IDENTIFICATION OF M-RAS/RAS-2 EXPRESSION PATTERNS IN *CAENORHABDITIS ELEGANS* AND CHARACTERIZATION OF M-RAS/RAS-2-NULL WORMS

by

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ABSTRACT

M-Ras is a member of the Ras superfamily of small-molecular weight-GTP binding proteins. It is a close relative of the oncoprotein p21Ras and shares 50% overall amino acid sequence identity. *ras-2* is the *Caenorhabditis elegans* ortholog of the human M-Ras. It shows strong conservation of structure in worms and vertebrates. In mammals, endogenous M-Ras acts downstream of the EGF and FGF receptors to activate ERK (MAPK) signaling. The M-Ras is predominantly expressed in the mammalian central nervous system and the heart. Many human cancers including breast and colonic cancers exhibit dramatic elevations of M-Ras mRNA.

Receptor tyrosine kinase /RasGTPase/ERK signaling pathway is evolutionary conserved. In *C. elegans* this highly conserved signaling pathway controls many biological processes including vulval induction. *C. elegans* is a good model system to understand the role of M-Ras in RTK/Ras/ERK pathway.

To understand the expression patterns of RAS-2 in *C. elegans,* I generated a transgenic *C. elegans* that expresses GFP driven by the endogenous *ras-2* promoter. To investigate the effect of over expressed or activated RAS-2, I also generated transgenic lines over expressing WT or activated RAS-2. I observed that RAS-2 is expressed in various tissues and organs of *C. elegans* including amphid sensory apparatus, proximal myoepithelial sheaths, spermatheca and in developing gonads. Based on the *ras-2* expression patterns, I chose and performed appropriate behavioral assays to characterize *ras-2* null worms. The phenotypic characterization and behavioral observations on *ras-2* null worms revealed several phenotypes including smaller body size and faster locomotion. Furthermore, based on the behavioral assays, I found that *ras-2* mutants have a normal ability to sense inputs and reproduce.

To investigate the importance of RAS-2 in pathological signaling of activated LET-23 (EGFR) or activated LET-60 (p21Ras) which cause multivulval phenotype in *C. elegans,* I examined the different vulval phenotypes in crosses between *ras-2* null worms and *let-23* or *let-60* activated mutants. This revealed that *ras-2* null background do not suppresses multivulval phenotype caused by activated *let-23* or *let-60*. However, the activated *let-60* exhibits a higher frequency of protruding vulva in the absence of *ras-2.*

PREFACE

I have performed all experiments and generated the data and figures presented in this thesis with the following exceptions:

- The microinjection procedure to generate transgenic *C. elegans* was performed in the lab of Dr. Don Moerman at the University of British Columbia, by Dr. Teresa Rogalski.
- The high-throughput behavioral analyses using the multi worm tracker were performed in the lab of Dr. Catherine Rankin at the University of British Columbia, by Evan Ardiel.
- The strains DM2409 – [*let-60 (n1046) iv; ras-2 (ok682) iii*] and DM2411 – [*let-23 (sa62) ii; ras-2 (ok682) iii*] were generated in the lab of Dr. Don Moerman at the University of British Columbia by Drs. Vidhu Sharma and Teresa Rogalski
- The cloning of *ras-2* promoter and cDNA and the site directed mutagenesis were performed with the help and guidance of Dr. Annette Ehrhardt

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LIST OF ABBREVIATIONS

ADF	amphid neurons with dual ciliated sensory endings
ASER	amphid neurons single ciliated endings
AWA	amphid wing “A” cell
bp	base pair
CalDAG-GEF	calcium and diacylglycerol regulated-GEF
CI	chemotaxis index
CSF	colony stimulating factor
Da	Dalton
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal regulated kinase
FGF 2	fibroblast growth factor 2
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
Grb 2	growth factor receptor bound protein 2
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
IL-3	interleukin-3
ISI	inter stimulus interval
L1	larval stage 1
L2	larval stage 2
L3	larval stage 3
L4	larval stage 4
M	molar
M9	minimal media salt solution 9
MEK	mitogen activated protein/ERK kinase
Muv	Multivulva
mRNA	messenger ribonucleic acid
mSOS1	mammalian son of sevenless 1
MWT	multi worm tracker
NGF	nerve growth factor
NGM	nematode growth medium
ORF	open reading frame
PCR	polymerase chain reaction
PI-3K	phosphatidylinositol kinase
Pvul	protruding vulva
Ral-GDS	Ral guanine nucleotide dissociation stimulator
RasGRF	Ras guanine nucleotide releasing factor
RNA	ribonucleic acid
RT	room temperature

RTK	receptor tyrosine kinase
SAGE	serial analysis of gene expression
SDM	site directed mutagenesis
SOS	son of sevenless
TRP	transient receptor potential
USA	united states of America
WT	wild type

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Most importantly I am extremely grateful for my parents, Chandradasa and Jayanthika, my sister, Geethika and my husband, Gunam.

DEDICATION

For my parents, Chandradasa and Jayanthika

CHAPTER 1: INTRODUCTION

1.1 An overview of the superfamily of Ras proteins

The discovery of Ras proteins dates back to the 1960s when scientists found that some murine retroviral isolates are able to produce tumors in rodents. First, in 1964, Jennifer Harvey found that murine leukemia virus, which originated from leukemic rats, rapidly produced sarcomas when introduced into newborn rats (Harvey, 1964). This observation was followed by the identification of three other murine retroviruses namely Kirsten-murine sarcoma virus, BALB-murine sarcoma virus and the Rasheed strain of rat sarcoma virus, that carry transforming viral genes, in the late 1960s and 1970s (Kirsten & Mayer, 1967; Peters et al., 1974; Rasheed et al., 1978). Due to the oncogenic properties of these viral genes, they were named **ra**t **s**arcoma (Ras) oncogenes. Nucleotide sequence analysis revealed that these genes were rat genes that have orthologs in humans, with single, activating point mutations (Reddy et al., 1982; Tabin et al., 1982). By the early 1980s, it had been reported that Ras genes encode ~21,000-Da polypeptides that are guanosine triphosphatases (GTPases) with intrinsic enzymatic property (Shih et al., 1979). Since then Ras proteins have been extensively studied in signal transduction and in oncogenesis.

To date, 167 proteins have been identified as members of human Ras superfamily. They can be subdivided in to five subfamilies namely Ras, Rho, Rab, Ran and Arf/Sar (reviewed in Rojas et al., 2012). These small GTPases or guanosine triphosphate (GTP) binding proteins are involved in regulating a complex and diverse range of cellular

functions. Ras and Rho proteins are involved in passing extracellular stimuli to the interior of the cell to regulate fundamental cellular functions. Ras has roles in cell growth, proliferation, differentiation and cell migration, whereas Rho is involved in actin organization, cell cycle progression and gene expression. Rab and Arf families are known to be involved in regulation of intracellular vesicular trafficking. Finally, Ran is implicated in nucleocytoplasmic transportation (reviewed in Wennerberg et al., 2005). Ras proteins are evolutionarily conserved and orthologs are found in *C. elegans, S. cerevisiae, S. pombe, Dictyostelium* and in plants (Colicelli, 2004).

