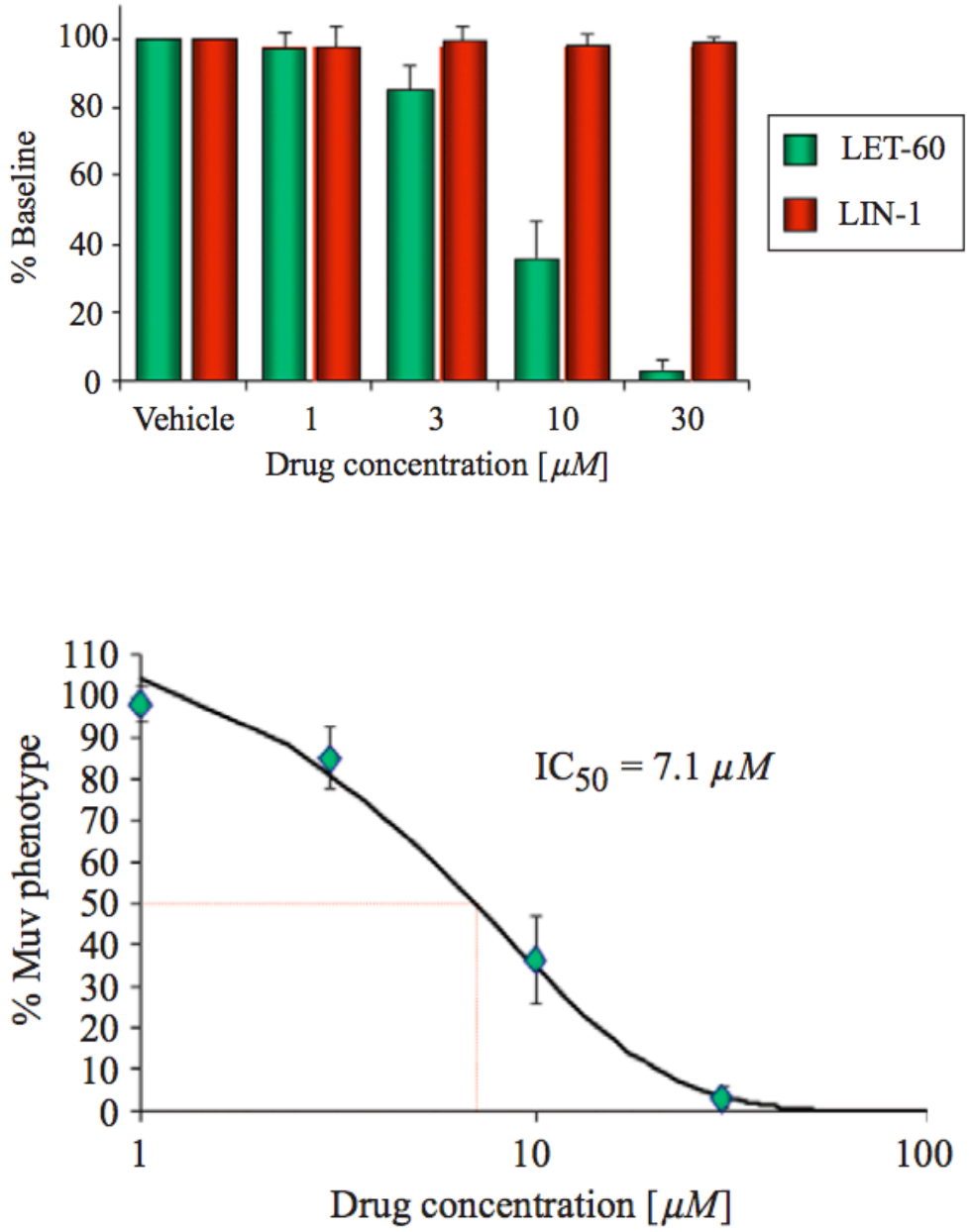

Figure 2.5 The MEK inhibitor U0126 dose dependently reduces the Muv phenotype induced by constitutively activated Ras in *let-60(n1046gf)* worms (top, green), but does not affect the Muv phenotype induced by loss of the downstream Ets family transcription factor in *lin-1(null)* animals (top, red).‘‘Percent baseline’’ refers to the percentage of animals displaying a Muv phenotype in the presence of inhibitor compared to vehicle-only control. The dose-response curve (bottom) demonstrates that the IC_{50} for Muv inhibition in *let-60(gf)* worms is approximately 7.1 μM, and essentially maximal inhibition can be achieved at the 30 μM dose to which *lin-1(null)* worms are insensitive.

VI. Conclusions and future directions

Despite intensive efforts by researchers and the pharmaceutical industry to develop inhibitors of Ras for molecularly-targeted therapeutics, to date no anti-Ras therapeutics have made the successful passage through the long and winding road of drug discovery. The inability to identify pharmacologic approaches that selectively target mutationally activated Ras directly has contributed to this failure, with the most advanced efforts targeting proteins that are involved in the posttranslational lipid modification of Ras (FTase) or signaling components downstream of Ras (Raf and MEK). Because FTase activity is critical for the function of >50 other human proteins (Reid *et al.*, 2004), and since the Raf>MEK>ERK pathway is not the simple linear cascade that we once imagined (McKay and Morrison, 2007), inhibitors of these proteins may have considerable off-target activities and cellular consequences. Our validation and application of inhibitor analyses of Ras signaling in *C. elegans* provides another model system for ongoing and future development of anti-Ras inhibitors. Overall, this genetic system, together with more physiologically relevant preclinical models of cancer, including new human cell culture and genetically-engineered mouse models (Hahn and Weinberg, 2002; Sharpless and Depinho, 2006), will produce improved preclinical analyses to facilitate greater clinical success for target-based drug discovery.

ACKNOWLEDGEMENTS

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CHAPTER III:

GENETIC AND FUNCTIONAL CHARACTERIZATION OF PUTATIVE RAS/RAF INTERACTION INHIBITORS IN *C. ELEGANS* AND MAMMALIAN CELLS

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Data shown in Figures 4C and 4D of this chapter were obtained in collaboration with Jamie K. Alan. Statistical analyses were performed in collaboration with Cicely Mitchell and Lloyd J. Edwards

ABSTRACT

Background

In mammals, activation of the Ras-Raf-MEK-ERK MAPK signaling cascade promotes cellular proliferation, and activating mutations in Ras are implicated in the onset and maintenance of numerous cancers. In *C. elegans*, this pathway is highly conserved and is required for proper development. Gain-of-function mutations in the Ras homolog LET-60 (*let-60(gf))* lead to constitutive signaling through this pathway and result in a multivulva (Muv) phenotype. MCP compounds were originally identified in a yeast two-hybrid screen for their ability to disrupt Ras-Raf interactions in that system, and have been shown to block Ras- and, in some cases, Raf-mediated biological activities in mammalian cells. Whether their biological activity is a consequence of interfering with the Ras-Raf interaction has been unclear. The purpose of this study was to use the easily-scored Muv phenotype as an accurate *in vivo* readout to characterize the selectivity of MCP110 and its analogs, which are novel putative Ras/Raf interaction inhibitors, for blockade of Ras-Raf function, and to determine whether impaired output of the Ras-MAPK pathway upon MCP treatment is linked to impaired interaction between Ras and its effector Raf.

Results

We first evaluated the effects of MCP compounds on the Muv phenotype in worm strains harboring mutations in genes at different levels of the Ras-MAPK pathway. Our genetic analyses showed that MCP compounds caused a significant dose-dependent reduction of Muv in worm strains with activating mutations in the

homologs of Ras (LET-60) or Raf (LIN-45), but not in worm strains with activating mutations in the MAP kinases MEK-2/MPK-1 or the Ets-like transcription factor LIN-1. Thus, these inhibitors selectively impair pathway function downstream of Ras and upstream of or at the level of Raf, consistent with disruption of a protein-protein interaction between Ras and Raf leading to decreased signaling from the Ras-MAPK pathway. Inhibition of the protein-protein interaction has not been confirmed in mammalian cells with full length, fully processed proteins. Therefore, in complementary studies, we also analyzed the ability of MCP compounds to disrupt Ras-Raf interactions in mammalian cells. First, in pulldown assays with the Ras-binding domain of c-Raf-1 (Raf-RBD), MCP110 treatment caused a reduction in pulldown of Ras when using the Raf-RBD, confirming that MCP110 blocks the physical interaction of Ras and Raf. In addition, we demonstrated MCP110-mediated disruption of the Ras/Raf-RBD interaction by a dose-dependent displacement of a fluorescent-tagged Raf-RBD probe, from plasma membrane locations of active Ras, to the cytosol and other compartments. Finally, decreases in active, phosphorylated ERK1/2 upon MCP treatment of the same cells demonstrated MCP-mediated functional impairment of Ras-Raf-MEK-ERK signaling.

Conclusions

We have effectively utilized the worm as an *in vivo* genetic system to evaluate the activity and selectivity of inhibitors designed to target the Ras-Raf-MAPK pathway. We have demonstrated the ability of MCP110 to disrupt, at the level of Ras/Raf, the Muv phenotype induced by chronic activation of this pathway in *C.*

elegans as well as the physical interaction between Ras and Raf-RBD in mammalian cells. Thus, pharmacological inhibition of the interaction between Ras and Raf is an effective means of altering a worm developmental program that depends on Ras-Raf-MAPK signaling.

INTRODUCTION

Over the past two decades, there have been many attempts to isolate and characterize pharmacological inhibitors targeting Ras-dependent signaling pathways. The small GTPase Ras normally transmits signals downstream of diverse inputs and is a critical signaling node for many cellular activities. Aberrant Ras activity leads to the deregulation of numerous cellular processes including proliferation, survival, cell adhesion and migration, that in turn can contribute to cellular transformation, invasion and metastasis (Campbell and Der, 2004), and Ras is mutationally activated in ~30% of cancers (Bos, 1989). Among the downstream effectors of Ras, the most well-characterized is the Ras-Raf-MAPK signaling pathway, in which Ras interaction with the serine/threonine kinase Raf causes a cascade of kinase activation, with Raf activating the mitogen-activated protein kinase kinases (MAPKK, or MEK) and MEK activating the ERK MAPK, which then translocates to the nucleus to phosphorylate and activate transcription factors to carry out the commands of Ras. The B-Raf isoform is mutationally activated, most commonly at V600E, in tumors including colorectal cancer, malignant melanoma and thyroid cancer (Davies *et al.*, 2002; Garnett and Marais, 2004), in a manner mutually exclusive with oncogenic Ras. Aberrant activation of MAPK has also been associated with various cancers (Wagner and Nebreda, 2009).

Given the relevance of the Ras-Raf-MAPK signaling pathway to a wide array of malignancies, there has been a great deal of interest in developing anti-cancer therapeutics by targeting specific elements of this pathway (Cox and Der, 2002; Khazak *et al.*, 2007; Roberts and Der, 2007; Sebolt-Leopold and Herrera, 2004).

Despite intensive efforts (Blum *et al.*, 2008), it has proven very difficult to selectively target Ras itself, which at present is widely viewed as “undruggable” due to the picomolar affinity of GTP for Ras. Pharmacological inhibition of the Raf and MEK kinases has been seen as more tractable, and several putative Raf inhibitors have reached clinical trials, including both antisense and kinase inhibitors. The most prominent of these, BAY43-9006 (sorafenib), was originally described as a Raf kinase inhibitor (Lee and McCubrey, 2003; Lyons *et al.*, 2001), but its activity in cancer patients did not correlate with Raf activation or mutational status. Instead, it demonstrated additional activity towards the pro-angiogenic vascular endothelial growth factor receptors (VEGFR)-2 and -3, and to other receptor tyrosine kinases such as PDGFR-beta that are also involved in tumorigenesis (Wilhelm *et al.*, 2004) (Wilhelm *et al.*, 2006). In a phase I clinical trial, the partial responses seen in renal cell carcinoma (RCC) patients, whose tumors have high VEGFR expression and are dependent on VEGFR signaling, led to additional testing of this inhibitor in this selected group of patients and eventually to its approval for the treatment of patients with advanced RCC (Wilhelm *et al.*, 2006) (Khazak *et al.*, 2007). Thus, the anti-tumor effects of sorafenib, now known as a “multikinase inhibitor”, are at least partly mediated by blockade of VEGFR kinase rather than Raf kinase. Newer Raf kinase inhibitors such as PLX4032 (Sala *et al.*, 2008) and its later derivatives, intended to be selective for mutationally activated B-Raf (V600E) are also under development (Tsai *et al.,* 2008). Extensive investment has also been made in MEK inhibitors including CI-1040, AZD6244 and others (Friday and Adjei, 2008; Khazak *et al.*,

2007) (Cox and Der, 2002) (Roberts and Der, 2007), although none has yet proven efficacious as single agent therapy.

Another approach to inhibit the Ras-Raf-MAPK signaling pathway is through protein-protein interaction (PPI) inhibitors. Given that an individual kinase generally has multiple substrates, an advantage of PPI inhibitors over kinase inhibitors is that they may be more selective towards a specific target, although this may be less of a problem for Raf proteins, which target primarily MEK. Challenges arise for PPI inhibitor development due to the relative lack of small-molecule starting points for drug design, the typical flatness of the protein-protein interface, the difficulty of distinguishing real from artefactual binding, and the size and character of typical small-molecule libraries (Arkin and Wells, 2004). Despite these challenges, there are ongoing efforts both to use traditional screening methods and also to design better strategies by using computer-generated systems to assess the targeting potential of protein-protein interactions and thence to isolate novel PPI inhibitors (Sugaya and Ikeda, 2009). Such efforts have led to the successful identification of several PPI inhibitors, of which only a few have reached clinical evaluation. Examples of protein-protein interactions for which inhibitors have been identified include the cytokine interleukin-2 (IL-2) with the α chain of IL-2 receptor (IL-2Rα), Bcl-2/Bcl-X_L with the pro-apoptotic molecule BAK, the human protein double minute 2 (HDM2) interaction with the tumor suppressor p53, and the ATP-dependent chaperone protein HSP90 with the cochaperone Hsp organization protein (HOP) (Arkin and Wells, 2004; Arkin and Whitty, 2009; Wells and McClendon, 2007). Concurrent with these developments has been the search for PPI inhibitors of the

interaction between the small GTPase Ras and the serine/threonine kinase Raf (Khazak *et al.*, 2007).

Here, we characterized the activity of a novel family of putative Ras/Raf interaction inhibitors derived from such a search. MCP compounds such as MCP1, MCP110 and MCP122 were originally isolated from a small molecule library using a dual-bait two-hybrid system to probe the interaction between Ras and Raf (Kato-Stankiewicz *et al.*, 2002; Khazak *et al.*, 2005; Lu *et al.*, 2004). Earlier reports characterizing the activity of these agents showed their ability to inhibit Ras signaling and Ras-mediated cell proliferation and anchorage-independent growth in cell-based systems, as well as transformed growth in nude mouse xenografts (Campbell *et al.*, 2007; Kato-Stankiewicz *et al.*, 2002; Skobeleva *et al.*, 2007). However, the mechanism of action of these putative Ras/Raf interaction inhibitors is not completely understood. The ability of MCP1 and later analogs such as MCP110 to inhibit Ras- but not Raf-mediated transformation in fibroblasts, colorectal cancer cell lines and melanoma cell lines suggested that their action was at the level of Ras rather than Raf, but more recent evidence indicated that MCP compounds also have activity towards melanoma cell lines in which B-Raf is mutated (Hao *et al.*, 2007). Therefore it is unclear whether the anti-transformation activity of MCP compounds is due to blocking Ras, Raf or yet another target. Whether MCP compounds directly disrupt the physical interaction between Ras and Raf, as shown by the yeast two-hybrid assay in which they were originally identified, has not been confirmed in mammalian cells.

Characterizing the precise mechanism of action of PPI inhibitors is a challenging task, especially given the difficulty of determining whether a given compound is interacting with the interface of one protein versus the other. There are no structural analyses available to reveal whether MCP compounds bind physically to Ras, to Raf, or to both. We therefore set out to determine at what level in the pathway MCP compounds act, by using epistasis analyses in the nematode *Caenorhabditis elegans*, a tractable genetic model system for the *in vivo* evaluation of Ras pathway drug activity

C. elegans has served as a very useful model organism to study development, neurobiology and many other biological processes. Recently it has also been useful in pharmacogenetic studies to identify the targets of pharmacological agents (Lackner *et al.*, 2005). The *C. elegans* Ras-Raf-MAPK signaling pathway is highly conserved, from the EGF ligand to the transcriptional output (Moghal and Sternberg, 2003; Reiner *et al.*, 2008). LET-60, the worm ortholog of Ras, is critical to regulate vulval development (Beitel *et al.*, 1990), and excessive activation at any level of the pathway results in hyperinduction of vulval tissue, leading to a Multivulva (Muv) phenotype. For example, a glycine to glutamic acid mutation at residue 13 (G13E) of LET-60, the worm ortholog of Ras, results in a gain-of-function that produces a constitutively activated LET-60 protein, analogous to the well known Ras(G12V) mutation in mammalian cells. Not surprisingly, then, LET-60(G13E) is well documented to induce the Muv phenotype (Beitel *et al.*, 1990), as do transgenes bearing activated Raf (LIN-45) or MEK/MAPK (MEK-2/MPK-1) and loss-of-function mutations in the downstream Ets-like transcription factor LIN-1 (Ferguson and

Horvitz, 1985) (Chong *et al.*, 2001) (Rocheleau *et al.*, 2005) (Beitel *et al.*, 1995; Lackner and Kim, 1998). Previous work by our group and others has validated these transgenes and the Muv phenotype of *C. elegans* as *in vivo* readouts to evaluate the activity of pharmacological inhibitors of the Ras-Raf-MEK-ERK pathway (Hara and Han, 1995; Reiner *et al.*, 2008) and to identify pharmacological targets (Lackner *et al.*, 2005). We therefore selected this system to characterize the activity and selectivity of known and novel MCP compounds.