1.1.1 Biochemistry and regulation of Ras proteins

The primary biochemical property of a Ras protein is its ability to act as a molecular switch by shifting between an active GTP-bound state and an inactive guanosine diphosphate (GDP)-bound state (Vetter & Wittinghofer, 2001). The GDP-bound inactive Ras proteins are activated by guanine nucleotide exchange factors (GEF) (Lenzen et al., 1998), which stimulate dissociation of GDP. Upon releasing GDP, Ras proteins bind to GTP, which is abundant in the cytosol. Binding to GTP results in conformational changes in two regions, known as switch I and switch II (Milburn et al., 1990) that are highly conserved and responsible for binding of activators and effectors. The GTP-bound Ras conformation possesses high affinity for effector targets and allows Ras to interact with and activate downstream effectors. The inactivation of a GTP-bound Ras protein requires GTPase activating proteins (GAP), which accelerate the low intrinsic GTPase activity of Ras proteins (Scheffzek et al., 1998). The majority of Ras super family proteins

undergo post-translational lipid modifications, which anchor them to the cell membrane, where they exert their function in propagating extracellular signals to the cell interior (reviewed in Wennerberg et al., 2005).

1.2 The Ras subfamily of proteins

The Ras subfamily, which is one of the major groups within the Ras superfamily, consists of 39 members (reviewed in Rojas et al., 2012). This family of proteins comprises the p21Ras, R-Ras, Rap and Ral proteins. The "classical", or p21Ras proteins, H-Ras, N-Ras and K-Ras 4A/4B, are the founding and best studied members of the family. These four proteins are closely related and share about 85% sequence identity. The p21Ras proteins gained notoriety in 1982 with the discovery that these proteins are highly implicated in human oncogenesis. For example, mutations in p21Ras can be found in 90% of pancreatic, 50% of colonic, 30% lung, 50% thyroid cancers and 30% of myeloid leukemia (Bos, 1989). A wide variety of extracellular stimuli, such as growth factors; epidermal growth factor (EGF), and nerve growth factor (NGF) (Buday & Downward, 1993; Burchill et al., 1995) and cytokines; interleukin-3 (IL-3) and colony-stimulating factor-1 (CSF-1)(Duronio et al., 1992), can cause activation of p21Ras proteins through activation of cell surface receptors of these factors.

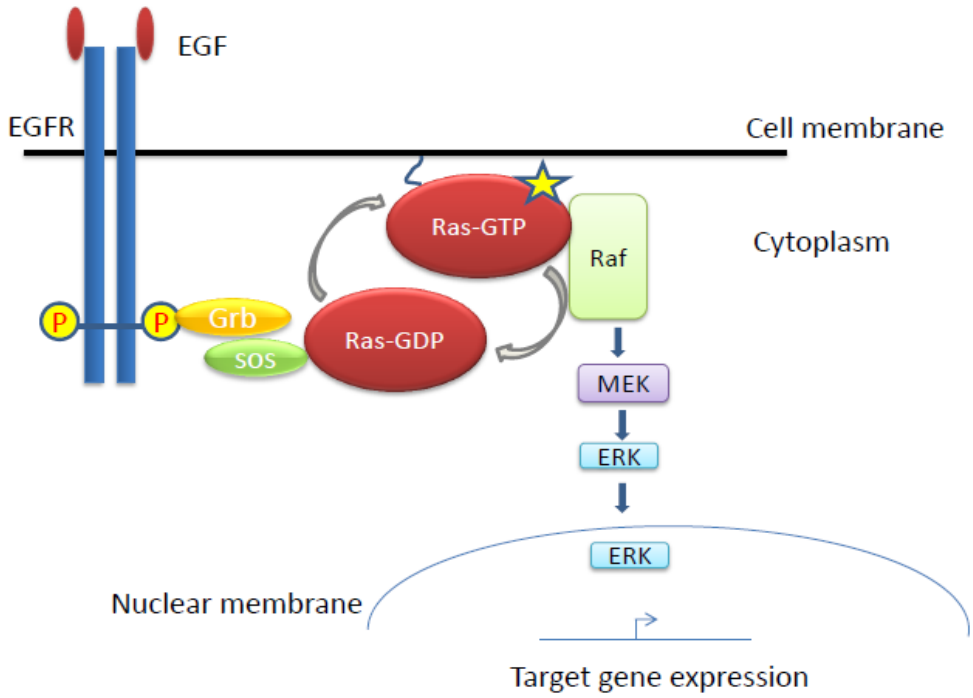

Figure 1.1. Schematic of RTK/Ras/ERK pathway in mammals including its major components.

Upon binding of growth factors such as EGF, the receptor tyrosine kinase (RTK), epidermal growth factor receptor (EGFR) dimerizes and autophosphorylates a tyrosine residue at its C-terminal. This phosphorylated tyrosine residue serves as a docking site for the adaptor protein, growth factor receptor-bound protein 2 (Grb2), which can recruit the guanine nucleotide exchange factor Son-of-Sevenless (SOS). SOS in turn facilitates loading of GTP in to Ras protein causing the activation of Ras.

Activated p21Ras is known to interact with various downstream effectors, including serine/threonine kinases (Raf-1, A-Raf, and B-Raf), the p110 catalytic subunit of phosphatidylinositol (PI)-3 kinase, and Ral guanine nucleotide dissociation stimulator (Ral-GDS). In the case of Raf-mediated signaling of Ras, Raf then activates mitogen

activated protein/ extracellular signal regulated kinase (MEK), which in turn activates extracellular signal regulated kinase (ERK), which are cytoplasmic kinases. Phosphorylated ERK translocates to the nucleus where it activates transcription factors. Ultimately, changes in gene transcription lead to modulation of a variety of cellular functions such as cell growth and proliferation, or motility. Thus, p21Ras is a key player in signaling pathways downstream of many extracellular stimuli.

M-Ras, R-Ras, TC21 (R-Ras2) are members of the R-Ras subfamily of Ras proteins (Kimmelman et al., 1997; Ehrhardt et al., 1999). These proteins share 40%-50% amino acid sequence identity overall with p21Ras and are virtually identical in effector binding switch I and switch II regions. M-Ras is recognized by the monoclonal anti-Ras antibody Y13-259, which binds to p21Ras via an epitope in switch II region (Ehrhardt et al., 1999). TC21 and R-Ras are known to function in cell proliferation and survival as well as in actin cytoskeleton dynamics. There is also evidence that these two proteins have roles in oncogenesis. The activating mutations of TC21 have been detected in human ovarian carcinoma (Chan et al., 1994), leiomayosarcoma (Huang et al., 1995) and in breast carcinomas (Barker & Crompton, 1998). R-Ras mutations also reported to promote cellular transformation, adhesion and survival (Osada et al., 1999).

1.3 M-Ras

M-Ras was first discovered as a small GTPase that participates in reorganization of actin cytoskeleton and was cloned from muscle cells, therefore termed as "muscle and microspike Ras" or M-Ras (Matsumoto et al., 1997; Kimmelman et al., 1997; Ehrhardt et

al., 1999). The *Mras* gene encodes a protein of 209 amino acids, which is 29kDa in molecular weight. In mice, M-Ras is most highly expressed in brain, but lower levels are found in most tissues including heart, liver, lung, pancreas, placenta and kidney (Ehrhardt et al., 1999). It was also identified as R-Ras3 due to high sequence similarity to subfamily member TC21 with which it shares overall secondary structure in possessing an N-terminal extension of 10 amino acids which is not common to the p21Ras (Kimmelman et al., 1997). Moreover, its overall homology with H-Ras and TC21 is 47% and 52% respectively and the effector binding switch I region are identical. In the switch II region M-Ras shows only 3 conserved exchanges (Ehrhardt et al., 1999). At the C-terminus, M-Ras - like p21Ras and R- Ras contains a 4 amino acid CAAX motif (a cysteine residue followed by two aliphatic amino acids and one random amino acid) (Figure 1.2). This feature causes Ras proteins to be farnesylated at the cysteine residue, which enables anchoring to membranes. M-Ras, like K-Ras4B, additionally features a poly-lysine motif at the C-terminus. The presence of these positively charged residues causes M-Ras and K-Ras4B to localize to the disordered plasma membranes, whereas other Ras proteins without them localize to lipid rafts (Ehrhardt et al., 2004).

M-Ras, like p21Ras, can be activated by growth factors like EGF and CSF-1, which are ligands of receptor tyrosine kinases. Due to unique membrane localization of the M-Ras and K-Ras4B, growth factors like EGF and CSF-1 were found to preferentially activate M-Ras and K-Ras4B over H-Ras or N-Ras (Ehrhardt et al., 2004). The activation of M-Ras by NGF and fibroblast growth factor 2 (FGF 2) also been reported (Kimmelman et al., 2002; Sun et al., 2006). Although there are quantitative differences in the levels of activation,

this data implies that M-Ras shares activators with p21Ras. In fact, M-Ras is activated by many GEFs that activate p21Ras, namely mSOS1, RasGRF, CalDAG-GEFII and CalDAG-GEFIII (Ohba et al., 2000). M-Ras also shares negative regulators with classical Ras, namely p120GAP, NF-1, and Gap-1m (Ohba et al., 2000). In addition, M-Ras is known to share most downstream effectors with the other Ras subfamily members, although there are also some specific targets. Two Rap-GEFs, MR-GEF (Rebhun et al., 2000) and RA-GEFII (Gao et al., 2001), were reported to selectively bind to GTP-loaded M-Ras and hence are potential M-Ras specific effectors.

Shoc-2, in complex with the catalytic subunit of the protein phosphatase 1 (PP1), has also been reported as an M-Ras specific effector in mammals (Rodriguez-Viciana et al., 2006). It was suggested that the M-Ras/Shoc-2/PP1 complex is an important regulatory element in p21Ras signaling. As an M-Ras specific effector, Shoc-2 and PP1 translocate to the membranes containing activated M-Ras, where PP1 can dephosphorylate the inhibitory phosphate of S259 residue of Raf (Rodreguez-Viciana et al., 2006). Activated Raf then acts on its downstream effector Erk to facilitate survival and proliferation. Thus, activated M-Ras may play an essential role in cancer formation by promoting primary oncogenic pathways and it will be of great interest to elucidate the specific role of the M-Ras in oncogenic signal transduction of RTK/p21Ras/Erk pathway. Shoc-2 is evolutionarily conserved and known as SUR-8 (SOC-2) in *C. elegans*, where it appears to be an essential component of Ras signaling (Sieburth et al., 1998).

Protein kinases such as Raf-1 are well-known effectors of Ras proteins. However, the interaction of M-Ras with Raf-1 is weak (Ehrhardt et al., 1999; Kimmelman et al., 1997)

which may explain the relatively weak activation of the ERK pathway by M-Ras. Furthermore, a study based on yeast two-hybrid assays also showed that interaction of M-Ras was poor with c-Raf-1, A-Raf, and B-Raf (Quilliam et al., 1999). The same study reported that M-Ras also weakly binds to another known Ras effector RalGDS and Rin 1. PI3K is known to be a downstream effector of M-Ras and its activation by M-Ras is GTP-dependent (Kimmelman et al., 2000). Activation of PI3K by M-Ras results in stimulation of the serine/threonine kinase Akt to promote cell survival signaling. RPM/RGL3 was identified as another shared effector of M-Ras and H-Ras. It belongs to the Ral-GDS family (Ehrhardt et al., 2001). In addition, both M-Ras and H-Ras possess pro-apoptotic properties through binding to Nore1 and regulate cell junction formation through AF-6 (Ehrhardt et al., 2001; Khokhlatchev et al., 2002)

1.3.1 Significance of M-Ras as an oncogene

Although mutations in *MRAS* are very rare and no known activating mutations have been reported in human tumors yet, M-Ras mRNA was found to be over-expressed in many human cancers of the breast, prostate, thyroid, ovary, stomach, rectum, colon and uterus (Yang et al., 2005). *In vitro*, higher expression of M-Ras has been reported to result in transformation of murine melanocyte lines Mel-a-6 and gamma3 (Wang et al., 2000). Continuous activity of M-Ras induced epithelial-to-mesenchymal transition and tumorigenesis in murine mammary epithelial cells (Ward et al., 2004). A constitutively activated mutant of M-Ras (Q71L M-Ras) was sufficient to induce factor independent growth and differentiation of normal murine bone marrow into colonies of

macrophages and neutrophils and immortal mast cells (Guo et al., 2005). These immortalized mast cells generated fatal mast cell leukemia in mice. Furthermore, the expression of activated mutant of M-Ras in purified hematopoietic stem cells (HSCs) showed leukemogenic transformation, immortalization and preferential generation of mast cells. It was concluded that pluripotent HSC generated mast cells through M-Ras dependent signals and that activated M-Ras was sufficient to immortalize and transform hematopoietic stem cells to leukemia stem cells (Guo et al., 2006). Expression of another constitutively active M-Ras mutant (G22V M-Ras) resulted in morphological transformation and increased proliferation in NIH3T3 fibroblasts (Ehrhardt et al., 1999). Furthermore, the expression of this mutant resulted in factor independent growth and survival in interleukin-3 dependent mast cells, indicating, again, a potential mechanistic role for M-Ras in oncogenesis.

1.3.2 Other functions of M-Ras

M-Ras is among the genes identified as aberrantly expressed in induced pluripotent stem cells derived from schizophrenic individuals, suggesting that M-Ras has a pathological significance in neuronal disorders (Brennand et al., 2011). Its elevated expression levels in hippocampal tissues of rats after a food search memory test (Robles et al., 2003) suggests that M-Ras may have a role in hippocampal synaptic plasticity. As well, M-Ras expression in hypothalamus was found to be induced after both heat and cold conditioning in 3-day old chicks, indicating that M-Ras may function in hypothalamic synaptic plasticity as well (Labunskay & Meiri, 2006). It is important to

note that M-Ras is highly expressed in brain tissue of mammals (Kimmelman et al., 2002) and chickens (Labunskay & Meiri, 2006). M-Ras was also reported to mediate the regulation of actin cytoskeleton in dendrites (Tasaka et al., 2012) and was involved in the normal development of cortical dendrites (Saito et al., 2009). In PC12 neuronal cells, M-Ras was activated by nerve growth factor and exogenous expression of the activated M-Ras mutant G22V-M-Ras resulted in neuronal differentiation and neuritogenesis via sustained activation of ERK pathway (Sun et al., 2006).