We first confirmed that MCP110 acts downstream of Ras/LET-60 and upstream or at the level of Raf/LIN-45, as would be expected for an inhibitor of the Ras/Raf interaction. In addition, we demonstrate here that the previously uncharacterized MCP110 analog, MCP116, but not MCP146, also inhibits Ras/LET-60 signaling and displays specificity comparable to MCP110. Finally, for the first time we show evidence in mammalian cells that MCP110 disrupts not only signaling from Ras to ERK but also the physical interaction between Ras and Raf, and have narrowed the interface on Raf to the Ras-binding domain.

RESULTS

The *C. elegans* Ras signaling pathway as a platform for analysis of small molecule inhibitors

We have previously established the multivulva (Muv) phenotype of the nematode worm *C. elegans* as an *in vivo model* system to study the action of pharmacological inhibitors targeting Ras-induced signaling cascades. Specifically, we used the well-characterized selective MEK inhibitor, U0126 (Khazak *et al.*, 2005;

Reiner *et al.*, 2008) to demonstrate that effective pharmacological inhibition of the Ras-Raf-MEK pathways restored a normal phenotype in worms that would otherwise display a Muv phenotype based on their genetic background. The Ras-Raf signal that controls vulval cell fate in *C. elegans* is well described at the molecular genetic level. Consequently, many genetic reagents, including both *in situ* mutations and transgenic constructs, are available for pharmacological dissection of the Ras pathway. In this study, we exploited activated Ras, activated Raf, combined activated MEK/ERK, and loss of an Ets transcription factor, all of which result in excessive vulval induction. For clarity we refer to these reagents, which are described further in the Methods section, as Ras, RafAA, MEK/ERK and Ets.

In this system, wild type worms have a normal vulva accompanied by no ventral protrusions, whereas worms with excessive Ras pathway activity have hyperinduction of epithelial cells that result in a Muv phenotype, characterized by ectopic nonfunctional pseudovulvae that are visible as ventral protrusions. Both phenotypes can be scored under a dissecting stereomicroscope, and can be quantified either in a binary manner as Muv or non-Muv(WT), or by the number (0-3) of ectopic pseudovulvae. Thus, animals with one or more ventral protrusions are scored as Muv, whereas worms with a fully developed vulva but no protrusions are scored as wild-type. Because of the timing of the *C. elegans* life cycle, the consequences of treating worms with pharmacological inhibitors can be quantified precisely by scoring the phenotypes of the progeny of the treated worms (Reiner *et al.*, 2008). Here we used this validated system to test the activity and target selectivity of small molecules that are putative Ras/Raf interaction inhibitors,

MCP110 (Lu *et al.*, 2004) (Kato-Stankiewicz *et al.*, 2002; Khazak *et al.*, 2005; Lu *et al.*, 2004) and its novel analogs MCP116 and MCP146.

To illustrate the phenotypes described above and quantitated in our study, we show images of animals grown under different drug conditions (Figure 1A). Wild-type animals have a normal vulva (white arrow) and an undisrupted ventral surface. Animals expressing activated Ras display the expected Muv phenotype when treated with vehicle (DMSO) only. In these worms (center), both the functional vulva (white arrow) and three additional ventral protrusions (black arrows) are identifiable. In contrast, these animals do not display the typical Ras-induced Muv phenotype when treated with MCP110, but rather have a single properly developed vulva and no protrusions (right). Thus, MCP-treated Ras animals have the same appearance as wild type animals, consistent with disruption of Ras-Raf-MEK-ERK signaling (Reiner *et al.*, 2008).

MCP110 and MCP116, putative Ras/Raf interaction inhibitors, reverse the hyper-induced Muv phenotype of worms expressing activated Ras

We scored the Muv phenotype of animals expressing activated Ras that were grown in the presence of MCP inhibitors or DMSO vehicle. Developmentally synchronous animals were collected from each treatment group (see Methods for details) and the Muv phenotype scored according to the presence and number of ectopic pseudovulvae. Animals displaying a Muv phenotype when drug-treated were normalized to the level of hyper-induction of Muv seen in vehicle-treated animals, with the baseline for Muv established separately for each genotype.

We expected the Muv phenotype to be sensitive to MCP compounds if the Ras-Raf interaction was successfully inhibited, and therefore that the progeny of these treated worms would display normal vulval development. As expected, we observed (Figure 1B) that animals expressing activated Ras/LET-60 treated with MCP110 (20 μM) were approximately 50% less likely than vehicle-treated animals to display a Muv phenotype. Delivery of drug concentrations higher than 20 μM was not possible due to MCP compound precipitation. Additionally, we observed that the previously uncharacterized MCP110 analog, MCP116, showed inhibitory activity similar to that of MCP110. Effects of both MCP110 and MCP116 were dose-dependent. In contrast, a third derivative, MCP146, showed no significant activity at any tested concentration. As an additional negative control, we show that treatment with the poorly active analog, MCP122 (Hao *et al.*, 2007) (Campbell *et al.*, 2007; Kato-Stankiewicz *et al.*, 2002)), had no effect. Together, these results indicate that both MCP110 and MCP116 inhibit the Ras-Raf-MAP kinase pathway downstream of Ras activation. This conclusion is consistent with the reported ability of MCP110 to inhibit Ras/Raf interactions in yeast and with its biological activities in mammalian cells.

MCP compound inhibition of the Muv phenotype is specific to the Ras pathway

We have shown previously (Reiner *et al.*, 2008) that the well-characterized MEK inhibitor U0126 suppressed the activated Ras Muv phenotype, but not the Muv phenotype conferred by loss of the Ets-like transcription factor. Therefore, as a

control for pathway specificity, to ensure that suppression of the Muv phenotype is not indirect, for example by inhibiting the cell cycle, we tested whether MCP compounds also inhibited the Muv phenotype of Ets worms. As expected, Ets animals were resistant to both MCP110 and MCP116, with no response at any dose (Figure 2A).

MCP compounds act upstream of MEK

To determine the pathway level at which MCP compounds act, we continued our analysis with MEK/ERK (Lackner and Kim, 1998). Epistasis analysis has shown that MEK/ERK is independent of upstream signaling from Ras or Raf (Lackner and Kim, 1998). Furthermore, inhibition of MEK alone is sufficient to block the Muv phenotype of MEK/ERK (Reiner *et al.*, 2008). Thus, MEK/ERK animals should also be resistant to MCP compounds, which are believed to act by disrupting the Ras/Raf interface. As predicted, these animals were also resistant to MCP110, MCP116 and MCP146 (Figure 2B), with no significant differences in Muv seen in worms treated with MCP compounds versus vehicle treatment. These results show that MCP110 and MCP116 target the Ras-Raf-MAPK pathway downstream of Ras and upstream of MEK.

MCP compounds inhibit activated Raf

To determine if MCP compound activity is due to blocking Raf, we compared the Muv phenotype of RafAA animals grown in the presence of MCP compounds or vehicle. Surprisingly, treatment of these worms showed that their progeny were sensitive to the action of MCP110, and, to a lesser extent, MCP116 and the poorly active derivative MCP146 (Figure 3). Reversion of the Muv phenotype in these

animals by MCP110 was dose-dependent and occurred with similar potency as in the Ras strain. This result suggested that MCP110 inhibition of Muv induction occurred at the level of Raf, rather than Ras. RafAA has been speculated to be Ras-independent (Rocheleau *et al.*, 2005; Yoder *et al.*, 2004). However, this Raf ortholog, although constitutively activated and sufficient to drive the Muv phenotype, still includes the Ras-interacting domains RBD and CRD (Chong *et al.*, 2001). It is possible that, in *C. elegans*, full activation of LIN-45AA requires a contribution from endogenous Ras. Further, given that MCP116 robustly inhibited Ras but not Raf induction of the Muv phenotype, it is possible that they do not bind in exactly the same manner to the Ras/Raf [Ras/RafAA] interface.

To determine whether Ras binding to Raf is required for Raf activation, we attempted several different approaches to developing new activated Raf transgenes that are Ras-independent. First, we attempted to engineer a transgenic strain expressing LIN-45 that was constitutively activated by the addition of the hypervariable and CAAX sequences from *C. elegans* LET-60/Ras, which is a validated method for constitutive activation of Raf (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). However, this transgene induced no Muv activity (data not shown). We also attempted to generate a *C. elegans* version of the Raf(22W) N-terminally truncated protein, which is Ras-independent because it lacks both the RBD and CRD domains and which is known to be constitutively active in mammalian cells (Stanton *et al.*, 1989). However, the protein products of this and other N-terminally truncated *lin-45* mutants were not expressed, for reasons that remain unclear but may be related to decreased protein stability (data not shown). Therefore we cannot

yet definitively answer the questions of whether MCP110 reverts the Muv phenotype of RafAA-expressing animals by acting at the level of Ras or Raf, or whether MCP110 and MCP116 bind to Ras or Raf in the same manner. These questions will likely require structural information that is not yet available.

MCP110 inhibits the physical interaction between Ras and Raf in mammalian cells

Another possibility to explain the ability of MCP110 to inhibit the Muv phenotype of LIN-45AA-expressing worms is that this action does not occur as a consequence of disruption of the Ras/Raf interface. To confirm that MCP110 can in fact disrupt the Ras-Raf interaction, we turned to mammalian cells where biochemical analyses are more tractable.

The initial screening strategy for MCP compounds relied on the ability of the screened library components to separate the interaction of Ras and Raf in a yeast-two hybrid assay utilizing full-length versions of H-Ras and Raf-1 (Kato-Stankiewicz *et al.*, 2002; Khazak *et al.*, 2005). It remains unclear if the activity of MCP1, the originally identified MCP pharmacophore, relied on interaction with the Ras or the Raf protein interface. To answer this question for MCP110, we took advantage of the fact that activated, GTP-bound Ras binds to Raf via interaction between its own effector domain (core residues 32-40 as well as flanking sequences) and the Ras binding domain (RBD) and cysteine-rich domain (CRD) of Raf. The affinity of the Raf RBD for active Ras-GTP has been exploited to generate a widely used probe for this interaction, designated Raf-RBD, which is composed of residues 51-131 in the

amino-terminal regulatory region of Raf-1. A GST-fusion protein of Raf-RBD [GST-Raf-RBD] has long been used as an affinity for pulldown assays to retrieve and quantitate the levels of activated Ras in cell lysates (de Rooij and Bos, 1997; Taylor and Shalloway, 1996). More recently, we have used YFP- or GFP-tagged Raf-RBD as a visual probe for the subcellular localization of active Ras (Bivona *et al.*, 2006; Chiu *et al.*, 2002). In each case, the readout is dependent on the physical interaction between Ras and Raf-RBD. Thus, to further understand the mechanism of action of MCP110 we analyzed its ability to disrupt the interaction between activated Ras and the Raf-RBD in a cell-based system.

We first performed pulldown assays in NIH 3T3 cells transiently transfected with both a constitutively active form of Ras [H-Ras(G12V)] and with GST-Raf-RBD. Briefly, GST-Raf-RBD coupled to GSH-agarose beads was used to retrieve active Ras from lysates of cells treated with vehicle or MCP110 (see Methods), and the pulled-down Ras was then detected by immunoblot analysis. We observed that the physical interaction between Ras and the Raf-RBD interaction was disrupted by MCP110 in a dose-dependent manner, but not by the vehicle negative control (Figure 3.4A, top panel). To confirm that less Ras was retrieved in the presence of MCP110 due to less effective interaction of Ras with the Raf-RBD rather than due to poor expression, we also assessed the total levels of Ras from equivalent amounts of lysates. We observed that Ras protein expression did not decrease upon MCP110 treatment (Figure 3.4A, lower panel), indicating that Ras was still available for pulldown but was not retrieved.

If MCP110 decreased the physical interactions between Ras and Raf, it should also decrease downstream signaling through the Ras-Raf-MAPK pathway. We therefore examined the levels of phosphorylated ERK1/2 (p-ERK1/2) by immunoblotting with a phospho-specific antibody for ERK1/2 proteins that are phosphorylated at threonine 202 and tyrosine 204. Consistent with the dose-dependent inhibition of the Ras-Raf interaction, p-ERK levels (Figure 3., upper panel) were also reduced in a dose-dependent manner upon addition of MCP110 but not vehicle, while the total levels of ERK remained unaffected (Figure 3.4B, lower panel). Together, these results indicate that MCP110 can inhibit the physical interaction between Ras and Raf, as well as at least one functional consequence of that interaction, namely signaling to the downstream effector MAP kinases, ERK1/2. They also demonstrate that interaction of Ras with the Raf-RBD alone can be impaired by MCP110, consistent with the possibilities that the MCP110-mediated inhibition of the Muv phenotype induced in *C. elegans* by the LIN-45AA mutant Raf ortholog that retains the RBD may be due to MCP110 binding to either Ras/LET-60 or to Raf/LIN-45. These possibilites cannot be distinguished at present.

MCP110 impairs localization of Raf-RBD to the plasma membrane in cells expressing constitutively active Ras

To corroborate our findings that MCP110 disrupted the physical interaction of Ras with the Raf-RBD, with a consequent functional impairment of downstream signaling, we wished to evaluate this interaction by another approach. As mentioned above, one biologically relevant method for doing so is to visually monitor the

localization of a fluorescently tagged Raf-RBD. We have previously utilized Raf-RBD tagged with yellow fluorescent protein (YFP-Raf-RBD) to probe the subcellular localization of activated Ras (Bivona *et al.*, 2006). We therefore used this probe in NIH 3T3 cells treated with vehicle or MCP110 to compare the localization of the Raf-RBD with that of a constitutively active, HA-tagged Ras, which was detected by Alexa-Fluor594-conjugated secondary antibody directed against the epitope tag. Cells were scored according to whether the YFP-Raf-RBD probe was localized to one of three major subcellular distributions: primarily membranes including plasma membrane; internal membranes and cytosol; or cytosol and nucleus.

In cells expressing the YFP-Raf-RBD probe along with empty vector, YFP-Raf-RBD displayed a diffuse distribution throughout the cytosol and nucleus (representative images are shown in Figure 3.4C and quantitation is shown in Figure 3.4D). In stark contrast but as expected (Bivona *et al.*, 2006), co-expression of constitutively activated Ras resulted in exclusion of YFP-Raf-RBD from the nucleus and strong recruitment of Raf-RBD to membrane sites of Ras localization such as the plasma membrane and internal membrane compartments (Figures 3.4C and 3.4D, vehicle treatment). Consistent with the ability of MCP110 to dose-dependently reduce the amount of Ras pulled down by GST-Raf-RBD (Fig. 4A, upper panel), it also dose-dependently impaired the recruitment of YFP-Raf-RBD to sites of activated Ras (Figure 3.4C, MCP110 treatment). Indeed, with increasing doses of MCP110, YFP-Raf-RBD was restored to the cytosol and the nucleus (Figure 3.4C, top row) even as Ras remained membrane bound and nuclear-excluded (Figure

3.4C, middle row). These results are also consistent with MCP110 disruption of the physical interaction between Ras and Raf.

DISCUSSION

MCP110 and MCP116 act downstream of Ras/LET-60 and upstream or at the level of Raf/LIN-45

Putative Ras-Raf interaction inhibitors such as MCP1 or derivatives based on MCP1, such as MCP110, have been shown previously to inhibit Ras-induced transcriptional reporter activity, cell migration, morphological and growth transformation as well as tumorigenicity in nude mice (Campbell *et al.*, 2007; Hao *et al.*, 2007; Kato-Stankiewicz *et al.*, 2002). However, although these small molecules were originally identified via a yeast two-hybrid screen for inhibitors of interactions between H-Ras and Raf-1 (Kato-Stankiewicz *et al.*, 2002; Khazak *et al.*, 2005; Lu *et al.*, 2004), whether their biological activities in mammalian cells are due to physical disruption of this interaction has not been shown. Further, there have been conflicting reports in the literature as to whether the presence of mutationally activated and therefore Ras-independent B-Raf [B-Raf(V600E)] confers resistance to inhibition by MCP compounds (Hao *et al.*, 2007; Kato-Stankiewicz *et al.*, 2002). However, these studies focused on different MCP analogs (MCP1 vs MCP110) and evaluated their actions in distinct B-Raf(V600E)-expressing melanoma lines, which could explain the different results. Therefore it was also not certain whether MCP compounds as a group act at the level of Ras or Raf.

Here we have used *C. elegans* as a genetic tool to investigate the level of the Ras-Raf-MAPK pathway at which MCP110 and its novel derivatives MCP116 and MCP146 act. The *C. elegans* orthologs in this pathway are highly conserved with those of mammals, but drive hyper-induction of vulval development in the worm, which is read out as a Muv phenotype. We have shown here that MCP110 and MCP116 but not MCP146 inhibit the Muv phenotype driven by a constitutively activated LET-60, which is the worm ortholog of Ras, but fail to inhibit the Muv phenotype driven by downstream elements of the pathway including MEK/MAPK and the Ets-like transcription factor, LIN-1. These results are consistent with but do not prove MCP inhibition of Ras-Raf interactions. The *lin-1 null*-driven Muv phenotype has been used previously by others to demonstrate specificity of first-generation farnesyltransferase inhibitors (FTIs) gliotoxin and manumycin, which target an enzymatic process that occurs upstream of LIN-1 and therefore should not, and did not, block Muv driven by LIN-1 deficiency (Hara and Han, 1995). We have used this transgene previously to validate epistatically the actions of the MEK inhibitor U0126 in this model (Hara and Han, 1995; Reiner *et al.*, 2008), thus supporting the use of the Muv-driven phenotype caused by genetic lesions in elements of the Ras-Raf-MAPK pathway as a readout for pharmacological inhibition of the pathway. We also showed here that worms expressing gain-of-function transgenes for MEK-MAPK were resistant to inhibition of Muv by MCP analogs, consistent with their proposed targeting of the Ras-Raf interface, which is upstream of MEK. However, whether they act at the level of Ras or of Raf still remained to be determined.