1.4 M-Ras is conserved in phylogeny

Ras proteins are evolutionarily conserved and are present in all eukaryotes from yeast to human. Phylogenetic data analyses indicate that M-Ras has orthologs in nematodes (*C. elegans*), ascidians (*C. intestinalis* and *H. roretzi*), amphioxus (*B. floridae*) and in zebrafish (Keduka et al., 2009). But there is no reported M-Ras ortholog in fruit fly (*D. melanogaster*). Importantly, in ascidians, which lack p21Ras, the RTK/Ras/MAPK pathway, which regulates neural system induction, is mediated by M-Ras. This suggests that M-Ras evolved independently of other Ras family members.

1.4.1 M-Ras in *C. elegans- ras-2*

M-Ras is conserved in the nematode *C. elegans* and the ortholog is known as RAS-2. Human and worm M-Ras/RAS-2 share about 51% overall amino acid identity and are identical in switch I (shaded light gray in Figure 1.2) region. Even though not identical, these two proteins display a high degree of homology in switch II region (shaded dark

gray in Figure 1.2). The gene *ras-2* encodes 211 amino acids with an estimated molecular weight of 24KDa (http://www.wormbase.org). Moreover, the C-terminus of the protein is uniquely conserved among mammals and the *C. elegans* (Ehrhardt et al., 1999). In contrast to human p21Ras and other human R-Ras subfamily members, M-Ras and its *C. elegans* orthologue lack the cysteine residues that are necessary for palmitoylation (highlighted dark yellow in Figure 1.2). Furthermore, human and *C. elegans* M-Ras do not contain a proline rich motif known as "R-Ras box" which is common to the R-Ras group (underlined in Figure 1.2). These conserved features in mammalian and *C. elegans* M-Ras suggest that it has distinct and unique functions apart from other Ras family members. Even though M-Ras is highly conserved in *C. elegans* its function in the nematodes has yet to be investigated.

```
hH-Ras          --------------------------MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDP 34
hN-Ras          --------------------------MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDP 34
hK-Ras4B        --------------------------MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDP 34
CeLET-60        --------------------------MTEYKLVVVGDGGVGKSALTIQLIQNHFVEEYDP 34
hR-Ras          MSSGAASGTGRGRPRGGGPGPGDPPPSETHKLVVVGGGGVGKSALTIQFIQSYFVSDYDP 60
hTC21           ---MAAAG------WRDGSG------QEKYRLVVVGGGGVGKSALTIQFIQSYFVTDYDP 45
CeRAS-1         --MGG----------RSNSATTAAQQNAVLRIVVVGGGGVGKSALTIQFIQRYFVQDYDP 48
hM-Ras          ------------MATS--AVPSDN--LPTYKLVVVGDGGVGKSALTIQFFQKIFVPDYDP 44
CeRAS-2         ------------MSNGGKRPPEDDSKLPYYKLVVIGDGGVGKSSLTIQFFQKQFVDYYDP 48
                                            ::**:* ******:****::*  **  ***

hH-Ras          TIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIH 94
hN-Ras          TIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNSKSFADIN 94
hK-Ras4B        TIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIH 94
CeLET-60        TIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLLVFAVNEAKSFENVA 94
hR-Ras          TIEDSYTKICSVDGIPARLDILDTAGQEEFGAMREQYMRAGHGFLLVFAINDRQSFNEVG 120
hTC21           TIEDSYTKQCVIDDRAARLDILDTAGQEEFGAMREQYMRTGEGFLLVFSVTDRGSFEEIY 105
CeRAS-1         TIEDSYTKQCFVDEDLCKLEILDTAGQEEFSTMREQYLRTGSGFLIVFAVTDRNSFEEVK 108
hM-Ras          TIEDSYLKHTEIDNQWAILDVLDTAGQEEFSAMREQYMRTGDGFLIVYSVTDKASFEHVD 104
CeRAS-2         TIEDQYIQHCEIDGNWVIMDVLDTAGQEEFSAMREQYIRGGRGFLLVFSVTERKSFEEAH 108
                ****.* :   :*     :::********:.:**:**:* * *** *:::.:  ** .

hH-Ref          QYREQIKRVKDSDDVPMVLVGNKCDLAA-RTVESRQAQDLARSYGIPYIETSAKTR-QGV 152
hN-Ras          LYREQIKRVKDSDDVPMVLVGNKCDLPT-RTVDTKQAHELAKSYGIPFIETSAKTR-QGV 152
hK-Ras4B        HYREQIKRVKDSEDVPMVLVGNKCDLPS-RTVDTKQAQDLARSYGIPFIETSAKTR-QGV 152
CeLET-60        NYREQIRRVKDSDDVPMVLVGNKCDLSS-RSVDFRTVSETAKGYGIPNVDTSAKTR-MGV 152
hR-Ras          KLFTQILRVKDRDDFPVVLVGNKADLESQRQVPRSEASAFGASHHVAYFEASAKLR-LNV 179
hTC21           KFQRQILRVKDRDEFPMILIGNKADLDHQRQVTQEEGQQLARQLKVTYMEASAKIR-MNV 164
CeRAS-1         KLHELICRIKDRDDFPIILVGNKADLENERHVARHEAEELAHRLSIPYLECSAKIR-KNV 167
hM-Ras          RFHQLILRVKDRESFPMILVANKVDLMHLRKITREQGKEMATKHNIPYIETSAKDPPLNV 164
CeRAS-2         KLYNQVLRVKDRSEYPVLLVANKVDLINQRVVSEQEGRELAAQLKLMYIETSAKEPPVNV 168
                     : *:** .. *::*:.** **   * :        .    :  .: ***    .*

hH-Ras          EDAFYTLVREIRQHKLRK---LNPPDESGPGC-----MSCKCVLS 189
hN-Ras          EDAFYTLVREIRQYRMKK---LNSSDDGTQGC-----MGLPCVVM 189
hK-Ras4B        DDAFYTLVREIRKHKEKM---SKDGKKKKKKS-----K-TKCVIM 188
CeLET-60        DEAFYTLVREIRKHRERH---DNNKPQKKK----------KCQIM 184
hR-Ras          DEAFEQLVRAVRKYQEQE-LPPSP-PSAPRKK----GGGCPCVLL 218
hTC21           DQAFHELVRVIRKFQEQE-CPPSPEPTRKEKD----KKGCHCVIF 204
CeRAS-1         DEAFFDIVRLVRKYQHDERMPIHPHDDRKLESPIKLKKKKNCRIQ 212
hM-Ras          DKAFHDLVRVIRQQIPEKSQKKKKKTKWRGDRATG-THKLQCVIL 208
CeRAS-2         DAAFHELVRIVRSFPSDEGDHEASMASVPRTKKR--KDKGKCLIS 211
                : **  :** :*.                              * :
```

Figure 1.2. Full-length amino acid sequence alignment of classical and R-Ras subfamily proteins of human (h) and *C. elegans* (Ce). Gaps are indicated by a hyphen (-). Switch I and switch II regions are boxed in red and blue respectively. The CAAX motifs are boxed in black. R-Ras box in human R-Ras and TC21 is underlined. Cysteine residues that might be palmitoylated are double underlined.

1.5 *C. elegans* as a model system

Since 1974, when Sydney Brenner introduced the nematode *C. elegans* as a model system for scientific studies, worms have been used widely in the field of science as a model organism. These free-living soil nematodes are about 1mm in length and easily grow on agar plates under laboratory conditions. They can be fed on *Escherichia coli (E.coli*), and thus are cost effective to culture, and they can also be easily stored at -80°C. Use of both males and self-fertilizing hermaphrodites are ideal for genetic studies as they can produce both inbred and crossbred progenies. An adult male worm is comprised of 1031 somatic nuclei while hermaphrodite comprises 959. Its life cycle is as short as 3 days, which permits the study of different developmental stages during a very short time frame. The semi-transparent nature of the body also facilitates *in vivo* observations in detail (Brenner, 1974).

More importantly, the fully sequenced genome (*C. elegans* Sequencing Consortium 1998) and practical and convenient forward and reverse genetics make the worms a powerful tool for a variety of research studies. Characterization of genes by reverse genetics can be done very easily and efficiently by microinjection of DNA into the germ line cells of the *C. elegans* (Mello et al., 1991). The injected DNA is stably maintained as an extra-chromosomal array, which can be transferred through generations and allows us to analyze the function of a gene over generations after a single injection. Mutagenesis of genes is a fundamental feature in genetic analysis and is very practical in *C. elegans*. Chemical mutagens, ionizing radiation and transposon hopping are some of the methods for mutagenesis. Using chemical reagents or double stranded RNA, gene

expression can be specifically reduced or removed (Fire et al., 1998) in worms. Among available protein sequences about 83% of the *C. elegans* proteome has human homologous genes (Lai et al., 2000), which enables scientists to scrutinize gene functions in *C. elegans* and extrapolate to humans.

1.6 RTK/Ras/ERK pathway in *C. elegans*

C. elegans has been widely used as a model system for cellular signal transduction studies. Some of the mammalian signaling pathways are highly conserved in this species. The RTK/Ras/ERK pathway is one of the highly conserved and extensively studied pathways in nematodes. In *C. elegans*, this pathway regulates many developmental processes including viability, vulval induction, uterine development, ovulation and spicule development (Moghal & Sternberg, 2003). In addition, this pathway has also been involved in induction of excretory duct cell fate, axon guidance, male tail fate specification, control of sex myoblast migration, promotion of germ line meiosis (Reviewed in Sundaram, 2006) and olfaction (Hirotsu et al., 2000).

Many of RTK/Ras/ERK pathway genes were first identified in *C. elegans*. Two different RTKs, LET-23 (ortholog of mammalian EGFR) and EGL-15 (ortholog of mammalian FGFR), are known to activate *C. elegans* Ras protein LET-60 (Reviewed in Sundaram, 2006). LET-60 is the only known p21Ras protein in *C. elegans* and is orthologous to mammalian K-Ras. LET-60 stimulates LIN-45 (*C. elegans* Raf), which in turn activates MEK-2 (*C. elegans* MEK), which will then activate MPK-1 (*C. elegans* Erk).

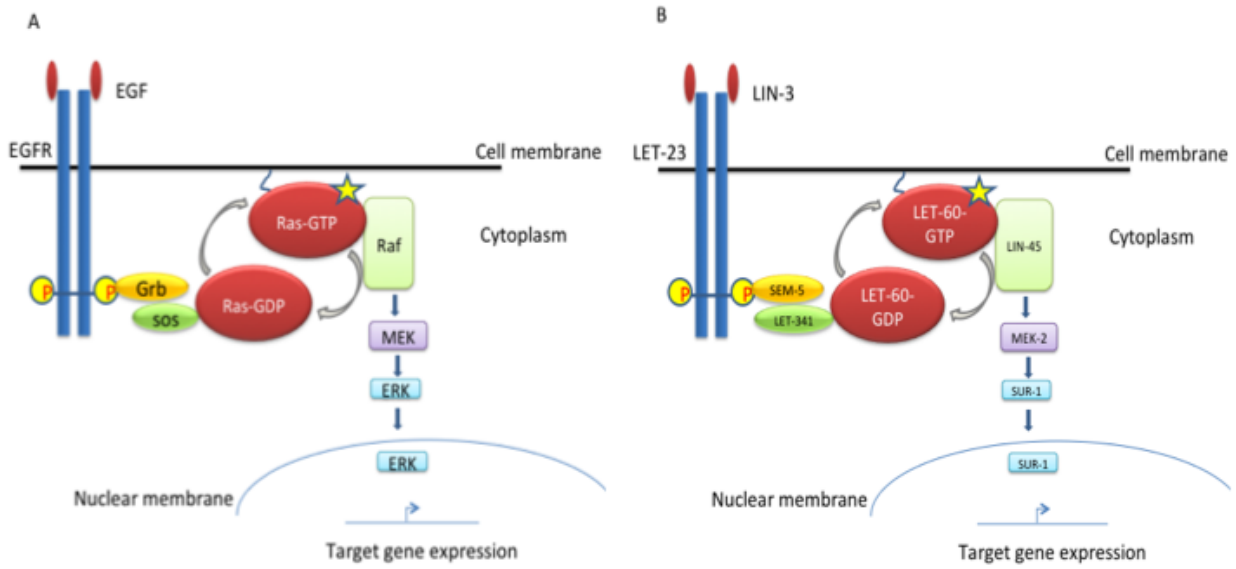

Figure 1.3. The components of the RTK/Ras/ERK pathway are conserved in mammals (A) and *C. elegans* (B)

Even though this pathway regulates many biological functions in worms, the best studied is vulval development. Many known Ras pathway components have been identified based on the vulval phenotypes. Therefore, the *C. elegans* vulval developmental process has become a good tool for discovering new genes involved in the Ras pathway. For example, the gene *sur-8* was first identified as a positive regulator of the Ras-mediated signal transduction during vulval development in *C. elegans* (Sieburth et al., 1998). The *loss-of-function* mutations of *sur-8* strongly suppress the Muv phenotype caused by *let-60 (gf)* mutation. Shoc-2, the mammalian ortholog of *sur-8* (*soc-2*) is found to be a specific effector of the activated M-Ras but not of activated p21Ras and its function was essential for the activation of Ras pathway by growth factors (Rodriguez-Viciana et al., 2006). Because Shoc-2 and M-Ras are both highly

conserved from *C. elegans* to human, the interaction of *Sur-8* (*soc-2*) with RAS-2 would also likely to be conserved.

1.7 Hypotheses

Given that M-Ras/RAS-2 is highly conserved between mammals and *C. elegans*, I postulated that RAS-2 may have an important role in *C. elegans*. Having specific effectors like Shoc-2, which is an essential component of the RTK/Ras/ERK pathway, I postulated that RAS-2 may be an essential component or modulator for signaling through this pathway by interacting with *sur-8* (*soc-2*).