Our analysis of worms expressing an ortholog of constitutively activated Raf, LIN-45AA, showed that MCP110 and MCP116 exert activity towards the Muv phenotype caused by this activated form of Raf, which was unexpected since it has been reported (Yoder *et al.*, 2004) to be Ras-independent and therefore should not be sensitive to disruption of the Ras-Raf interaction. Epistasis analysis involving *lin-45AA* transgenic worms, in combination with loss-of-function mutations in elements upstream of LIN-45 in the pathway, suggested that the Muv phenotype of *lin-45AA*-expressing worms is independent of Ras activity (Rocheleau *et al.*, 2005), but this was not proven conclusively because Ras/LET-60 itself was still present and functional. The mechanisms by which Raf and LIN-45 are activated have been extensively reviewed elsewhere (Chong and Guan, 2003; Chong *et al.*, 2001; Harding *et al.*, 2003), and it is clear that human and worm orthologs have similar regulatory mechanisms. However, the significant pharmacological inhibition of the activated Raf/LIN-45-driven Muv phenotype by MC110 seen here implies that the mechanisms by which LIN-45 is activated may still require Ras-Raf interaction. Indeed, this protein retains both the Ras-binding domain and CRD and thus has room for MCP action on the Ras-Raf interface. Our attempts, like those of many others in the *C. elegans* field, to generate more informative *lin-45* transgenes were unsuccessful, so whether a truly Ras/LET-60-independent form of Raf/LIN-45 would be resistant to inhibition by MCP110 or MCP116 remains to be determined.

The discrepancies in previous observations of MCP activity leaves room to consider that the selectivity of these compounds for either Ras or Raf may rely in

part on the model system or cell context. The ability of MCP110 and MCP116 to inhibit the Muv phenotype in worms expressing activated Ras/LET-60 may be due to the 86% identity of LET-60 shared with N-Ras in the first 164 amino acids, which allows LET-60 to possess all the biochemical functions of Ras proteins in mammals (Han and Sternberg, 1990). Conversely, some subtle isoform differences may also account for apparently discordant results between studies that do not evaluate precisely the same players (Fischer *et al.*, 2007).

While the worm does not replace mammalian cell culture models or higher organisms for in *vivo* studies, the use of a living organism for pharmacological studies, especially one that, like *C. elegans*, has been extensively characterized at the developmental and behavioral levels, can also lead to the detection of toxicity and off-target activity early in the drug discovery process, as well as to genetic identification of the target of unexpected biological activities (Lackner *et al.*, 2005).

MCP110 is a true protein-protein inhibitor of the Ras-Raf-RBD interaction

The original screen for MCP compounds, described in detail in (Kato-Stankiewicz *et al.*, 2002; Khazak *et al.*, 2005), involved a modification of the yeast two-hybrid assay, which is a standard and powerful technique to detect protein-protein interactions and was the first method used to identify the interaction between Ras and Raf in live cells (Vojtek *et al.*, 1993). The technique, performed in this case in a hyperpermeable strain of yeast to enhance penetration of the cell wall by small molecules (Khazak *et al.*, 2005), detects these interactions by the transcriptional transactivation of a dual reporter system, and thus it is important for the interactions

being analyzed to take place in the cell nucleus. Normally, Ras is post-translationally modified by farnesylation at its C-terminus for membrane targeting and biological activity, but in order to produce more functional Ras (bait) in the nucleus, the C-terminal modification motif was mutated to become insensitive to plasma membrane targeting (Khazak *et al.*, 2005). The alteration of Ras localization to fit the purpose of the screen may have had an impact on the outcome, especially since it is thought that Raf interacts differently with farnesylated vs nonfarnesylated Ras proteins (Williams *et al.*, 2000). In addition, compounds registering positive in this screen may have had allosteric effects on regions of Raf not directly interacting with Ras.

To add another layer of complexity to the potential mechanism of action (MOA) of MCP110 and related compounds, as well as to experimental approaches to identifying inhibitors of Ras-Raf and to testing and validating inhibitor MOA, the activation of Raf-1 involves a series of steps involving membrane translocation, dephosphorylation at negative regulatory sites, and subsequent phosphorylation at activating sites in the kinase domain (Kolch, 2000). Activation of B-Raf is similar but not identical, and currently there is much attention being paid to possible influences of Raf-1 on B-Raf and vice versa (Marais *et al.*, 1997; Rajakulendran *et al.*, 2009; Trakul *et al.*, 2005; Wellbrock *et al.*, 2004). Given that localization of Ras and the complex regulation of Raf are key determinants for activation of the signaling cascade, it is also possible that the original screen could have selected lead candidates affecting Ras or Raf interaction with other proteins that are positive regulators of the pathway. Several scaffolding proteins interact with members of the

Ras-Raf-MAPK pathway to regulate the pathway by effects on protein localization or protein-protein interactions (Kolch, 2005). For example, Sur-8 is an evolutionarily conserved scaffold protein that is a positive regulator required for optimal Ras-MAPK signaling (Sieburth *et al.*, 1998). Sur-8 facilitates Ras-Raf complex formation (Li *et al.*, 2000), whereas reduction of Sur-8 suppresses activated Ras-mediated signaling in *C. elegans* (Li *et al.*, 2000; Sieburth *et al.*, 1998). The formation of a ternary complex of Sur-8 with activated Ras and Raf suggests that Sur-8 could also be a potential target of MCP110 activity, although the ability of MCP110 to impair the interaction of Ras with just the Raf-RBD interaction indicates that Sur-8 would not be an exclusive target.

An important finding of this study was therefore our detection of MCP110-mediated disruption of the physical Ras-Raf interaction, providing evidence for the first time that MCP110 significantly disrupts the protein-protein interface involving full-length H-Ras and the Raf-RBD in mammalian cells. This indicates that MCP110 can act as a true protein-protein interaction inhibitor. Whether it also disrupts the interaction of K-Ras and N-Ras with Raf-RBD remains to be determined. Also remaining to be determined is whether it shows selectivity for disruption of interactions of Ras with the different Raf isoforms.

The strong association of Raf-RBD with Ras-GTP versus Ras-GDP (Herrmann *et al.*, 1995) supports MCP110 disruption of the Ras-Raf complex, but the selectivity of MCP110 to disrupt interactions between Ras and Raf versus other GTPase/RBD pairs has also not yet been determined. Given that it is presently unclear whether MCP110 binds to Ras, to Raf-RBD or both, it would also be of interest to evaluate

the ability of MCP110 to disrupt the interaction of the RBDs of other GTPases and their effectors. For example, RBDs of Ral GEFs (RalGDS, Rgl1-3) can interact with Ras as well with the Rap1A GTPase (Esser *et al.*, 1998; Spaargaren and Bischoff, 1994; Wolthuis *et al.*, 1996). Whether MCP110 can also disrupt the interaction of Ras with RalGEF RBDs or Rap1A with Raf-RBD will be important to determine. Additionally, the effector domain of the Ras-related GTPase Rit provides a similar surface to that of Ras (Fischer *et al.*, 2007), and may thus also be disruptable by MCP110, MCP116 or related compounds. Finally, why MCP110 and MCP116 did not display the same ability to inhibit the Muv phenotype induced by the *C. elegans* Raf ortholog LIN-45AA is currently unclear. The availability of structural information on complexes of MCP110 and of MCP116 with Ras-Raf would be of great assistance in making predictions about the most fruitful avenues to pursue in these directions.

CONCLUSIONS

Here we used both mammalian cell culture studies and the genetically tractable *C. elegans in vivo* model to investigate the activity of putative Ras/Raf interaction inhibitors. We dissected the pathway and were able to determine that MCP compounds act downstream of Ras/LET-60 and upstream or at the level of Raf/LIN-45, thereby providing additional proof-of-principle for the use of *C. elegans* as a simple and attractive model for the characterization of novel or already isolated Ras pathway inhibitors. The work presented here has contributed to a better understanding of the mechanism of action of putative Ras/Raf interaction inhibitors

based on the MCP110 pharmacophore. Besides supporting previous conclusions that MCP110 significantly inhibits the signals caused by activated Ras *in vitro* and *in vivo,* we have been able to narrow the requirements for its activity by successfully using it to disrupt the Ras/Raf-RBD interaction. In future, MCP110 can be further analyzed to test its selectivity towards other Ras effectors harboring RBDs. Perhaps the existing MCP110 analog can be further improved to increase its potency and target selectivity for further testing in additional pre-clinical models. Moreover, it would be interesting to see if additional screens involving Ras and the Raf-RBD could be developed to isolate novel Ras/Raf interaction inhibitors.

METHODS

C. elegans strains, strain maintenance and culturing conditions

Strain maintenance and nomenclature are as described (Brenner, 1974; Horvitz *et al.*, 1979). Expression of activated Ras was from the *n1046* allele in which an *in situ* mutation in LET-60/Ras causes a G13E change equivalent to that in human Ras, which is functionally similar to the well known G12V activating mutation (Beitel *et al.*, 1990). For activated Raf, we used *lin-45AA* (*kuIs57*) (Yoder *et al.*, 2004). Activation of Raf is a multistep process in which several regulatory residues are modified to regulate its kinase activity. Transgenic alteration of the conserved Akt negative regulatory sites from serine to alanine at residues 312 and 453 (“AA”) in Raf leads to a hyper-induced phenotype comparable to that conferred by activated Ras (Chong *et al.*, 2001; Yoder *et al.*, 2004). Activated MEK/ERK resulted from transgenic expression of both activated *Drosophila* MEK (MEK-2) and activated *C.*

elegans ERK (MPK-1), all driven by a heat-shock promoter (*gaIs37* (Lackner and Kim, 1998)). Consequently, the MEK/ERK Muv phenotype is temperature-sensitive, such that animals are grown at 25˚C to induce a Muv phenotype, but are wild type at 15˚C (Lackner and Kim, 1998). Finally, as used herein, “Ets” refers to loss of the LIN-1/Ets transcription factor function (*lin-1 null* (*sy254*)). LIN-1 inhibits vulval fate, so LIN-1 loss results in hyper-induction (Beitel *et al.*, 1995; Lackner and Kim, 1998). Strains were cultured on 2% NG agar plates seeded with *E. coli* strain OP50. The SD418 *gaIs37* (*mek-2*(gf)+*mpk-1*(gf)) strain was maintained at 15°C and switched to 25°C to induce its conditional hyper-induced phenotype.

Drug assays and quantification of the multivulva (Muv) phenotype

The experimental procedures for *C. elegans* drug treatments and phenotype quantification were previously described in detail (Reiner *et al.*, 2008). Briefly, experiments were performed in 6-well tissue culture plates in which only the four corner wells were filled with 3 ml of 2% NG agar. Either vehicle (dimethyl sulfoxide; DMSO) or experimental drug (MCP110, MCP116, or MCP146) was diluted in M9 buffer and applied in a defined volume to the agar in each well to achieve the final dose. Plates absorbed the drug overnight, then were seeded with 90 µl of OP50 overnight culture and allowed to grow for 24 hours to ensure a suitable bacterial lawn.

To obtain a population of treated animals that was developmentally synchronous, we harvested embryos during a narrow time frame. For each strain

and drug, 12-15 adult hermaphrodites laid eggs for 3 hours, after which the parents were removed.

Animals to be assayed were exposed to drug throughout development. *let-60(*gf*), kuIs57* and *gaIs37* animals were scored as early adults using the dissecting microscope. *lin-1(sy254)* vulval invaginations were scored at the 4th larval stage (L4) because adult pseudovulvae were too distorted to quantify clearly (Reiner *et al.*, 2008). For DIC microscopy, *lin-1* animals were mounted on slides in M9 buffer containing 5 mM sodium azide.

To reproducibly score the outcome of drug assays, we used a specific set of phenotypic criteria. First, we categorized animals in a binary assay as Muv or non-Muv, depending on the presence of the ectopic pseudovulvae that indicate hyper-induction of vulval tissue. Second, we quantified the number of ectopic pseudovulvae. Each genotype assayed had a different baseline for degree of hyper-induction, and therefore for each genotype the baseline was re-established such that animals treated with the experimental drug were normalized to the level of hyper-induction in animals treated with vehicle.

Statistical analyses

Two-way ANOVA interaction analyses were performed to characterize the effect of each drug at each concentration on each treated strain. A p value of < 0.05 was considered significant.

Cell culture and transfections

NIH3T3 mouse fibroblasts were grown in DMEM (Sigma-Aldrich) supplemented with 10% GCS calf serum (Gibco) and 1% penicillin/streptomycin (Gibco) and maintained in 5% CO_2 at 37°C. Cells were plated the day before transfection at a density of 200,000 cells per 60 mm dish or 100,000 cells per 35 mm dish (or in a 6-well plate), for the pulldown and co-localization assays, respectively. For pulldown assays, pCDNA3.1 (vector only, v.o.) or pCDNA3.1 encoding activated H-Ras(12V) were transfected transiently into cells using *Trans*IT-LT1 Transfection reagent (Mirus) according to the manufacturer's specifications. For co-localization assays, pEYFP-Raf-RBD was cotransfected with either empty pCGN-HA vector (v.o.) or pCGN-HA encoding activated H-Ras(12V). Immediately after transfection, a designated amount of either DMSO vehicle or MCP110, MCP116 or MCP146 was added at 3, 10 or 30 μM and further assays were performed after 48 h.

Pulldown assays and immunoblotting

Transiently transfected NIH3T3 cells (see above) were lysed in 400 μl of freshly prepared Magnesium Lysis Buffer (MLB) combined with protease inhibitors cocktail (BD BaculoGold, BD Biosciences Pharmingen). Lysates were cleared by centrifugation at 12,000 RPM for 10 minutes at 4°C, and protein concentration was measured in a Bradford Assay (Bio-Rad laboratories, Hercules, CA). GST fusion proteins of the Raf-1 Ras binding domain (GST-Raf-RBD) were prepared from pGEX2T encoding Raf-RBD as described previously (Smith and Johnson, 1988; Taylor and Shalloway, 1996). Empty vector pGEX2T plasmid encoding GST alone

was a negative control. A total of 100 µg of MCP110 pre-treated protein lysate was incubated with 10 µl of glutathione agarose beads (Sigma) previously coupled to GST alone or to GST-Raf-RBD. Parallel with the implementation of the lysate and the bead fusions, additional drug was added and the pulldown reaction was performed in a final volume of 500 µL, rocking for 1 h at 4°C. Protein bound to beads were collected, washed three times in lysis buffer and eluted in non-reducing protein sample buffer. Pulldown samples and total protein were analyzed by SDS-polyacrylamide gel electrophoresis and western blotting.

For immunoblotting, membranes were blocked in 5% non-fat dry milk and incubated overnight with primary antibodies diluted to 1:3,000 for H-Ras, 1:500 for p-ERK (Cell Signalling) or 1:2,000 total ERK (Cell Signalling) dilution, overnight at 4°C. Membranes were washed and incubated for 1 h in a 1:30,000 dilution of anti-mouse or anti-rabbit IgG-horseradish peroxidase antibody (Amersham), washed extensively with TBS-T and developed with SuperSignal West Dura Extended Duration substrate (Thermo Scientific, Rockford, IL).

Inmunofluorescence for recruitment of Raf-RBD probe

NIH 3T3 cells were grown on coverslips, transiently transfected as above, and treated with MCP110 for 48 h before fixation in 3.7% formaldehyde, permeabilization with 0.5%Triton and blocking in 2% BSA for 1 h at room temperature. Coverslips carrying fixed cells were incubated in a 1:200 dilution of anti-HA antibody (Covance) for 1 h, followed by two washes in 1X Phosphate Buffered Saline (PBS), an additional incubation with AlexaFluor594 anti-mouse conjugated secondary antibody

(Invitrogen, 1:1,000) for 30 min and washed three times with 1X PBS. Coverslips were mounted into a glass microslide with ProLong Gold antifade mounting medium and cells were visualized by confocal microscopy.

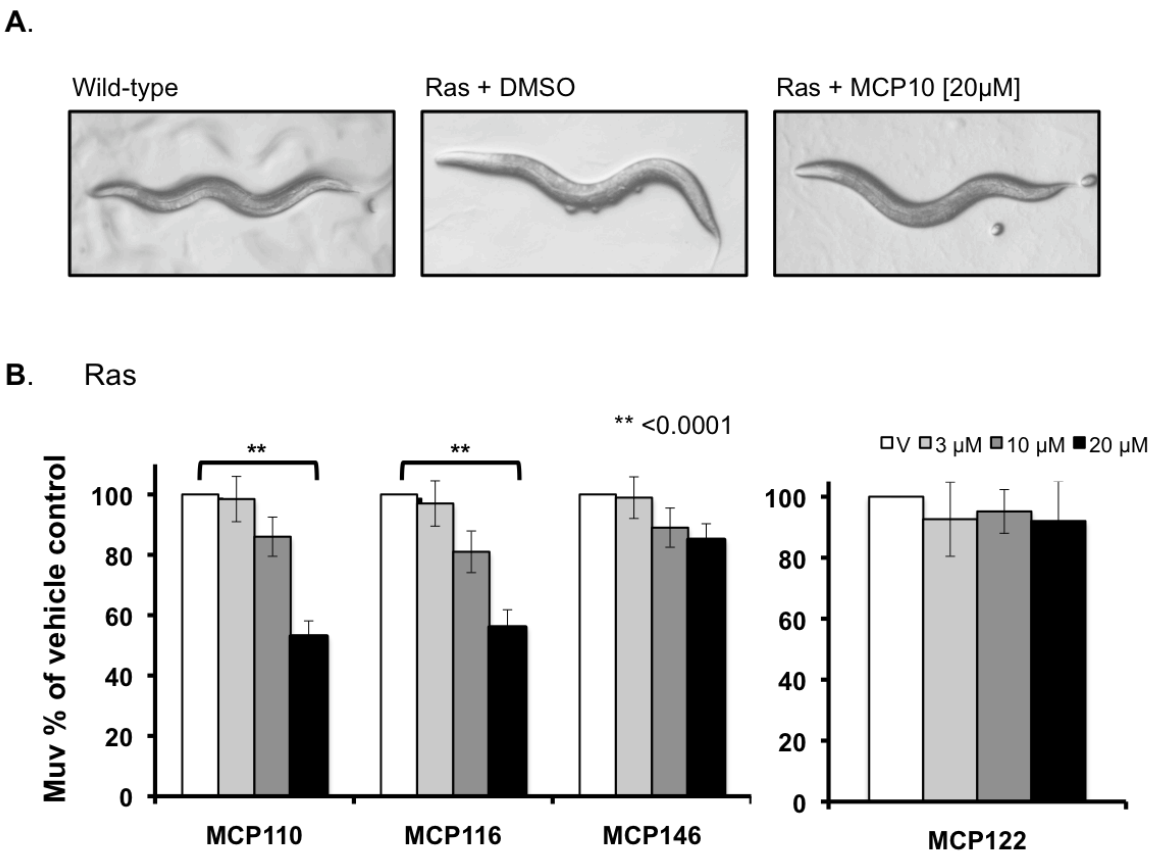


Figure 3.1 MCP110 and MCP116 but not MCP146 inhibit the Ras/LET-60-induced Muv phenotype. A. Representative images of: left, untreated wild-type animals; center, progeny of animals harboring constitutively activated Ras treated with vehicle only (Muv phenotype; ventral protrusions from pseudovulvae); right, progeny of the same worm strain shown in the center panel, following treatment of parents with 20 μM of MCP110 (reversal of Muv to WT, as indicated by lack of ventral protrusions). **B.** Animals harboring constitutively activated Ras as in panel A were treated with either vehicle (DMSO) or MCP110, MCP116 or MCP146 (3, 10 or 20 μM; higher concentrations precipitated out of solution). The Y-axis indicates the percentage of treated animals with hyper-induced Muv phenotype, normalized to the number of vehicle-treated animals with Muv phenotype. Data were analyzed by two-way ANOVA. (**) indicates a *p* value of less than 0.0001.