I hypothesized that the lack of RAS-2 in *C. elegans* result in phenotypes that are caused by less activation of the pathway, and that lack of RAS-2 similar to *sur-8* (*soc-2*), may supress phenotypes caused by activation of components of it, such as *gain-of-function* p21Ras/LET-60 and *gain-of-function* EGFR/LET-23.

1.7.1 Objectives

The overall objective of my study was to identify the expression patterns of *ras-2* in *C. elegans* and characterize *ras-2* null worms based on the expression patterns and to determine whether RAS-2 is essential to pathological signaling of LET-60 and LET-23.

Aim 1. Identification of the expression patterns of *ras-2* in *C. elegans* by means of generation of transgenic *C. elegans* using a green fluorescent protein (GFP) reporter fused to the endogenous *ras-2* promoter, or fused to wild-type (WT) or constitutively activated *ras-2* genes.

Aim 2. Characterization of *ras-2* null *C. elegans* by performing assays related to the cells or tissues where *ras-2* is expressed.

Aim 3. Investigation of the pathological signaling of activated *gain-of-function* p21Ras/LET-60 and *gain-of-function* EGFR/LET-23 in the absence of RAS-2 in *C. elegans* by analyzing worms expressing activated LET-60 or activated LET-23 on a *ras-2* null back ground.

CHAPTER 2: MATERIALS AND METHODS

2.1 *C. elegans* strains

Worms were cultured and maintained on nematode growth medium (NGM) seeded with *Escherichia coli* (OP50) according to standard conditions (Brenner, 1974). N2 (Bristol) and VC2010 were the two wild-type (WT) worm strains used in this study. VC2010 was used for microinjection of plasmids and as the WT control in the egg-laying assay. For all other assays in this study, the N2 strain was used as the WT control. RB852 *ras-2*(ok682) was obtained from the *C. elegans* Gene Knockout Consortium (Robert Barstead laboratory, Oklahoma Med Research Foundation, Oklahoma City, OK). The ok682 mutation is a 977 bp deletion in the *ras-2* gene that includes the 3rd coding exon and flanking introns. The transgenic strain DM7442 (*raEx442 [rol-6 (su1006)+ras-*2p::GFP*]*) produces green fluorescence protein (GFP) driven by the *ras-2* promoter, and was used to determine RAS-2 localization in *C. elegans*. The transgenic strains, DM7443 (*raEx443 [rol-6 (su1006)+ras-*2p::GFP::*ras-*2*])* and DM7447 (*raEx447 [rol-6 (su1006)+ras-*2p::GFP::*ras-*2G26V]) produce GFP-tagged WT RAS-2 or activated RAS-2, respectively, and were used to identify RAS-2 expression patterns, to analyze any phenotype caused by Ras pathway activation, and were used in the egg-laying assay. VG387 *dpy-5*(e907) and CB312 *unc-13*(e312) hermaphrodites were used for the male reproductive efficiency assay and in the male mating behavior assay respectively. CX10 *osm-9*(ky10) and CX3410 *odr-10*(ky225) were used as negative controls in the avoidance and chemotaxis assays, respectively. To score vulval phenotypes DM2409 *let-60*(n1046);*ras-*

2(ok682), DM2411 *let-23*(sa62);*ras-2*(ok682), MT2124 *let-60*(n1046) and PS1839 *let-23*(sa62) were used.

2.2 RNA isolation and cDNA synthesis

C. elegans total RNA was extracted from WT N2 worms using TRIzol® reagent (Ambion, Rockford, IL) according to the manufacturer's instructions. Briefly, 60-70 worms were collected in a 1.5 ml centrifuge tube after adding minimal media salt solution 9 (M9) buffer to the worm plate. Worms were washed three times with M9 buffer in order to remove bacteria. After the final wash, 1 ml of TRIzol® was added and incubated at room temperature (RT) for 5 minutes. Next, 200 µl of chloroform was added to the tube and the tube was shaken for 15 seconds followed by 2-3 minutes incubation at the RT. Tubes were centrifuged at 14,000 rpm for 15 minutes at 4°C. The upper-phase aqueous solution was transferred to a new centrifuge tube without disturbing the phenol containing lower layer. Then 500 µl of isopropanol was added to the separated upper-phase and mixed well before it was let incubate at RT for 10 minutes. Next, the resulting solution was centrifuged at 14,000 rpm for 10 minutes in 4°C. After centrifugation, the RNA pellet was washed once with 1ml ice-cold ethanol by spinning at 12,000 rpm for 5 minutes at 4°C. Finally, the RNA pellet was air dried and dissolved in RNAase-free water followed by incubation at 55°C for 10 minutes. The RNA concentration was measured by NanoDrop (ThermoFisher Scientific Inc., Wilmington, DE) using ND 1000 software program. Complementary DNA (cDNA) was synthesized from 1000 ng RNA using reverse

transcriptase reaction with SuperScript® First- Strand (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

2.3 Construction of *ras-2*p:: GFP transcriptional fusion vector (pDM#1218)

In order to construct a plasmid encoding GFP driven by the *ras-2* promoter, an 820 bp stretch upstream of the initiating ATG of *ras-2* was amplified from *C. elegans* genomic DNA by PCR with the primers F17C8.4-forward and Ras-2promo-reverse, containing BamHI restriction sites. The PCR product was gel purified using the QIAquick® gel extraction kit (Qiagen, Valencia, CA) after agarose gel electrophoresis. It was then cloned in to the BamHI site of the multiple cloning site of the promoter-less GFP expression vector, pPD95.75 (provided by Andrew Fire, Stanford University, USA) to generate pDM#1218. Directional insertion and correct sequence of the *ras-2* promoter were confirmed by restriction digest and DNA sequencing.

2.4 Construction of *ras-2*p::GFP::*ras-2* (pDM#1222) and *ras-2*p::GFP::*ras-2*G26V (pDM#1223) translational fusion vectors

We modified the *ras-2*p::GFP plasmid (see above) to drive expression of GFP-tagged RAS-2 from the endogenous *ras-2* promoter. First, we removed the Stop codon of GFP and introduced BglI and NotI sites at the 3' end of GFP. We then inserted cDNA of either wild-type (WT) or activated RAS-2 (RAS-2G26V) that were obtained by amplification of N2 cDNA. WT *ras-2* cDNA was amplified by nested PCR. First, the WT *ras-2* cDNA was amplified using primers Ras-2-Nru1-F and Ras-2-nest-AS, followed by a secondary PCR,

using the primers Ras-2-sense (containing a BamHI site) and Ras-2-AS (containing NotI site). In order to construct the *ras-2*p::GFP::*ras-2*G26V plasmid, the 26th codon (GGA) of WT *ras-2* cDNA was mutated to GTA. The mutation was generated by site directed mutagenesis by standard PCR.