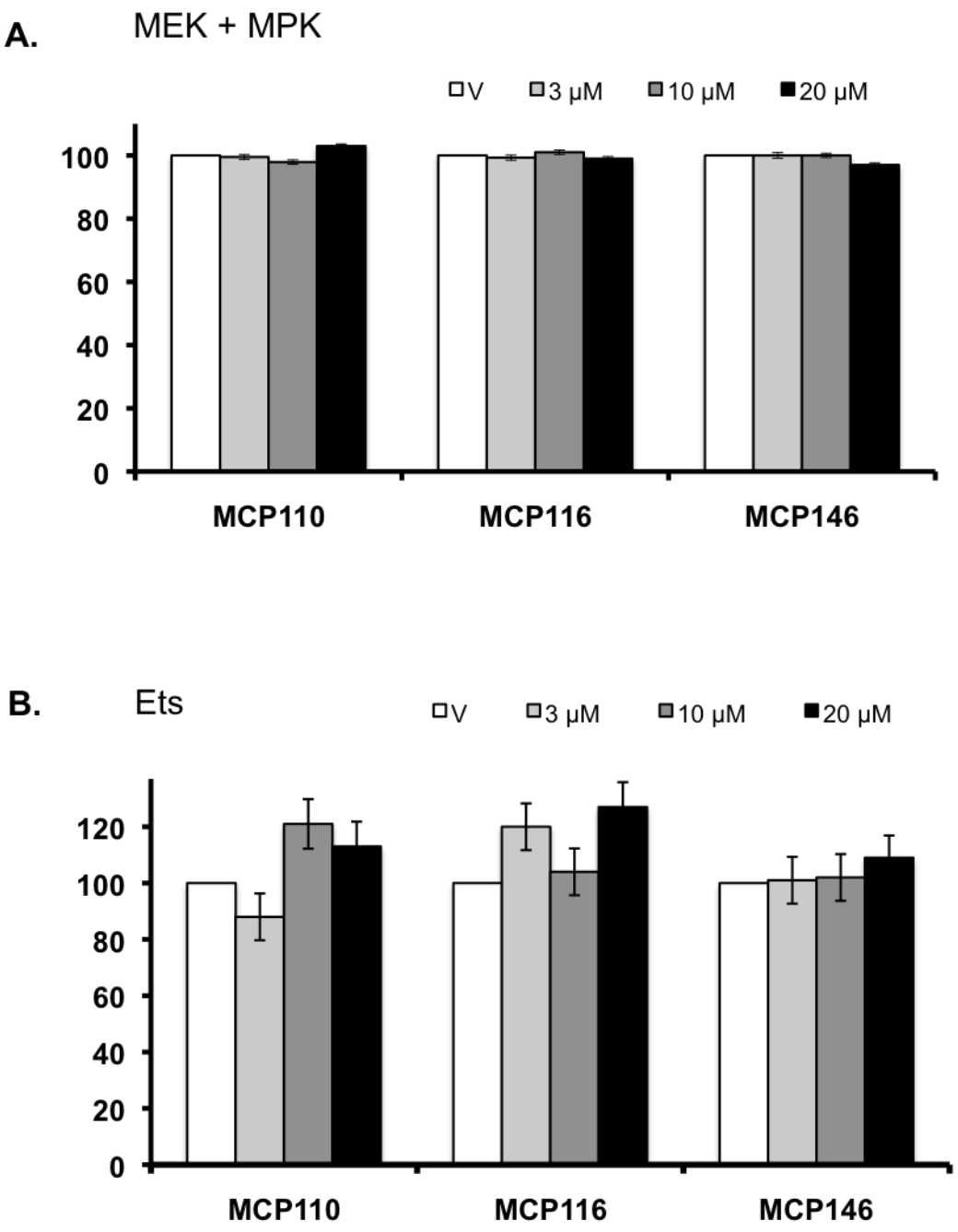


Figure 3.2. MCP110 and derivatives do not inhibit the Muv phenotype induced in worm strains harboring mutations in components of the Ras-Raf-MAPK pathway downstream of Raf. Animals were treated and results shown as for Figure 1B: **A.** Ets and **B.** activated MEK/ERK.

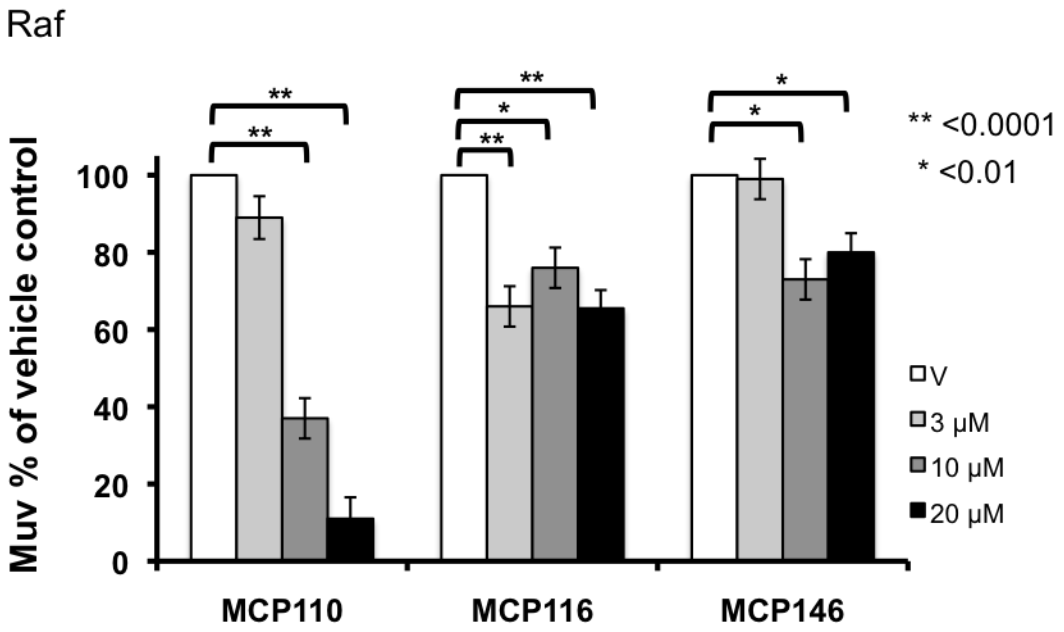
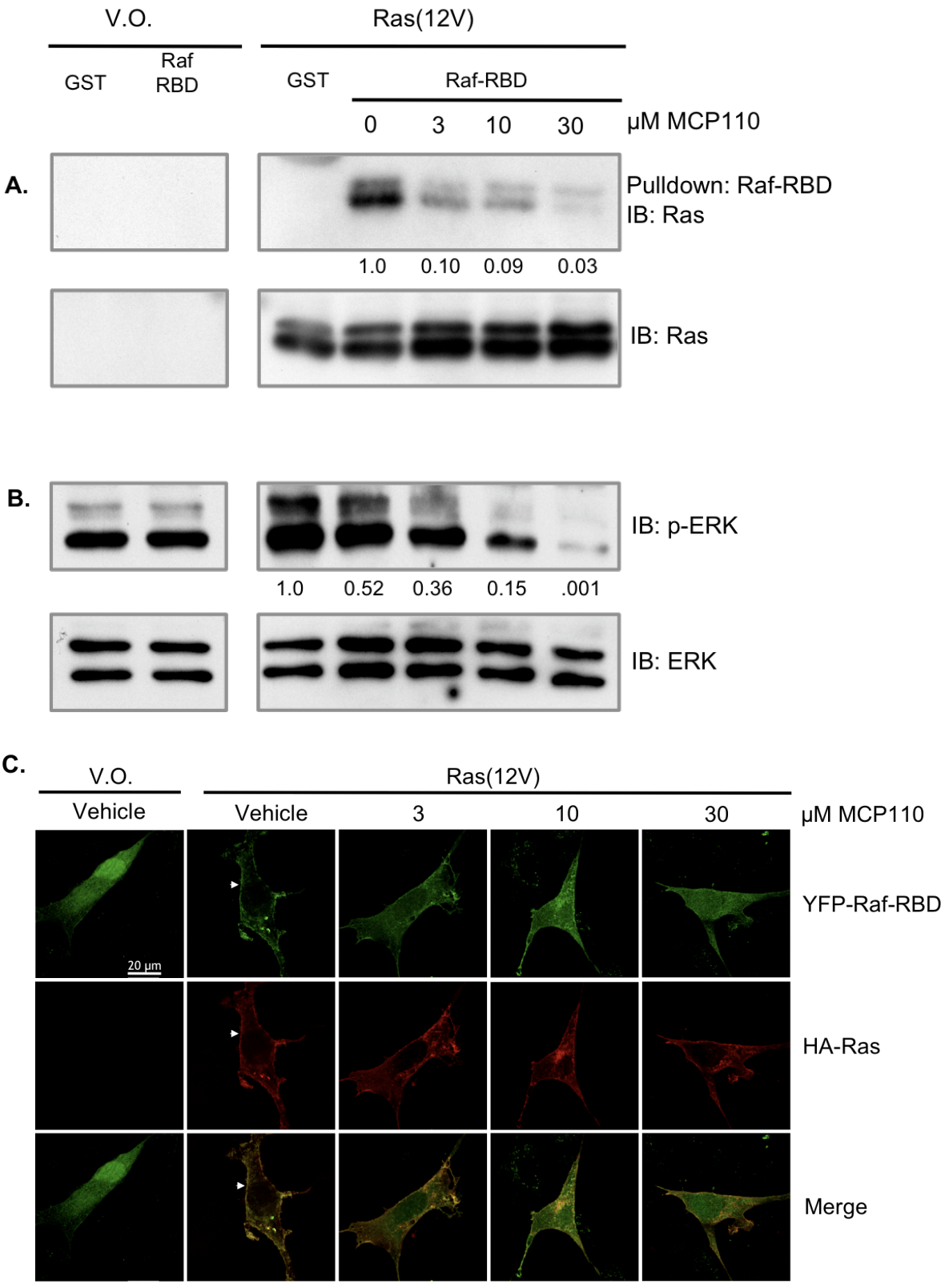


Figure 3.3. MCP110 inhibits the Muv phenotype of worms expressing activated Raf/LIN-45. kuIs*57* (*lin-45AA*) transgenic animals expressing a constitutively activated Raf/LIN-45 protein were treated with 3, 10 or 20 µM doses of vehicle (DMSO) or MCP110. The Y-axis indicates the percentage of treated animals with hyper-induced Muv phenotype, normalized to the number of vehicle-treated animals with Muv phenotype. Data were analyzed by teo-way ANOVA. animals. (**) indicates a p-value of <o.0001 and (*) denotes a p≤0.01.)


V.O.
Ras(12V)
GST
Raf RBD
GST
Raf-RBD
0
3
10
30
μM MCP110
A.
Pulldown: Raf-RBD
IB: Ras
1.0
0.10
0.09
0.03
IB: Ras
B.
IB: p-ERK
1.0
0.52
0.36
0.15
.001
IB: ERK
C.
V.O.
Ras(12V)
Vehicle
Vehicle
3
10
30
μM MCP110
20 μm
YFP-Raf-RBD
HA-Ras
Merge

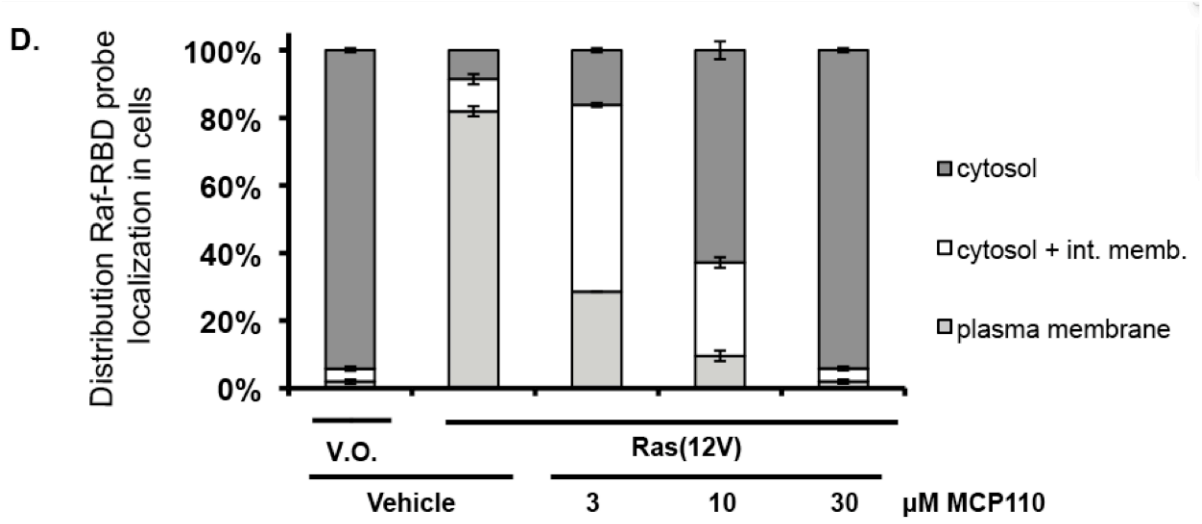


Figure 3.4. MCP110 inhibits Ras/Raf interaction in mammalian cells. *A. Pulldown assay for Ras-Raf interaction.* Pulldowns of active H-Ras(12V) were done using GST-Raf-RBD as described in METHODS. NIH 3T3 cells and subsequent lysates were treated with vehicle or MCP110 (3, 10 and 30 μM). Ras was detected by immunoblotting with a specific H-Ras antibody. Both Raf-RBD-bound Ras (upper panel) and total Ras in the lysates (lower panels) are shown. MCP110 disrupted the Ras/Raf interaction in a dose-dependent manner; numbers shown indicate quantitation of Ras pulldown by densitometry, normalized to vehicle control. *B. Western blot analysis for phospho-ERK.* The same lysates from cells expressing active H-Ras(12V) depicted in panel A above were immunoblotted for phospho-ERK1/2(S473) (upper panel) and for total ERK1/2 (lower panel) and quantitated by densitometry. MCP110 decreased ERK activation in a dose-dependent manner. *C. Immunofluorescence localization of YFP-Raf-RBD probe for active Ras-GTP.* NIH3T3 cells transiently expressing H-Ras12V and YFP-Raf-RBD were treated with vehicle or MCP110. Representative images of cells quantitated in Panel D below are shown here. In the absence of active Ras (v.o.), YFP-Raf-RBD was localized diffusely throughout the cytoplasm and nucleus, whereas in the presence of active Ras (H-Ras(12V)), the Raf-RBD probe was recruited to the plasma membrane and, like Ras, was nuclear-excluded (vehicle panels). Increasing concentrations of MCP110 increasingly shifted the YFP-Raf-RBD probe from the plasma membrane to the cytosol and to internal membranes and finally to both cytosol and nucleus, whereas Ras remained membrane-associated and nuclear-excluded, indicating dose-dependent disruption of the Ras/Raf-RBD interaction. D. Quantification of the distribution of YFP-Raf-RBD subcellular localization. Cells were binned according to whether the YFP-Raf-RBD probe accumulated primarily in the cytosol, or cytosol + internal membranes, or plasma membrane and was nuclear-excluded, as described in Methods and depicted qualitatively in panel C.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

Through the progress of this work we have learned valuable lessons about the activity of MCP compounds as putative Ras/Raf interaction inhibitors. We first validated the Muv phenotype that results from Ras-, Raf- or MEK-driven hyperinduction of vulval development in *C. elegans* as an *in vivo* readout to assess the potency and selectivity of pharmacological inhibitors targeting the Ras>Raf>MEK>ERK pathway, thus providing a stronger base for its further use to evaluate other such inhibitors.

Second, we evaluated the activity of putative Ras/Raf interaction inhibitors of the MCP class by taking advantage of a collection of worm strains harboring mutations at different levels of the Ras>Raf>MEK>ERK pathway (Table 2.1). Our results revealed the selectivity of MCP compounds inhibition of the Muv phenotype conferred by activating mutations at specific different levels of the pathway, and as a result of performing the assays in the genetically tractable worm model we were able to confirm that MCP acts downstream of Ras and upstream or at the level of Raf. Complementary results were obtained when testing the ability of MCP110 to interrupt Ras/Raf interaction in pulldown assays. This approach allowed us to show

for the first time that this putative Ras/Raf interaction inhibitor is really capable of physically disrupting the interaction between activated Ras and Raf in mammalian cells. Further, we narrowed down the site of interaction to Ras and the Raf-RBD. In these cells, we also were able to demonstrate impaired recruitment of Raf-RBD to sites of membrane-bound, active Ras. Although the mechanism of action of MCP compounds is still not completely known, in part because we do not know definitively if they bind to the Ras or Raf side of the interface, the weight of evidence suggests that they interact preferentially with Ras.

In addition to validating tools for the *in vivo* evaluation of known or novel inhibitors of the Ras>Raf>MEK>ERK signaling pathway, our studies support the idea that protein-protein interaction inhibitors of Ras/Raf interactions could target the pathway more effectively than FTIs or kinase inhibitors as discussed in the Introduction chapter, although this remains to be tested directly in the same system. Optimistically, in the near future there may be additional efforts to perform other screenings to look for novel protein-protein interaction inhibitors of Ras and Raf. For the last seven years, MCP compounds have undergone *in vitro* and *in vivo* evaluation. During this time these putative Ras/Raf interaction inhibitors have been of value in the laboratory for the inhibition of Ras-driven transformation in cells and the vulva formation in the worm. While the MCP110 compound itself, with its low-to-middling micromolar activity in cell-based assays and real but modest activity in nude mouse xenografts, will not become a successful clinical candidate, it, like many other failed clinical candidates including the MEK inhibitor U0126, will likely continue to be very useful to inhibit existing or novel Ras-dependent assays in the laboratory.

Perhaps efforts to chemically modify the MCP pharmacophore to improve its selectivity and potency may lead to better Ras/Raf interaction inhibitors from the family of MCP compounds.

As mentioned in the introduction to this dissertation, one of the advantages of using the worm as a model is the possibility to uncover compound-related off-target activity. In testing the activity of MCP110 and related derivatives against Ras signaling, we identified an unexpected, dose- and compound-dependent phenotype, indicative of potential off-target activity. This finding will be discussed on Future Direction 2.

My studies confirmed that the worm is not an ultimate model to fit all the needs for drug discovery strategies, but it can certainly be exploited as a model for disease-related pathways of interest that are highly conserved and in which functional readouts can be generated to measure their activity and the effects caused by inhibition of the pathway. In the following sections I will describe three future directions that represent natural outgrowths of the work that I have accomplished while pursuing the research directions described in this dissertation.

FUTURE DIRECTION I: Determine whether MCP110 interacts with the Ras or Raf interface

In worms

The analysis of MCP activity in *let-60(gf), lin-45AA(gf), mek-2*(gf) + *mpk-1*(gf) *and lin-1(null)* strains allowed me to test the selectivity of MCP towards the Ras>Raf><MEK>ERK pathway, but did not clarify whether MCP interacts with Ras or Raf to exert its anti-Muv activity. For that purpose, additional tools are required to

completely understand the mechanism of action of these compounds. I therefore attempted several strategies to generate new transgenic worms. Since we would like to determine if these small molecules are binding to Ras, to Raf, or to the Ras/Raf interface, it would be logical to test their activity in the absence of one or the other. This idea is based on several points – 1) the nature of the original MCP screen, in which a yeast-two hybrid assay was modified to select compounds that disrupt Ras interactions with Raf (Kato *et al.*, 1992; Khazak *et al.*, 2005), 2) the fact that the Raf/LIN-45AA mutant that is sufficient to induce a Muv phenotype, and that has been reported to be Ras-independent by virtue of S>A mutations at the Akt inhibitory sites, retains the RBD and may still depend on additional input from endogenous Ras, and 3) the fact that one of many critical steps for Raf activation is for the RBD in the N-terminus of Raf to make contact with the Ras effector domain, in part to promote phosphorylation that relieves autoinhibitory constraints on the kinase domain (Figure 1.7)(Leevers *et al.*, 1994; Vojtek *et al.*, 1993). Thus, our hypothesis is that the absence of a functional Ras binding domain in the otherwise constitutively active form of Raf/LIN-45AA or a similar construct would generate a truly Ras-independent form of Raf, and this would be (unlike LIN-45AA) insensitive to MCP inhibition.

As a result, we embarked on the generation of a new transgenic animal expressing a constitutively activated LIN-45, lacking the RBD whose activity would be independent of Ras interaction. In order to generate a construct with those specifications, many considerations were taken into account. First, as mentioned, Raf activation is complex and requires particular regions of the N-terminus (Figure

1.7). For that reason, I performed detailed sequence and computer-modulated secondary protein structure analysis to carefully select regions of LIN-45 that would be biologically functional, and then amplified by PCR the selected areas of LIN-45 including the kinase domain and the complete C-terminal region of Raf (Figure 4.1). Additional sequences upstream of the kinase domain were added to ensure the proper conformation of the protein product of Raf/LIN-45.

Our first idea, was to target the LIN-45 kinase domain to the plasma membrane by adding the plasma membrane targeting sequence from Ras/LET-60, as similarly described in (Leevers *et al.*, 1994). Given that human Ras variants have different hypervariable regions and CAAX motifs, the sequences of H-, N-, K- Ras and LET-60 were aligned and carefully analyzed, to reveal that the K-Ras hypervariable domain had the higher identity with LET-60 (Figure 4.1, blue). The LIN-45/LET-60 fusion constructs were made to express a fusion of LIN-45 kinase domain plus the hypervariable domain and the "CAAX"-related plasma membrane targeting sequence of LET-60 (Figure 4.1, yellow). However, many attempts to generate transgenic worms expressing this construct produced a high success rate of transgenics expressing the co-injection markers, but none showing the expected Muv phenotype indicative of expression of activated LIN-45-CAAX. It is possible that overactivation of LIN-45 may cause toxic effects in embryos and thus not allow the viability of such LIN-45-CAAX transgenics. It is also possible that, regardless of the sequence homology between mammalian and worm genes, this strategy, which was previously performed in a mammalian system (Leevers *et al.*, 1994), is not functional for the worm protein counterparts.

A second strategy to generate a Ras-independent, LIN-45 activated construct was designed to once again amplify the kinase domain region of LIN-45, but without the LET-60 plasma membrane targeting sequence. This strategy was based on reports in which truncation solely of the first 305 amino acids from the N-terminal region of Raf (Δ22W) was sufficient to obtain a constitutively activated gene product that displayed high transforming activity in NIH 3T3 cells (Stanton *et al.*, 1989) (Oldham *et al.*, 1996). Given the high conservation of the kinase domain between mammalian Raf and LIN-45 (Figure 4.2C), we hypothesized that a similar construct would also be functional when expressed in the worm. However, in control cell culture experiments we did not observe expression of the protein product of several versions of this mutant, perhaps due to issues with protein stability. Therefore one important future direction is to address other approaches to the generation of a transgenic worm expressing constitutively activated but Ras-independent LIN-45, which does not presently exist. In theory such a reagent would provide us with an excellent tool for the analysis of substrate requirements for MCP action. However, because we were later able to obtain data in mammalian cells on MCP110-mediated disruption of the Ras/Raf interaction, future studies specifically on the mechanism of action of MCP compounds would be better done in that system rather than in *C. elegans*.

A.

```
A-Raf           ----------------------MEPPRGPPANGAEPS---------------------- 15
B-Raf           MAALSGGGGGGAEPGQALFNGD-MEPEAGAGAGAAASSAADPAIPEEVWNIKQMIKLTQE 59
C-Raf           MEHIQG-------AWKTISNGFGFKDAVFDGSSCISPT---------------------- 31
LIN-45          MSRINFKK---SSASTTPTSPHCPSPRLISLPRCASSSIDRK------------------ 39
                                .      .    .:

A-Raf           ------------------------------------------RAVG--------------- 19
B-Raf           HIEALLDKFGGEHNPPSIYLEAYEEYTSKLDALQQREQQLLESLGNGTDFSVSSSASMDT 119
C-Raf           ---------------------------------------IVQQFG--------------- 37
LIN-45          --------------------DQASPMASPSTPLYPKHSDSLHSLSG-------------- 65
                                                    . ..

A-Raf           ------------------------------------TVKVYLPNKQRTVVTVRDGMSVYD 43
B-Raf           VTSSSSSSLSVLPSSLSVFQNPTDVARSNPKSPQKPIVRVFLPNKQRTVVPARCGVTVRD 179
C-Raf           -----------------YQRRASDDGKLTDPSKTSNTIRVFLPNKQRTVVNVRNGMSLHD 80
LIN-45          ----------------HHSAGGAGTSDKEPPKFKYKMIMVHLPFDQHSRVEVRPGETARD 109
                                                 : *.** .*:: * .* * :  *

A-Raf           SLDKALKVRGLNQDCCVVYRLI----KGRKTVTAWDTAIAPLDGEELIV--EVLEDVPLT 97
B-Raf           SLKKALMMRGLIPECCAVYRIQ----DGEKKPIGWDTDISWLTGEELHV--EVLENVPLT 233
C-Raf           CLMKALKVRGLQPECCAVFRLLH-EHKGKKARLDWNTDAASLIGEELQV--DFLDHVPLT 137
LIN-45          AISKLLKKRNITPQLCHVNASSDPKQESIELSLTMEEIASRLPGNELWVHSEYLNTVSSI 169
                .: * *  *.:  : * *        .. :     :   : * *:** *  : *: *.

A-Raf           MHNFVRKTFFSLAFCDFCLK-FLFHGFRCQTCGYKFHQHCSSKVPTVCVDMSTN------ 150
B-Raf           THNFVRKTFFTLAFCDFCRK-LLFQGFRCQTCGYKFHQRCSTEVPLMCVNYDQL------ 286
C-Raf           THNFARKTFLKLAFCDICQK-FLLNGFRCQTCGYKFHEHCSTKVPTMCVDWSNI------ 190
LIN-45          KHAIVRRTFIPPKSCDVCNNPIWMMGFRCEFCQFKFHQRCSSFAPLYCDLLQSVPKNEDL 229
                 * :.*:**:    **.* : : : ****: * :***::**: .*  *   .

A-Raf           -RQQFY------HSVQD---------LSGGSRQHEAPSNRPLNELLTPQGPSPRTQHCDP 194
B-Raf           -DLLFVSKFFEHHPIPQEEASLAETALTSGSSPSAPASDSIGPQILTSPSPSKSIPIPQP 345
C-Raf           -RQLLLFP---NSTIGD-------SGVPALPSLTMRRMRESVSRMPVSSQHRYSTPHAFT 239
LIN-45          VKELFGIASQVEGPDRSVAEIVLANLAPTSGQSPAATPDSSHPDLTSIKRTGGVKRHPMA 289
                    :        .  .          .                :              .

A-Raf           EHFPFPAPANAPL---QRIRSTSTPNVHMVSTTAPMDSN---LIQLTGQSFSTDAAGSRG 248
B-Raf           FRPADEDHRNQFG---QRDRSSSAPNVHINTIEPVNIDD---LIR--DQGFRGDGGSTTG 397
C-Raf           FNTSSPSSEGSLS---QRQRSTSTPNVHMVSTTLPVDSR---MIE--------DAIRSHS 285
LIN-45          VSPQNETSQLSPSGPYPRDRSSSAPNINAINDEATVQHNQRILDALEAQRLEEESRDKTG 349
                              * **:*:**::  .           :          :.  . .
```

```
A-Raf           ------------------------------------GSDGTPRGSPSPA----------- 261
B-Raf           ------------------------------------LSATPP--ASLPG----------- 408
C-Raf           ------------------------------------ESASPSALSSSPN----------- 298
LIN-45          SLLSTQARHRPHFQSGHILSGARMNRLHPLVDCTPLGSNSPSSTCSSPPGGLIGQPTLGQ 409
                                                    *  ..  .. *

A-Raf           --------------------SVS-SGRKSPHSKSPA-EQRERKSLADDKKKVKNLG-YRD 298
B-Raf           --------------------SLT-NVKALQKSPGPQ-RERKSSSSSEDRNRMKTLG-RRD 445
C-Raf           --------------------NLSPTGWSQPKTPVPAQRERAPVSGTQEKNKIRPRG-QRD 337
LIN-45          SPNVSGSTTSSLVAAHLHTLPLTPPQSAPPQKISPGFFRNRSRSPGERLDAQRPRPPQKP 469
                                    ::       :.  *   ..   *  :  .  :     :

A-Raf           SGYYWEVPPSEVQLLKRIGTGSFGTVFRGRWHGDVAVKVLKVSQPTAEQAQAFKNEMQVL 358
B-Raf           SSDDWEIPDGQITVGQRIGSGSFGTVYKGKWHGDVAVKMLNVTAPTPQQLQAFKNEVGVL 505
C-Raf           SSYYWEIEASEVMLSTRIGSGSFGTVYKGKWHGDVAVKILKVVDPTPEQFQAFRNEVAVL 397
LIN-45          HHEDWEILPNEFIIQYKVGSGSFGTVYRGEFFGTVAIKKLNVVDPTPSQMAAFKNEVAVL 529
                    **:  .:. :  ::*:******::*.:.* **:* *:*  **..*  **:**: **

A-Raf           RKTRHVNILLFMGFMTRPGFAIITQWCEGSSLYHHLHVADT--RFDMVQLIDVARQTAQG 416
B-Raf           RKTRHVNILLFMGYSTKPQLAIVTQWCEGSSLYHHLHIIET--KFEMIKLIDIARQTAQG 563
C-Raf           RKTRHVNILLFMGYMTKDNLAIVTQWCEGSSLYKHLHVQET--KFQMFQLIDIARQTAQG 455
LIN-45          KKTRHLNVLLFMGWVREPEIAIITQWCEGSSLYRHIHVQEPRVEFEMGAIIDILKQVSLG 589
                :****:*:*****:  .  :**:**********:*:*: :.  .*:*  :**: :*.: *

A-Raf           MDYLHAKNIIHRDLKSNNIFLHEGL-TVKIGDFGLATVKTRWS--GAQPLEQPSGSVLWM 473
B-Raf           MDYLHAKSIIHRDLKSNNIFLHEDL-TVKIGDFGLATVKSRWS--GSHQFEQLSGSILWM 620
C-Raf           MDYLHAKNIIHRDMKSNNIFLHEGL-TVKIGDFGLATVKSRWS--GSQQVEQPTGSVLWM 512
LIN-45          MNYLHSKNIIHRDLKTNNIFLMDDMSTVKIGDFGLATVKTKWTVNGGQQQQQPTGSILWM 649
                *:***:*.*****:*:***** :.: **************::*:  *.:  :* :**:***

A-Raf           AAEVIRMQDPNPYSFQSDVYAYGVVLYELMTGSLPYSHIGCRDQIIFMVGRGYLSPDLSK 533
B-Raf           APEVIRMQDKNPYSFQSDVYAFGIVLYELMTGQLPYSNINNRDQIIFMVGRGYLSPDLSK 680
C-Raf           APEVIRMQDNNPFSFQSDVYSYGIVLYELMTGELPYSHINNRDQIIFMVGRGYASPDLSK 572
LIN-45          APEVIRMQDDNPYTPQSDVYSFGICMYEILSSHLPYSNINNRDQILFMVGRGYLRPDRSK 709
                *.******* **:: *****::*: :**:::. ****:*. ****:*******  ** **

A-Raf           ISSNCPKAMRRLLSDCLKFQREERPLFPQILATIELLQRSLPKIERSASEPSLHR----- 588
B-Raf           VRSNCPKAMKRLMAECLKKKRDERPLFPQILASIELLARSLPKIHRSASEPSLNRAG--- 737
C-Raf           LYKNCPKAMKRLVADCVKKVKEERPLFPQILSSIELLQHSLPKINRSASEPSLHR----- 627
LIN-45          IRHDTPKSMLKLYDNCIMFDRNERPVFGEVLERLR--DIILPKLTRSQSAPNVLHLDSQY 767
                :  : **:* :*  :*:   ::***:* ::*  :.     ***: ** * *.: :

A-Raf           --------------------TQADELPACLLSAARLVP--------- 606
B-Raf           ------------------FQTEDFSLYACASPKTPIQAGGYGAFPVH 766
C-Raf           ------------------AAHTEDINACTLTTSPRLP----VF--- 648
LIN-45          SVMDAVMRSQMLSWSYIPPATAKTPQSAAAAAAAANKKAYYNVYGLI 813
                                    :     :.  . :
```

B.

```
A-Raf          -----GTVKVYLPNKQRTVVTVRDGMSVYDSLDKALKVRGLNQDCCVVYRLI----KGRK 51
C-Raf          PSKTSNTIRVFLPNKQRTVVNVRNGMSLHDCLMKALKVRGLQPECCAVFRLLH-EHKGKK 59
B-Raf          -----PIVRVFLPNKQRTVVPARCGVTVRDSLKKALMMRGLIPECCAVYRIQ----DGEK 51
LIN-45         -----KMIMVHLPFDQHSRVEVRPGETARDAISKLLKKRNITPQLCHVNASSDPKQESIE 55
                     : *.** .*:: * .* * :  *.: * *  *.:  : * *       .. :

A-Raf          TVTAWDTAIAPLDGEELIVEVL 73
C-Raf          ARLDWNTDAASLIGEELQVDFL 81
B-Raf          KPIGWDTDISWLTGEELHVEVL 73
LIN-45         LSLTMEEIASRLPGNELWVHSE 77
                    :   : * *:** *.
```

C.

```
A-Raf          YYWEVPPSEVQLLKRIGTGSFGTVFRGRWHGDVAVKVLKVSQPTAEQAQAFKNEMQVLRK 60
C-Raf          -YWEIEASEVMLSTRIGSGSFGTVYKGKWHGDVAVKILKVVDPTPEQFQAFRNEVAVLRK 59
B-Raf          ---EIPDGQITVGQRIGSGSFGTVYKGKWHGDVAVKMLNVTAPTPQQLQAFKNEVGVLRK 57
LIN-45         ----ILPNEFIIQYKVGSGSFGTVYRGEFFGTVAIKKLNVVDPTPSQMAAFKNEVAVLKK 56
                   :  .:. :  ::*:*******::*.:.* **:* *:*  **..*  **:**: **:*

A-Raf          TRHVNILLFMGFMTRPGFAIITQWCEGSSLYHHLHVADT--RFDMVQLIDVARQTAQGMD 118
C-Raf          TRHVNILLFMGYMTKDNLAIVTQWCEGSSLYKHLHVQET--KFQMFQLIDIARQTAQGMD 117
B-Raf          TRHVNILLFMGYSTKPQLAIVTQWCEGSSLYHHLHIIET--KFEMIKLIDIARQTAQGMD 115
LIN-45         TRHLNVLLFMGWVREPEIAIITQWCEGSSLYRHIHVQEPRVEFEMGAIIDILKQVSLGMN 116
               ***:*:*****:  .  :**:**********:*:*: :.  .*:*  :**: :*.: **:

A-Raf          YLHAKNIIHRDLKSNNIFLHEGL-TVKIGDFGLATVKTRWS--GAQPLEQPSGSVLWMAA 175
C-Raf          YLHAKNIIHRDMKSNNIFLHEGL-TVKIGDFGLATVKSRWS--GSQQVEQPTGSVLWMAP 174
B-Raf          YLHAKSIIHRDLKSNNIFLHEDL-TVKIGDFGLATVKSRWS--GSHQFEQLSGSILWMAP 172
LIN-45         YLHSKNIIHRDLKTNNIFLMDDMSTVKIGDFGLATVKTKWTVNGGQQQQQPTGSILWMAP 176
               ***:*.*****:*:***** :.: *************::*:  *.:  :* :**:****.

A-Raf          EVIRMQDPNPYSFQSDVYAYGVVLYELMTGSLPYSHIGCRDQIIFMVGRGYLSPDLSKIS 235
C-Raf          EVIRMQDNNPFSFQSDVYSYGIVLYELMTGELPYSHINNRDQIIFMVGRGYASPDLSKLY 234
B-Raf          EVIRMQDKNPYSFQSDVYAFGIVLYELMTGQLPYSNINNRDQIIFMVGRGYLSPDLSKVR 232
LIN-45         EVIRMQDDNPYTPQSDVYSFGICMYEILSSHLPYSNINNRDQILFMVGRGYLRPDRSKIR 236
               ******* **:: *****::*: :**::::. ****:*. ****:*******  ** **:

A-Raf          SNCPKAMRRLLSDCLKFQREERPLFPQILATIELL---- 270
C-Raf          KNCPKAMKRLVADCVKKVKEERPLFPQILSSIELLQ--- 270
B-Raf          SNCPKAMKRLMAECLKKKRDERPLFPQILASIELLARS- 270
LIN-45         HDTPKSMLKLYDNCIMFDRNERPVFGEVLERLRDIILPK 275
                : **:* :*  :*:   ::***:* ::*  :. :
```

Figure 4.1 Raf protein sequence alignments
A) Protein sequence alignment of full-length human A-Raf, B-Raf, c-Raf and LIN-45. Also shown are alignments of B) ras-binding domains (Raf-RBD) [blue] and C) kinase domains [green]. Sequence alignments were performed in ClustalW2 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). As in Figure 1.4, (*) = identical, (:) = conserved substitutions and (.) = semi-conserved substitutions.