Table 2.1. Primer pairs and thermocycler program for PCR

PCR Fragment	**Primer pair**	**PCR conditions**			
		Step	Temperature (° C)	Time (seconds)	Number of cycles
ras-2 promoter	F17C8.4-Forward TATGTCGACTCGAGGATCCAACCAGTGAAGAAGACGACG Ras-2promo-reverse TATAGGATCCGTTCTTTAATCTATGAATTG	1. Initial heating 2. Denaturation 3. Annealing 4. Extension	95 95 55 70	120 20 60 35	35
WT *ras-2* cDNA (Primary PCR with nested primers)	Ras-2-Nru1-F CAATTCCGGATGATTCATTC Ras-2-nest-AS GGTCCAACTTCTCACATCAAACGG	1. Initial heating 2. Denaturation 3. Annealing 4. Extension	95 95 54 70	120 25 20 120	25
WT *ras-2* cDNA (Secondary PCR)	Ras-2-sense TATAGGATCCTCAAACGGAGGTAAGAGACCG Ras-2-AS TATAGCGGCCGCCTAGGAAATTAAACATTTTCC	1. Initial heating 2. Denaturation 3. Annealing 4. Extension	95 95 60 70	120 25 20 60	25
SDM G26V Primary PCR with 1). Ras-2-Nru-1-F and G26V-SDM-R 2). G26V-SDM-F and Ras-2-Bsg1-R	Reaction 1). Ras-2-Nru1-F CAATTCCGGATGATTCATTC G26V-SDM-R TTCCCGACACCTACATCTCCGATCA Reaction 2). G26V-SDM-F TGATCGGAGATGTAGGTGTCGGGAA Ras-2-Bsg1-R CTCACGATATCTTACTATCG	1. Initial heating 2. Denaturation 3. Annealing 4. Extension	95 95 55 70	120 120 90 25	25
SDM G26V (secondary PCR)	Ras-2-Nru1-F CAATTCCGGATGATTCATTC Ras-2-Bsg1-R CTCACGATATCTTACTATCG	1. Initial heating 2. Denaturation 3. Annealing 4. Extension	95 95 55 70	120 120 90 25	25

2.5 Microinjection procedure

Microinjections were performed with test plasmid and pRF4 *[rol-6 (su1006)]*, which encodes a marker DNA that allows for identification of successful transformants based on their "roller" phenotype. Injection mixes were prepared containing 86ng/µl of pRF-4 and 4ng/ìl of *ras-2*p::GFPor 0.5ng/µl of *ras-2*p::GFP::*ras-2* or 0.5ng/µl of *ras-*2p::GFP::*ras*-2G26V plasmids. The mixes were injected into the gonad of adult hermaphrodites. All injections were carried out as previously described (Mello et al., 1991) using a microinjection set up with a Zeiss inverted compound microscope (IM35).

2.6 Dye filling assay

A plate with a synchronized adult worm population was collected into a 1.5 ml centrifuge tube with 1 ml of M9 buffer. Worms were spun down for 30 s at 2600 rpm and washed twice with M9 buffer and re-suspended in 1 ml of M9 buffer. Then, 1 µl of 1 mM DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) (Invitrogen, Carlsbad, CA) was added and quickly mixed by vortexing. The tube was stored in dark for 30 minutes and a quick vortex was done after 15 minutes. After staining, worms were spun and washed two times with M9 buffer. Following the last washing step, the M9 buffer was removed and worms were placed on to a freshly seeded NGM plate and allowed to feed on fresh bacteria. After 1-2 hours, a few worms were picked and mounted in 11 µl of M9 buffer drop on a glass slide and covered with a cover slip. Worms were observed under the fluorescence microscope.

2.7 Brood size assay

Individual L4 hermaphrodites were transferred to normal OP50-seeded NGM plates. Starting from the next day, the egg-laying hermaphrodites were transferred to fresh plates daily until the hermaphrodites stopped laying eggs. The progenies in each plate were incubated at 15°C and counted after three to four days. The total number of progenies from each plate was added up and the final brood size of each hermaphrodite was determined. 5-10 hermaphrodites were used in the assay for each strain.

2.8 Reproductive efficiency in adult males

L4 males were separated from the hermaphrodites 24 hours before setting up the mating pairs and allowed to mature at 20°C. The next day, one *dpy-5* L4 hermaphrodite was placed on a 1 cm diameter food patch on a NGM plate with one adult male and incubated at 20°C. After 24 hours the male was removed from the plate. Then, every day up to four days the hermaphrodite was transferred to a fresh plate to lay eggs. Both self and cross progenies were counted after three days when offspring are visible adults. Each plate was considered as a single mating trail. 10-15 plates per genotype were scored. The mating efficiencies were calculated as a percentage of number of cross progeny per number of total progeny.

2.9 Avoidance assay (Glycerol ring assay)

A 6 cm unseeded NGM plate was used as an assay plate. Five minutes before the assay, a bottle mouth (2 cm diameter) was dipped in a fresh 8M-glycerol/Congo red dye

solution, and a ring of this high osmotic solution was stamped on the agar and allowed to soak in. A synchronized population of three days old young adult hermaphrodites in a culture plate was washed twice with the M9 buffer to remove bacteria. Worms were then transferred into the middle of the glycerol ring in a small drop. Excess M9 buffer was absorbed with a Kimwipe. The assay plate then was placed on the platform of the multi worm tracker and snapshots were taken every 2 minutes for 10 minutes by the image acquisition camera of the multi worm tracker and the fraction that crossed the glycerol ring was analyzed.

2.10 Chemotaxis assay

The chemotaxis assay plates were prepared using 6 cm NGM plates. The underside of the plate was divided and marked into 4 equal quadrants. The center of the plate was marked and considered as the origin “O”. A circle of 0.5 cm radius was marked around the origin where worms were placed at the beginning of the assay. A single point was marked 2 cm away from the origin in each quadrant and the points in the opposite quadrants were named either Test “T” or Control “C” (Figure 2.1).

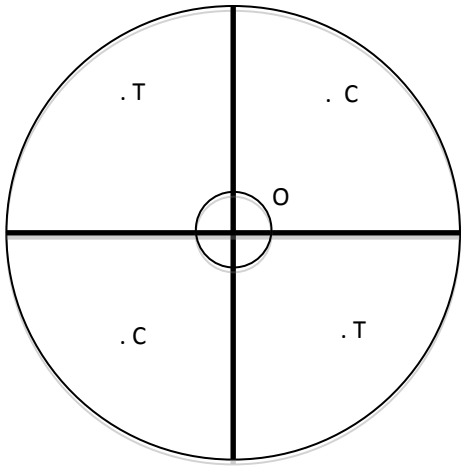

Figure 2.1. Schematic of the underside of the chemotaxis assay plate. The 6 cm NGM plate is divided into four quadrants and designated either as test "T" or control "C" area. The origin "O" is marked in the middle.

The test and control solutions were made by combining equal volumes of either 0.5 % diacetyl (butane-2, 3-dione) in ethanol or ethanol and 0.5 M sodium azide in M9 buffer, which is an anesthetic agent and immobilizes worms. A synchronized population of three day-old young adults were washed three times with M9 buffer to remove bacteria. After the final wash, M9 buffer was aspirated, leaving about 100 µl with worms. A 10 µl drop of worm solution (containing about 50-100 worms) was placed on the origin of the assay plate. Immediately after, 2 µl of test solution was placed in each test sites, and 2 µl of control solution was placed in the control sites. The lids were closed and the assay plates were kept at 20° C for 30 minutes and plates were visually scored under the dissection microscope. The number of worms in each quadrant that completely crossed the circle of origin was recorded. The chemotaxis index for an individual plate was obtained using the following equation.