In mammals

Our results upon testing the ability of MCP110 to inhibit the Ras/Raf-RBD interaction brought us a step closer to elucidate the protein interface requirements for the activity of the drug. Now that we have evidence that MCP110 can disrupt the interaction between Ras and Raf-RBD, the rising questions are: Does MCP110 also inhibit other Ras interaction with other effector pathways or is it specific for the Ras/Raf interaction in particular? Does MCP110 also disrupt the interaction of related small GTPases with the RBDs of their respective effectors?

To address the first question it would be necessary to assess the activation of other Ras downstream effector pathways in the same total lysate where Ras/Raf-RBD interaction is disrupted by MCP110. For example another key directly interacting effector of Ras is the lipid kinase, PI3-K, that causes phosphorylation of its own indirect downstream target, the serine/threonine kinase, Akt (also known as PKB, protein kinase B). The observation of steady levels of Akt phosphorylation with concomitant reduced levels of ERK phosphorylation upon MCP110 treatment would be indicative that the disruption of Ras and Raf-RBD interactions is accomplished in a manner as to lead only to effects in the Raf>MEK>ERK pathway and not to other Ras effector pathways. If seen, this might be more consistent with binding of MCP110 to Raf rather than to Ras, given the overlap in binding sites on Ras for PI3K (p85 subunit) versus for Raf. Alternatively, while we assume that MCP110 is dispersed uniformly throughout the cell and would therefore not be subject to localization effects, one way to rule this out would be to dye-label the inhibitor and monitor its subcellular localization in treated cells.

An alternative to discern the specificity of MCP110 towards disrupting the Ras/Raf-RBD complex, versus other related GTPases and their respective effector RBDs, would be to measure the ability of MCP110 to disrupt those interactions through pulldown assays. For example, it is known that the RBDs of Ral GEFs (RalGDS, Rgl1-3) can interact with Raf as well as with the Rap1A GTPase (Esser *et al.*, 1998; Spaargaren and Bischoff, 1994; Wolthuis *et al.*, 1996). Thus, it would be interesting to compare the ability of each of these RBDs to assess MCP110-mediated disruption of the interaction of Ras or Rap1A with Raf- or Rgl-RBD. These results could potentially help disclose the selectivity of MCP110 for a certain GTPase (Ras vs. Rap or others) or to discriminate between effector binding RBDs.

FUTURE DIRECTION II: Evaluate the specificity of MCP compounds at blocking only Ras/Raf interactions versus off-target activities

Previous studies in cell-based assays have evaluated the ability of the MCP compounds to inhibit cell-autonomous phenotypes caused by Ras activation, but the physiological effects of these compounds in an *in vivo* system were not determined previously. In selecting the worm as a model to characterize the activity of MCP compounds we already started the process to do so. Since the development and behavior of the worm is so well-characterized, any off-target effects causing lethality, arrested development, slow growth, paralysis or behavioral changes can not only be easily detected, but the abundant literature and thoroughly curated databases such as “Wormbase” [http://www.wormbase.org] can also help us identify rapidly whether a certain phenotype has been previously observed and traced to a particular

pathway or protein. Another more laborious, but effective approach, as discussed in Chapter I, would be to perform a chemical genetic screen to phenotypically screen for any phenotypes resembling the ones caused by the drug and then proceed to genetically map the target gene. Of course, it is also possible that MCP compounds may have some off-target effects that cannot be readily observed under the dissecting microscope (low resolution), which may cause false negative conclusions.

In the event, our results showed that, in addition to the ability of MCP compounds to selectively reverse the Muv phenotype of Ras and Raf but not MEK/ERK or Ets animals, they also caused changes in the social behavior of treated animals. Both wild type worms and animals mutant or transgenic for activation of the Ras>Raf>MEK>ERK pathway normally disperse and crawl randomly through the agar plates (Figure 4.2, left column); this “normal” behavior is often described as solitary behavior (de Bono and Bargmann, 1998). However, when exposed to MCP110 or MCP116, but not MCP146 or vehicle, Ras (*let-60*(gf), Ets (*lin-1* null*)* and even wild type animals changed their solitary behavior and instead adopted a social behavior called “clumping” in which groups of worms congregated at the edges of the bacterial lawn (Figure 4.2). Moreover, the strength of this clumping behavior was dose-dependent (data not shown). This conglomeration of worms was easily disrupted by the movement of the plates, and also was restored after the plates were settled down for more than 20 minutes.

The reproducibility of this conglomerating/social or clumping behavior suggests that MCP compounds are the source of this particular phenotype, since vehicle-treated worms behave normally and clumping is not dependent on the

genetic background of the strain being tested. However, the clumping behavior can also be uncoupled from inhibition of the Muv phenotype in several ways. First, MCP110 and MCP116 were equivalently potent at inhibiting Ras/LET-60-induced Muv (Figure 3.1), whereas MCP110 was significantly more potent at inducing clumping (Figure 4.2). Second, MCP110 but not MCP116 induced clumping in LIN-1-deficient worms (Figure 4.2), which have a Muv phenotype that is resistant to inhibition by either MCP compound (Figure 3.2). Third, MCP116 induced some clumping in N2 wild type worms but not in LIN-1-deficient worms, the latter of which is Muv and the former of which is not. Fourth, the MEK inhibitor U0126 robustly inhibits the Muv phenotype of animals expressinf Ras but has no effect whatsoever on clumping, indicating that clumping is not a necessary outcome of Muv inhibition. Together, these results indicate that MCP110 and MCP116 have two distinct activities, one of which is the intended inhibition of hyperinduced vulval formation ("on-target") and the other of which is clumping behavior ("off-target").

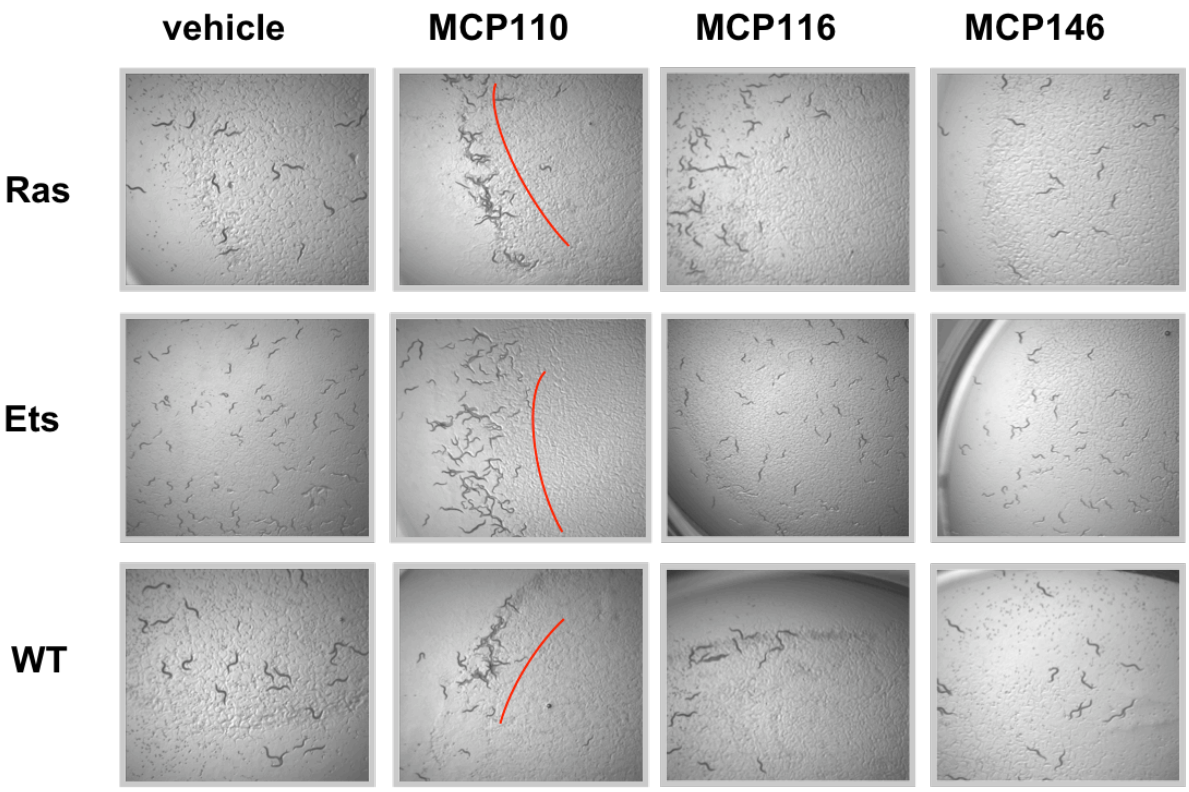


Figure 4.2. Off-target activity: changes in worm feeding behavior upon treatment with MCP compounds Treatment of Ras (*let-60 gf),* Ets (*lin-1 null)* or wild type (WT) strains with MCP110 and MCP116 caused a switch from normal solitary feeding behavior to social behavior (see red line). MCP146 did not cause behavioral changes, suggesting that this is not simply a previously unappreciated effect of Ras/Raf inhibition but an off-target activity selectively present in MCP110 and MCP116 but not MCP146.

This observation provides evidence that MCP compounds have alternate targets in addition to their activity towards inhibiting Ras/Raf interaction. This alternate activity was detected by the change in the feeding behavior of the animals treated with MCP compounds, but not DMSO vehicle. Moreover, the strength of the aggregation/clumping phenotype was analog- and dose-dependent, since it was associated with only two analogs (MCP 110 and MCP116) and was seen only mildly at 10 μM and strongly at 20 μM.

My colleague Dr. Reiner immediately recognized that this clumping behavior had been previously described in the literature (de Bono and Bargmann, 1998) as a natural variation of feeding behavior. This behavior is associated with a variant of the orphan G-protein coupled receptor (GPCR), NPR-1. The wild type worms used here (N2) and solitary strains found in nature have a valine at residue 215, while naturally occurring social strains have a phenyalanine at this same position. NPR-1 encodes a predicted G-protein coupled receptor similar to neuropeptide Y receptors (NPYR) in humans (de Bono and Bargmann, 1998). In humans, NPYRs regulate food consumption, mood, anxiety, memory retention, hippocampal excitability and blood pressure (Blomqvist and Herzog, 1997). Given the nature of our results, its possible that treatment with MCP-110 and MCP-116 may also be targeting the function of NPR-1, thus inducing the social/clumping behavior. Further testing of the ability of MCP110 and MCP116 to promote this behavior will require further behavioral assays using *npr-1* mutants. These assays may reveal the potential of MCP compounds to inhibit the GPCR, and the possibility to create new pharmacological agents that retain only anti-NPR-1 activity. Currently, the MCP-

driven clumping behavior is being evaluated by our collaborator Mario deBono (MRC, Cambridge, UK), who has expertise in the study of the *npr-1* phenotype (de Bono and Bargmann, 1998).

In summary, testing the activity of MCP compounds in *C. elegans* allowed the identification of an unexpected off-target effect that remains to be characterized. Altogether, our results support the idea that MCP 110 and MCP 116 may contain at least two different activities; 1) inhibition of Ras/Raf interaction and 2) inhibition of NPR-1. Our results also confirm the selectivity of this off-target activity, and show clearly that it can be uncoupled from the activity of inhibiting Ras/Raf interaction and from the strain being tested. Moreover, our evaluation of the MEK inhibitor U0126 supports the idea that disruption of the Muv phenotype is not sufficient to cause the switch to social behavior, confirming that these activities are separable and not simply nonspecific.

FUTURE DIRECTION III: Development of a novel screen for Rac pathway inhibitors - proof-of-principle using the Rac inhibitor EHT 1864

Given the experiences I have gained in evaluating inhibitors of the Ras>Raf>MEK>ERK pathway, the thought of developing alternate strategies to use *C. elegans* as a model for other drug discovery efforts seemed reasonable. The selection of the next target for investigation included evaluation of the current literature to identify proteins that are both currently validated as potential targets for anti-cancer therapy and conserved in the worm. Our interest in small GTPases

quickly narrowed the number of attractive targets and we selected the small GTPase, Rac.

BACKGROUND

Rac belongs to the family of Rho (Ras homologous) proteins, a subfamily of the Ras superfamily of small GTPases (Ellenbroek and Collard, 2007). (Figure 1.1). Rho small GTPases are regulated in a similar way to the Ras family of GTPases, where positive regulators including GEFs and negative regulators including GAPs control the "on" and "off" state of these proteins by modulating their GTP/GDP binding status. In addition, Rho proteins are also regulated by a third class of modulators, Rho guanine nucleotide dissociators (RhoGDIs). These ubiquitously expressed chaperone proteins have the capacity to retain most COOH-terminally processed Rho proteins (e.g., Cdc-42, Rac1, Rac2 and RhoA but not RhoB or TC10 in the cytosol (Michaelson *et al.*, 2001). The action of RhoGDI as a negative regulator prevents the localization of Rho proteins to their targeted membranes, thereby preventing their full activation and interaction with downstream effectors until release of RhoGDI. The best characterized members of the Rho subfamily are RhoA, Rac1 and Cdc42, and their best known role is to regulate actin dynamics (Ellenbroek and Collard, 2007). In particular activation of Rho GTPases by extracellular stimuli or by introducing activated Rho proteins causes the formation of cellular stress fibers, lamellipodia and filopodia, respectively (Hall, 1998; Nobes and Hall, 1999). In turn these changes in the actin structure also affect regulation of cell shape, cell adhesion and cell migration (Ridley and Hall, 1992a; Ridley and Hall,

1992b). The specific regulation of lamellipodia by Rac therefore provides a selective for activation or inhibition of Rac function.

RAC PATHWAY ACTIVATION IN TUMORIGENESIS

To date activating mutations have not been detected in Rho family proteins; however, aberrant signaling is frequently accomplished by alterations affecting their regulators (as summarized in Figure 4.3). For example the increased expression of a Rac-specific positive regulator, the GEF Tiam-1 (Mertens *et al.*, 2003), has been correlated with the progression of renal cell carcinoma, prostate and breast cancer (Adam *et al.*, 2001; Engers *et al.*, 2006; Engers *et al.*, 2000; Minard *et al.*, 2004). Moreover, there is additional *in vivo* evidence showing that mice deficient in Tiam1 are resistant to Ras-induced tumors (Malliri *et al.*, 2002), indicating that this RacGEF is required for full oncogenesis. Also, the loss of expression of a Rho GAP, DLC-1 or Rac-GAP β-chimaerin, leads to persistent activation of Rho GTPases in liver cancer (Ching *et al.*, 2003; Yuan *et al.*, 1998) and in breast cancer, respectively (Yang *et al.*, 2005). In addition to the deregulation of Rac modulators, Rac itself is also overexpressed in a subset of cancers; among these are included gastric cancer (Pan *et al.*, 2004), testicular cancer (Kamai *et al.*, 2004), breast cancer (Fritz *et al.*, 2002), oral squamous cell carcinoma (Liu *et al.*, 2004) and brain cancer (Hwang *et al.*, 2005a; Hwang *et al.*, 2005b). The deregulation of proteins that modulate Rac activation or the overexpression of Rac in various types of cancers provides substantial evidence that Rac is important for the onset and maintenance of cancer. Also, our laboratory showed that Rac1 and Rac3 are physiologically relevant targets

of the anti-transforming activity of geranylgeranyltransferase inhibitors (Joyce and Cox, 2003). Altogether the deregulation of several elements of Rac pathways in different types of cancer and the relevance of key regulators like Tiam1 for tumorigenesis begins to validate Rac as a good target for the development of new anti-cancer therapies. Additional direct validation comes from recent work showing that mice deficient in Rac1 are impaired in Ras-mediated lung cancer formation (Kissil *et al.*, 2007).

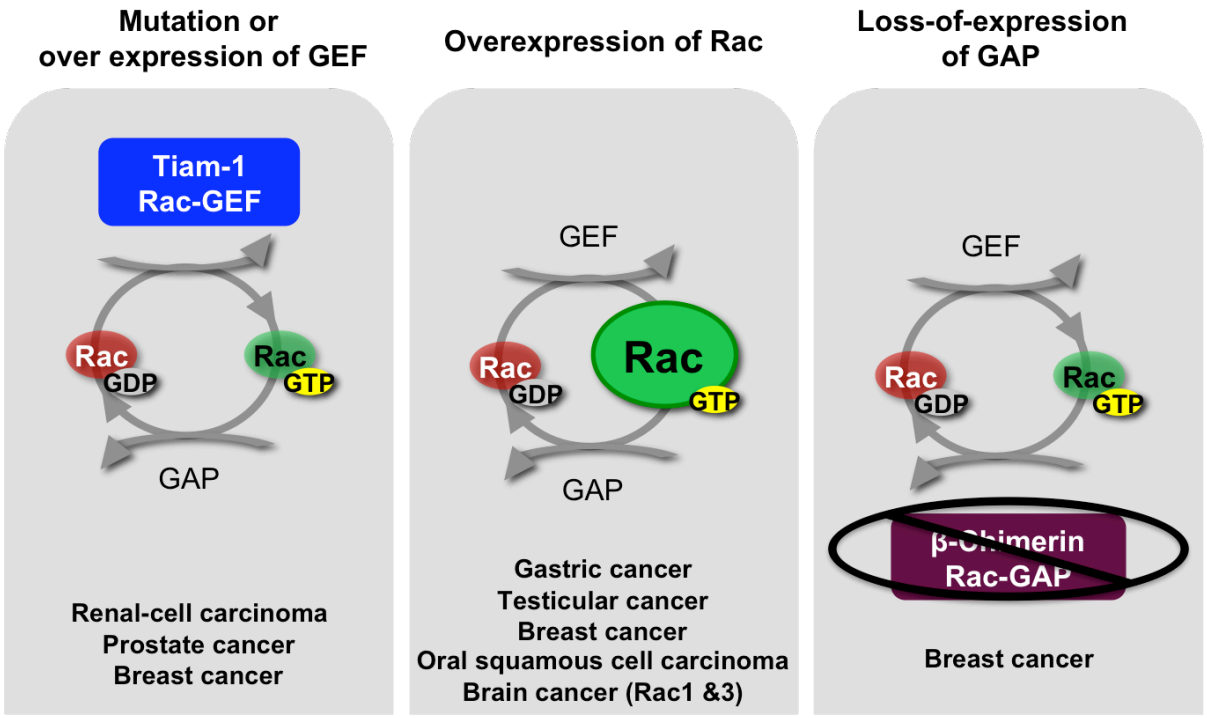


Figure 4.3. Rac activity is deregulated in many cancers. Rac itself does not harbor activating mutations; on the other hand deregulation of Rac caused by over expression of RacGEFs (e.g., Tiam1), decreased expression of GAPs (e.g., b-chimaerin) or overexpression of Rac itself has been reported for subsets of cancers.

RAC PATHWAY AS A TARGET FOR ANTI-CANCER DRUGS

One example of a promising Rac-specific inhibitor is EHT1864. Although the exact mechanism of action of EHT1864 is not yet understood, it is thought to act in an unusual manner by displacing guanine nucleotide from Rac, thereby allowing it to block both wild type and active Rac. Consistent with this, EHT1864 has been validated in cell-based assays to inhibit the lamellipodia formation caused by PDGF-mediated stimulation of wild type Rac (Shutes *et al.*, 2007) and the transformed phenotype caused by activating mutations in Rac (e.g., G12V) or Tiam1 (Desire *et al.*, 2005). Whether EHT1864 has activity *in vivo* is not known.

The p21-activated kinases (PAKs) are also attractive targets for anticancer drug development against the Rac pathway. PAKs are a highly conserved family of serine/threonine kinases with six human members, PAK 1-6, that serve as downstream effector proteins for Rac and related Rho family GTPases. Only PAK1-3 bind active Rac, whereas all of them can bind Cdc42 (Ellenbroek and Collard, 2007). Existing evidence supports PAK as an essential mediator for cell transformation caused by oncogenes like Ras. Moreover, there is evidence that PAK1 is also upregulated in breast cancers (Salh *et al.*, 2002; Vadlamudi *et al.*, 2000) and that other PAK family members are also associated with other tumor types. A detailed review of the biological role of PAK family members and their association with different types of cancer is provided elsewhere (Kumar *et al.*, 2006). Previous evidence supports that overexpression of mutationally activated PAK is sufficient for mammary gland tumor formation in mice (Wang *et al.*, 2006). Moreover, there is additional evidence that kinase-deficient PAK mutants inhibit cell

transformation mediated by the Ras-Rac pathway (Tang *et al.*, 1997). Altogether these results highlight PAK as a potential target for anticancer therapies. To date there are several compounds that inhibit PAK activity by targeting either its kinase activity or its interaction with other proteins (reviewed in (Kumar *et al.*, 2006)). In summary, there are considerable *in vitro* and *in vivo* data showing the efficacy of inhibitors targeting either Rac activity or the activity of one of its downstream effectors, PAK. The existence of such inhibitors is not discouragement to keep searching for additional and perhaps better Rac pathway inhibitors.

RAC PATHWAY IN *C. ELEGANS*

C. elegans has two Rac1 homologs: CED-10 and RAC-2, which both have 83% identity to the protein sequence of Rac1 (Lundquist *et al.*, 2001). However, analysis of sequence alignments of the worm Rac variants and their human counterparts showed that CED-10 shares the most similarities with Rac1. Previous studies using RNAi or loss-of-function mutants demonstrated that CED-10 is necessary for distal tip cell migration, axon guidance and axon pathfinding and cell corpse phagocytosis (Lundquist *et al.*, 2001). On the other hand, the expression of a gain-of-function *ced-10(G12V)* under the control of a neuron-specific *unc-115* promoter displayed premature CAN axon termination, misguidance and ectopic branching (Lundquist *et al.*, 2001). These results indicate the importance of CED-10 to worm function.

In addition to the conservation of CED-10 itself, other regulatory proteins and downstream effectors of Rac are also conserved in the worm. For example the specific mammalian Rac GEF, Trio, is conserved in the worm. Its counterpart, UNC-

73, functions through Rac to modulate cytoskeleton rearrangements (Hansson, 1983). Another regulator of Rac1 also conserved in worms is RhoGDI. The molecular characterization of *C. elegans* RhoGDI (ceRhoGDI), showed that ceRhoGDI has affinity for all counterparts of the worm RhoGTPases (Yap *et al.*, 1999). Also to date three homologs of the Rac downstream effector, PAK, have been found in the worm (Lucanic *et al.*, 2006). The conservation of these important elements of the Rac pathway in the worm highlights the potential to use *C. elegans* for the development of genetic *in vivo* tools for the screening of Rac pathway inhibitors.

GENERATION OF RAC/CED-10-DEPENDENT LETHAL PHENOTYPE FOR DRUG SCREEN BINARY READOUT

Based on the homology of Rac with CED-10, there is potential to perform genetic manipulations in the worm to generate an *in vivo* system where worms carrying activated CED-10 can be used to screen for Rac pathway inhibitors. The design of a powerful drug screen would involve the use of a phenotype that can be easily scored, where a dramatic and positive change occurs upon pathway inhibition, and preferably utilizes a simple binary readout such as dead vs. alive to reveal the activity of a candidate drug.

In a standard drug screen, wild-type animals would be treated and the endpoint would be to look for phenotypes reminiscent of genetic loss of function of CED-10. However, the phenotype of *ced-10(lf)* involves defects in cell corpse phagocytosis and axon pathfinding. These phenotypes are both weak and unconventional for the design of a high throughput screen, since scoring any of

these defects involves mounting animals on microscope slides and using DIC microscopy, which is very low throughput. Therefore, treatment of wild type animals with a Rac inhibitor would be expected to yield only incremental changes in these animals, making the screen complex, laborious and very low-throughput.

Another approach to sensitize the pathway for the development of a drug screen is by using gain-of-function mutations. As described in Chapters II and III of this dissertation, the increased signaling and aberrant phenotype caused by an activating mutation in Ras/LET-60 can be reversed to normal signaling and wild type phenotype by treatment with inhibitors targeting downstream effectors of LET-60. The use of a similar gain-of-function mutation in Rac/CED-10, to cause increased signaling and a robust aberrant phenotype, could then be reversed to normal by inhibitors of the Rac1/CED-10 pathway (Figure 4.5). A mutation from glutamine (Q) to leucine (L) in residue 61, located in the switch 2 region, leaves both Rac and CED-10 insensitive to negative regulation and constitutively active in mammals and worms, respectively. However, unlike Ras/LET-60, where a gain of function causes a hyper-induced Muv phenotype in adult worms, constitutive activation of Rac/CED-10 causes embryonic lethality. The expression pattern of CED-10 is located throughout the embryo early in development and at different specific cell types during the adult stages. CED-10 is a critical player in the migration of epithelial cells and is important to achieve the ventral enclosure of the embryo, which generates a structurally intact animal (Lundquist *et al.*, 2001). Enclosure of the embryo is a key step required to ensure that the embryo will reach the following developmental stage, which is elongation. Failure of these cells to migrate and enclose the embryo

therefore causes embryonic lethality. As a consequence, the overall expression of mutationally activated CED-10 results in catastrophic defects in the development of the worm, and transgenes could not be isolated.

However, it is possible to take this natural event and use it to our advantage. The goal is to avoid the overall toxicity caused by constitutive overexpression of activated Rac/CED-10, yet permit normal development of parental worms followed consistently by embryonic lethality under defined conditions unless the Rac pathway is interrupted successfully, for example by a pharmacological inhibitor. To achieve this goal, my collaborator, Dr. David Reiner (UNC-CH), devised and engineered a conditional expression system in which expression of activated CED-10 is driven by the epithelial-specific *lin-26* promoter and so CED-10 is expressed only in epithelial cells. In addition, the construct contains a 3' UTR engineered to be sensitive to nonsense-mediated decay (NMD) mRNA decay. NMD normally works as a cellular mechanism of mRNA surveillance, to detect premature nonsense mutation and prevent the expression of truncated or aberrant proteins.

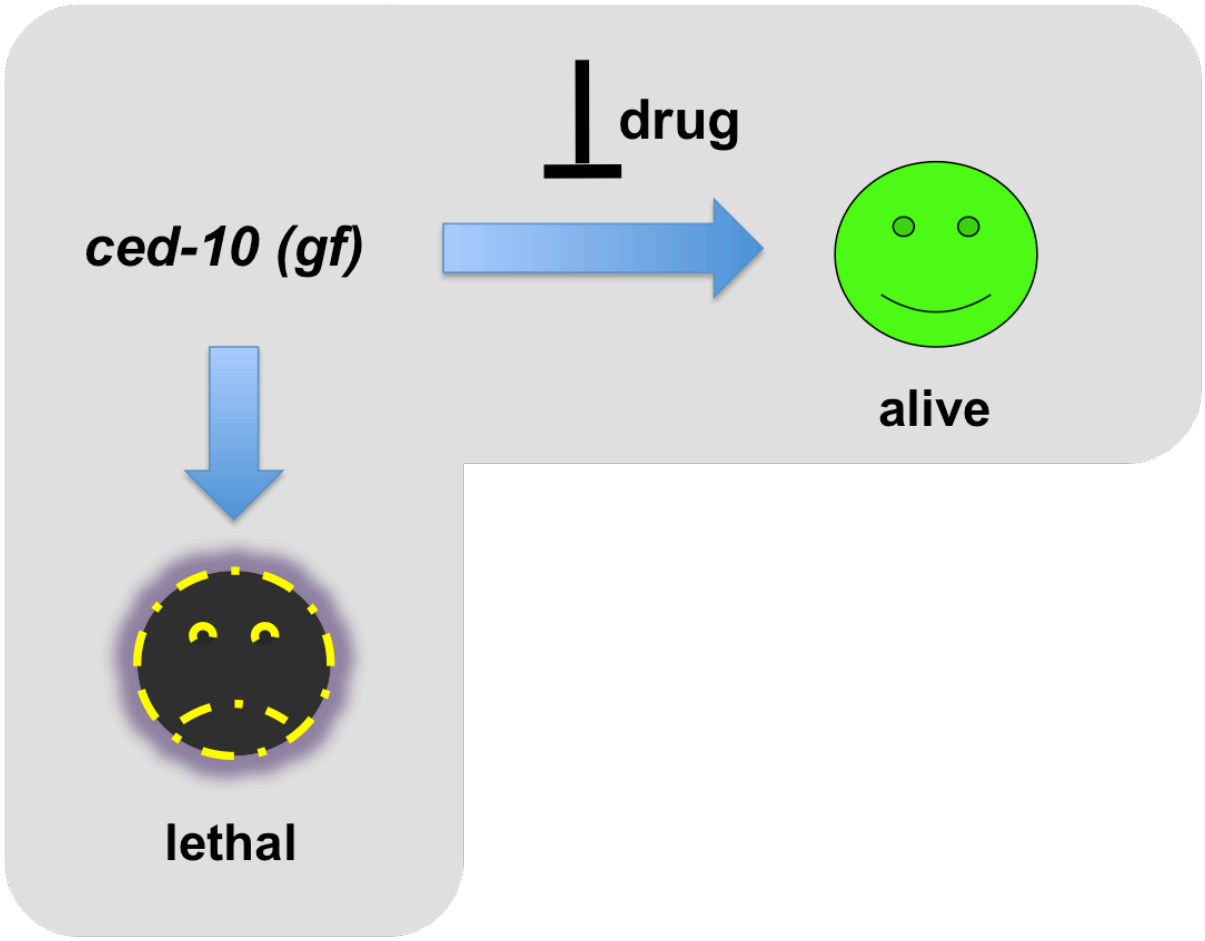


Figure 4.4 Overview of a positive binary readout for screening putative Rac pathway inhibitors. We generated a conditional system to allow expression of the otherwise embryonically lethal activated Rac/CED-10. This conditional system allows rescue of lethality by inhibitors of activated Rac or of the Rac pathway. Survival versus lethality thus acts as a positive and binary readout to identify candidate Rac pathway inhibitors.

A transgenic animal containing this construct was generated in a temperature-sensitive mutant background that conditionally disrupts the NMD system. As a consequence, in animals grown at 15°C, NMD is active, and the CED-10 transcript is degraded. On the other hand, in animals grown at 23°C, NMD is inactive, and the CED-10 transcripts are stably expressed. This conditional expression system has allowed the isolation and maintenance at 15°C of what would otherwise be a toxic transgene. Also it allowed the generation of a binary readout, indicative of Rac-specific expression, where at 15°C worms are alive and at 23°C worms are dead. If, in a screen, a given compound rescues the *ced-10*-driven lethality, then it would be a potential candidate for a Rac pathway inhibitor.

A successful screen will also require 100% lethality in the absence of a Rac inhibitor. Maintenance at 15°C ensures the conservation of this transgenic strain, but for experimental purposes I needed to determine the minimal temperature at which 100% lethality is reached. The relevance of finding this key temperature was to ensure that the lethal phenotype can be altered by minimal pharmacologic intervention. To determine this basal temperature I grew *ced-10(Q61L)* animals at a range of temperatures and scored their viability and lethality proportions at each temperature (Figure 4.5). This titration experiment allowed me to identify 23°C as the minimal temperature for 100% lethality, and was therefore selected as the screening temperature.

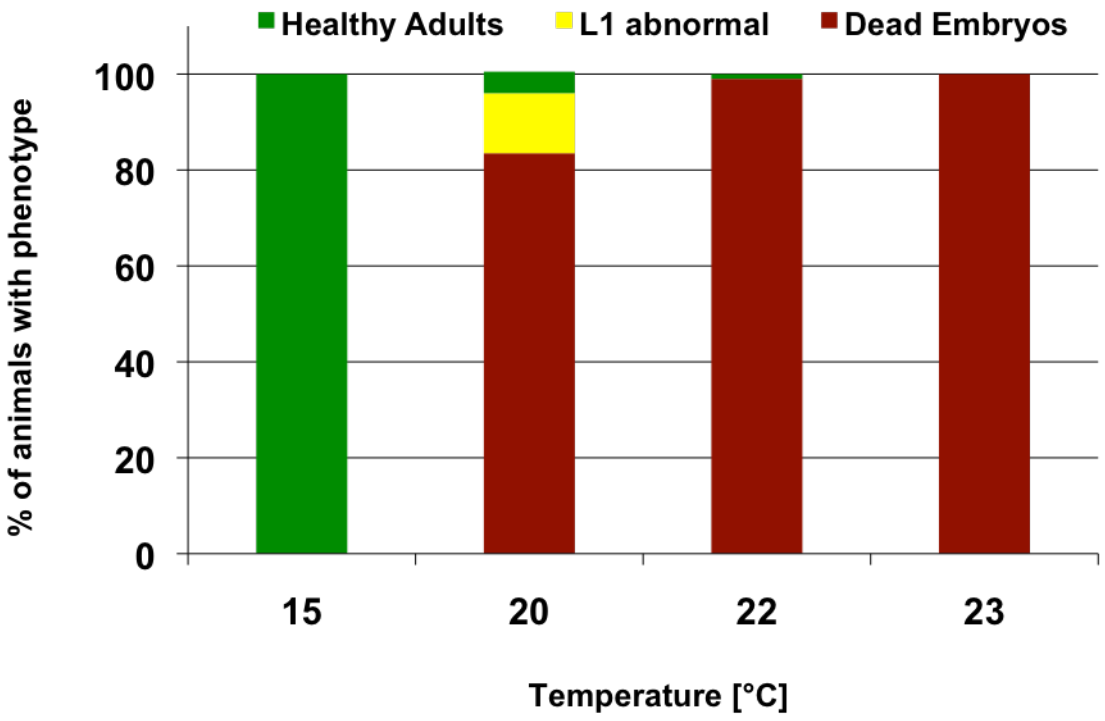
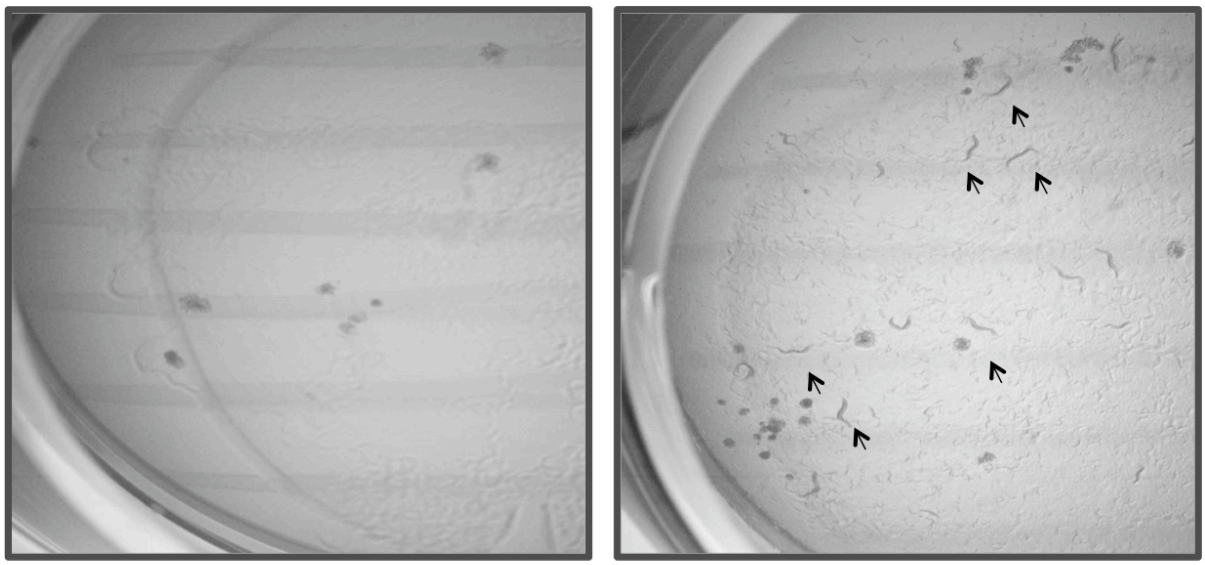


Figure 4.5 Temperature titration of Rac/CED-10-dependent lethal phenotype. The minimal temperature for 100% lethality of *ced-10 gf* transgenic animals was determined to be 23°C.

EVALUATE THE ABILITY OF A VALIDATED RAC INHIBITOR (EHT 1864) TO RESCUE THE LETHAL PHENOTYPE OF WORMS EXPRESSING ACTIVATED RAC/CED-10

EHT 1864 has been evaluated and characterized as a putative Rac inhibitor. The published evidence has shown that this inhibitor has *in vitro* and *in vivo* activity (in cells) towards both wild type and activated Rac. In these studies, the authors successfully showed that this compound can reduce both the lamellipodia formation caused by PDGF-mediated stimulation of wild type Rac (Shutes *et al.*, 2007) and the transformed phenotype caused by Tiam1 or by activating mutations in Rac(G12V) (Desire *et al.*, 2005). The mechanism of action of EHT 1864 is not well understood, but it is thought to act unconventionally, by displacing nucleotide from either active or wild type Rac. If this inhibitor does work at the level of Rac or perhaps even downstream, then it should rescue the lethal phenotype caused by the expression of activated CED-10. However if it works by inhibiting upstream regulators (e.g., GEFs), then these inhibitors should not be expected to rescue the lethal phenotype of these animals, unless activated CED-10 lethality also requires wild type Rac. At the same time that we use this inhibitor to validate our system, we could also unveil new information that could help understand better the mechanism of action of EHT1864. Given the nature of the proposed action of this Rac inhibitor, we expected to be able to validate our system by rescuing the CED-10-dependent lethal phenotype at 23°C in a dose-dependent manner.

A.



Vehicle
(dead embryos)

EHT 1864 25 µM
(viable worms)

B.

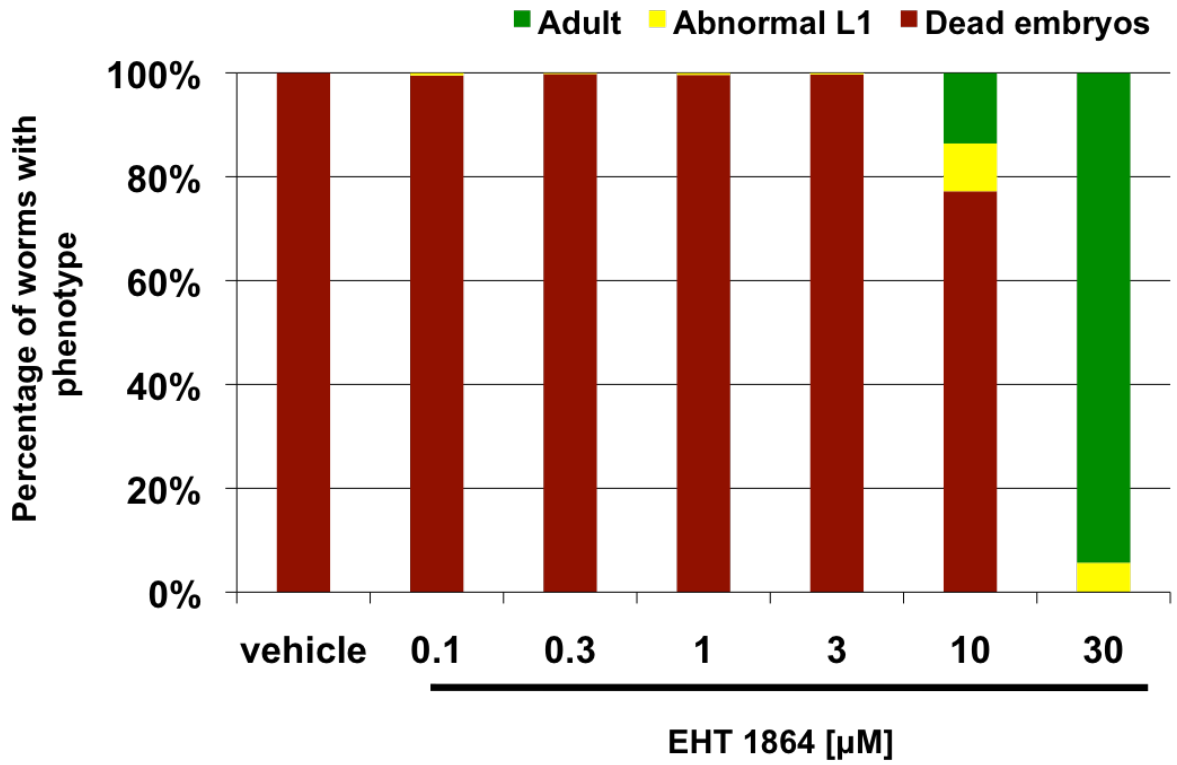


Figure 4.6 EHT 1864 rescues the lethal phenotype of CED-10 transgenic worms. A) Visual representation of lethality rescue by EHT 1864 of *ced-10*(gf) animals grown at 23°C. B) EHT 1864 rescued the lethal phenotype of CED-10 transgenic worms in a dose dependent manner.

Treatment of CED-10 transgenic worms showed that EHT 1864 rescued the CED-10-dependent lethality in a dose-dependent manner. The establishment of the system as a binary readout allowed the visual scoring of the positive effects of EHT 1864 as shown in Figure 4.6A. In addition to the visual scoring process, the number of worms surviving upon addition of EHT 1864, was quantified. I detected nearly 100% rescue at 30 μM EHT 1864 (Figure 4.6B). Additionally, worms were not only rescued from lethality, but many also progressed farther in development. For example, more worms advanced to the L1 stage when treated with EHT 1864 (data not shown). Additional experiments have shown that the rescue of CED-10-dependent lethality is reproducible and dose-dependent. Control experiments to confirm the selectivity of EHT 1864 for inhibition of Rac compared to other highly related small GTPases showed that treatment of NIH 3T3 mouse fibroblast cells with this Rac inhibitor reversed induction of lamellipodia formed upon stimulation of wild-type Rac by PDGF, but failed to reverse formation of stress fibers or filopodia, which are phenotypes caused by activation of Rho or Cdc42, respectively (data not shown). These results are consistent with rescue of *C. elegans* lethality as a specific consequence of inhibiting Rac activity.

The results obtained here validate the use of the CED-10-dependent lethal phenotype as the readout for the development of a screen for putative Rac pathway inhibitors. Rescue of the lethal phenotype was consistently reproducible and also strong. The ease with which the rescue of these animals can be detected supports the idea that this will be a viable and powerful way to screen for putative inhibitors of

Rac or the Rac pathway. By using this binary readout, the assay can be easily optimized to meet the requirements of a high throughput screen by using first 96-well plates and then 384-well plates in which thousands of compounds can be screened at once, under the dissecting microscope. In addition to validating this system for the development of a screen for Rac pathway inhibitors, our preliminary data opens the possibility of using this system to screen for inhibitors of other clinically relevant targets. In addition to detecting positive candidates, the use of an *in vivo* system would offer the advantage of detecting compounds with relevant biological activity and the ability to discard compounds with toxic properties earlier in the drug discovery process.

Designing a HTS to pursue screening

Given the positive validation of the CED-10-dependent lethal phenotype rescue by a Rac specific inhibitor (EHT 1864), we now have a system with which to design a screen for putative Rac pathway inhibitors. In such a screen, any compound that can rescue the lethal phenotype of worms with activated CED-10 would be considered a potential candidate for a Rac specific or Rac pathway inhibitor. Moreover, the binary readout offered by this system will allow the visual screening of thousands of compounds simultaneously, that can be executed even by inexperienced researchers.

Developing the HTS screen

In order to develop the worm culture conditions to meet the requirements of a high-throughput screen, I will need to switch my current protocol from solid agar to

liquid culturing media. This can be achieved by adopting similar culture conditions often used for the construction of deletion mutant libraries to generate *C. elegans* gene knockouts (§). Briefly, adult hermaphrodite worms are grown and synchronized using the bleaching protocol, described above in Chapter II. Newly hatched larvae are synchronously grown and titrated to control the amount of animals to be dispensed in each well of a 96-well plate. Performing these experiments in a 96-well plate would accelerate the screening process, making it a more powerful design by increasing the probabilities of screening more compounds per experiment and also finding positive candidate compounds. In my collaboration with the BRITE Institute (Biomanufacturing Research Institute and Technology Enterprise) at North Carolina Central University (NCCU, see below), we have discussed that 96-well plates are for piloting the screen, which when successful on a smaller scale will be transitioned to 384-well plates suitable for robotic handling. The conditions necessary to treat and grow animals in the screen will need to be carefully designed to ensure the assay will be proficient for its length. Each screening plate will include control (vehicle wells) and also 2-3 different concentrations of each compound in the library to be screened. As drug potency and solubility varies from compound to compound it will be necessary to test various concentrations simultaneously. This strategy will increase the probability of detecting activity. Additional logistics of the experimental design will need to be further developed as the process proceeds.

I expect that by screening a small molecule chemical library, I will be able to find compounds that could rescue the CED-10-dependent lethal phenotype. Also, I

expect some compounds to produce a strong rescue while others may show weak activity at rescuing the lethal phenotype of these worms. But regardless of the strength of the rescue, potential hit compounds can be selected for further testing. Given that this screen is based on worms expressing activated CED-10, I expect to detect inhibitors of either Rac itself or perhaps inhibitors of downstream elements. Some of these inhibitors can be kinase inhibitors, but this predisposition will depend on the nature of the chemical library being screened.

Another possibility is that the library tested does not contain any compounds capable of interfering in the pathway, or where that interference is linked either mechanistically or nonspecifically with toxicity that will prevent the rescue of the lethal phenotype. Some toxicity issues can be addressed by running parallel experiments at the permissive temperature of 15°C. If CED-10 transgenics show lower viability at this temperature, it could be an indication of the toxic effects of a particular compound. If I do not get any hits at all, it may mean that the library being tested does not cover the appropriate type of chemical entities.

In consultations with personnel from the drug discovery facilities at BRITE, there were suggestions about the optimization of our current experimental platform. As mentioned above, among these were to not only try to optimize the assay to a 96-well format, but if possible to 384-well format, since the current robotics utilized to dispense the compounds from their libraries are capable of this throughput, thus making any platform adjustment to work easier. Our current plan for the analysis of hits is to visually screen each well for the rescue of *ced-10*-driven lethality, and although this would realistically yield a throughput of approximately a 5,000

compounds per day, this readout would not yet fit the standards of a real high-throughput setting. Therefore, we need to work on generating a fluorescent readout that automated machines would be able to read to automatically collect the data from the screen. This fluorescent readout would have to be thoughtfully engineered so that it quantifies the worms whose lethality has been rescued by a screened compound. Based on the latter, a stage-specific developmental marker would be an option, in which only those worms reaching certain developmental stages due to rescue can express the fluorescent probe.

Our consultation sessions with the personnel at BRITE facilities are the beginning of a potential collaboration for the development of the Rac-inhibitor pilot screen transition into a HTS at BRITE. Their facilities possess all the equipment necessary to accommodate the needs of the screen, and moreover they have several small molecule compound libraries that could be used as the starting point to initiate the screen, initially for proof-of-principle and later for true searching. We foresee that the development of this screen into a HTS for Rac specific or Rac pathway inhibitors, we may uncover potential hits. Also, by using the worm as an *in vivo* tool this early in the drug discovery process we should be able to rapidly deliver answers to discovery problems like toxicity or off-target effects, thus improving the likelihood of selecting candidates more likely to be successful in further preclinical and later clinical evaluation stages.

EXAMINATION OF POTENTIAL LEAD COMPOUNDS

It is important to note that the goal of the proposed work is not to study Rac function in *C. elegans*, but to use it as an *in vivo* platform for drug discovery. In

order to further characterize the activity and selectivity of candidate compounds selected from the Rac or Rac pathway inhibitor screen, I would test selected hit compounds in a secondary screen, using cell-based assays. Evaluation of our candidate compounds in cell-based assays will allow us to determine the potency, selectivity and perhaps potential cytotoxic effects caused by these drugs. Moreover, testing in cell-based assays will allow the assessment of additional qualities of candidate compounds like their ability to alter specific biological effects caused by activated Rac, and to determine whether they block at the level of Rac or elsewhere. This secondary screen will complement the effects seen in the *in vivo* studies using *C. elegans* and will support or eliminate some hits based on their properties.

In order to test the selectivity of hit compounds we need to select endpoints that will be accurate for inhibition solely of the Rac pathway. Negative controls should include endpoints of neighboring pathway effectors as a way to measure the selectivity of each compound for the Rac pathway. One general endpoint is to measure the activation of Rac downstream effectors. In the past, our lab has evaluated the action of GGTIs towards cells expressing activated Rac1, by using a c-Jun luciferase reporter assay (Joyce and Cox, 2003). c-Jun is a downstream target of JNK, which in turn can be activated by Rac1 and Rac-3. To execute this, I would transiently transfect NIH3T3 cells with either vector encoding activated Rac1(61L) along with a c-jun luciferase reporter, then after 24 h analyze the cell lysates for luciferase activity. This cell-based assay would allow me to evaluate hundreds of compounds simultaneously, which will be ideal for the first round of secondary screen. However, it will not determine specificity for Rac.

If the number of candidate compounds from the primary screen is small (<50), an alternate and more selective way to measure the activity of these compounds is to visually screen for morphological changes in the cell. Expression of activated Rac1 causes the formation of lamellipodia, and our laboratory has shown that inhibition of activated Rac1 by GGTI can decrease lamellipodia formation (Joyce and Cox, 2003). Based on the ability of other Rac inhibitors to reverse this phenotype, I believe I can also use this type of assay to monitor the activity of hit compounds. However this procedure will be useful only if there are few hit compounds or at a later stage in the evaluation of candidate compounds, since the complex procedures to visually prepare the cells for imaging are time consuming, and so it may not be useful for the evaluation of hundreds of compounds.

Overall, I also expect that we can obtain candidate compounds from this secondary screen. The detection of positive candidates will be an indication of the success of this novel platform for drug discovery. And it is possible that given the nature of our primary screen we can obtain candidate compounds that can be further evaluated for their advancement to additional pre-clinical studies.

It is possible that the chemical properties of some of these compounds will limit their solubility or bioavailability of each compound to exert its activity. Since the primary screen is to be performed in *C. elegans*, it is possible that candidate compounds may have toxic effects in mammalian cells that contain additional targets, but this will not be appreciated until we perform these experiments. The assays proposed in this aim will help elucidate the activity of candidate compounds towards the Rac pathway, but will not reveal the specific target of selected

compounds. To approach this problem, additional biochemical and biological assays can be performed. For example, to address if candidate compounds are targeting the kinase activity of PAK, the *in vitro* kinase activity of PAK can be evaluated.

Overall we have the tools to develop a reliable and high throughput secondary screen. With these ideas I should be able to test the activity of candidate compounds to inhibit Rac or the Rac pathway. Moreover, the success of this secondary screen will support the generation of additional tools for the screening of other clinically relevant targets using the worm as an efficient and valuable *in vivo* model for drug discovery of Ras and Rac pathway inhibitors.

CONCLUSIONS

Altogether, my proposed future directions would lead to the development of better tools for the analysis of existing inhibitors of the Ras>Raf>MEK>ERK pathway and to the rise of a novel platform for the screening of novel Rac or Rac pathway inhibitors. By developing additional transgenic lines harboring new Raf/LIN-45 mutants, we would not only be able to further understand the mechanism of action of MCP compounds, but also increase the tools available for the evaluation of other inhibitors of the Ras>Raf>MEK>ERK pathway. Despite the wealth of genetic reagents freely available in the *C. elegans* community, there is surprisingly no *lin-45* mutant suitable for my studies of Ras/Raf interaction inhibitors; nor have I been successful to construct one. If I can develop a successful strategy to do so, this would be very useful to the field.

On the other hand, the further development of a high-throughput screen for novel Rac pathway inhibitors would showcase a novel approach for drug screening in *in vivo* systems as well as remove one of the problems of screening for molecularly targeted therapeutics, which is not knowing the best specific target in a given pathway to go after. The screen I propose is unbiased and should in principle identify any functionally useful inhibitor of the pathway. If successful, such a screen could potentially be modified for the development of additional screens for other cancer-related and validated targets.

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