Chemotaxis Index = (number of worms in both test quadrants - number of worms in both control quadrants)/ (total number of scored worms)

2.11 Obtaining a growth curve

Six to eight adult hermaphrodites were allowed to lay eggs for 1-2 hours in a seeded NGM plate and were removed. Developing larva and adult worms were measured under a 10X objective of a dissection microscope that has a reticle in the eyepiece. In order to measure the body length, larvae or adults were killed with 2 µl of bleach/NaOH solution and straightened using the worm pick. Nine to eleven worms of each strain were measured when they were 16, 24, 32, 40, 48, 56, 64, 72, 80, 88 and 96 hours old.

2.12 Male mating behavior assay

L4 males were separated 24 hours before the assay and incubated at 20˚C in order to use virgin males in the assay. Also one day before the assay, 35 mm NGM plates were seeded with 50 µl of OP50 in a 1 cm diameter spot. On the day of the assay 20 *unc-13* adult hermaphrodites were transferred to an assay plate and evenly distributed on the food spot. An adult male was placed in the middle of the food spot and observed for 15 minutes under a dissection microscope. Presence or absence of mating behaviors such as contact response, backing, turning and locating the vulva of hermaphrodite within 15 minutes were marked. 20 adult males from each strain were observed.

2.13 High-throughput behavioral analysis using the multi worm tracker (MWT)

Basic body measurements, spontaneous movements, and habituation response to tap stimuli were examined and analyzed by using the multi worm tracker system. This package consists of an image acquisition camera, real time image analysis software (MWT) and the offline behavioral parameter measurements software (Choreography). This is a convenient system that can analyze the behavior of the multiple worms on a petri dish at once (Swierczek et al., 2011).

2.13.1 Acquiring the basic body measurements and the spontaneous movements

A plate of a synchronized population of 4-day-old worms (50-60 worms) was set on the MWT platform and the lid was closed. The basic body measurements and the spontaneous movements on the food were recorded over a period of 5 minutes using the MWT. Acquired data was analyzed using the Choreography software. Offline analysis with Choreography software (version 1.3.0_r1035; Swierczek et al., 2011) engaged "--shadowless," "--minimum-time 20," and "--minimum-move-body 2" filters. The MeasureReversal plugin was used to identify reversals.

2.13.2 Response to tap stimuli and short-term habituation

Four to five adult hermaphrodites were allowed to lay eggs for 4 hours and removed from the plates. The synchronized populations of 4-day-old worms in the MWT plates were used in the assay. The plate was placed on a specially designed platform for MWT plates, which is under the camera. Worms were allowed to settle for 5 minutes before

the tap stimuli started. 30 stimuli were delivered at a 10 s inter-stimulus intervals (ISI) to the side of the plates via an automatic solenoid tapper. Behavior of the worms was recorded and analyzed as described previously (Swierczek et al., 2011). The MeasureReversal plugin was used to identify reversals occurring within 1 second of the tap.

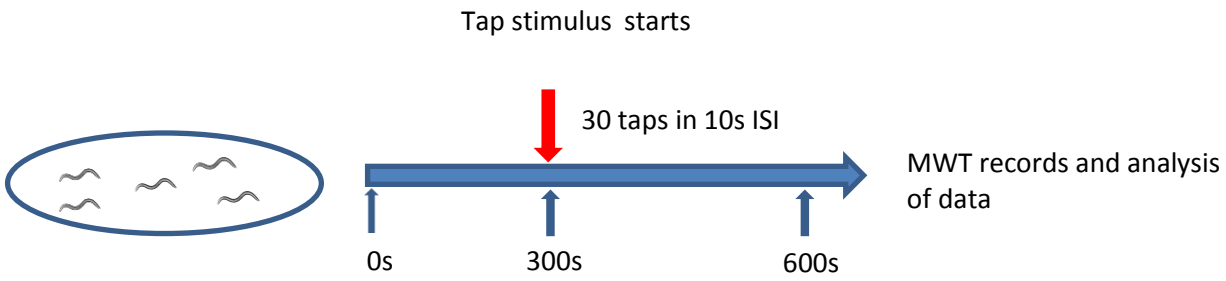

Figure 2.2. Schematic of short-term habituation experiment**.** Worms were allowed to settle for 300 s before the tap starts. 30 taps were delivered at 10 s ISI.

2.14 Scoring vulval phenotypes caused by activated LET-60 and LET-23 in *ras-2* null background

Three adult hermaphrodites were allowed to lay eggs for three consecutive days on freshly seeded NGM plates each day. The progenies were incubated in 15˚C for six days and were scored for different vulval phenotypes including multivulva, protruding vulva and wild-type vulva under a dissection microscope. The number of worms scored for each strain is indicated below (Table 2.2).

Table 2.2. Strains and number of worms scored for vulval phenotypes

Strain	**Number of worms scored**
DM2409 - *let-60 (n1046) iv; ras-2 (ok682) iii*	1044
MT2124 - *let-60 (n1046)*	1207
DM2411 - *let-23 (sa62) ii; ras-2 (ok682) iii*	680
PS1839 - *let-23 (sa62)*	633

2.15 Serial analysis of gene expression (SAGE) and microarray data screening for *ras-2* expression profiling

The SAGE and microarray data provided by *C. elegans resources* database (http://tock.bcgsc.ca/cgi-bin/sage190) were screened and a list of cells, tissues and developmental stages of *C. elegans* that express *ras-2* transcripts was generated. For the SAGE data filtering criteria following specifications were used: Sequence quality of 0.99, removal of all duplicate ditags, ambiguous tags and antisense tags. In addition to SAGE data I also screened the microarray data provided by the *C. elegans resources* database for *ras-2* expression. The microarray data has been generated from Affymetrix GeneChip™ (http://elegans.bcgsc.ca/perl/affymetrix/index).

2.16 Statistical analysis

Statistical analysis using two-way ANOVA and Chi squared test was performed using Prism 6 (GraphPad Software). For the Student's t test Microsoft office excel software

was used. A p value < 0.05 was considered to be statistically significant. P < 0.05 *, P < 0.001 **, P < 0.0001 ***

CHAPTER 3: RESULTS

In this chapter I report the results of my studies on *ras-2* expression profiling in *C. elegans* and the phenotypic and behavioral analysis of *ras-2* null worms. Also in an attempt to evaluate the role of *ras-2* in pathological signaling through Ras pathway, I present the results on vulval phenotypes caused by activated *let-23* or *let-60* on a *ras-2* null background.

3.1 Cloning of *ras-2* promoter and cDNA and generation of transgenic *C. elegans* lines

In order to interpret the phenotypes of *ras-2* null worms, we needed to know the normal expression pattern of RAS-2. I did this by generating a transgenic worm that expresses GFP under endogenous *ras-2* promoter. Firstly, I cloned the 820 bp long endogenous *ras-2* promoter sequence into the promoter-less worm expression vector pPD95.75 and produced the *ras-2*p::GFP (pDM#1218) plasmid construct. This plasmid was injected into worms and one stable transgenic line (DM7442) was successfully established. Secondly, I cloned WT or activated *ras-2* cDNA into this *ras-2*p::GFP construct, so that *ras-2* was fused at its 5' end with GFP, and obtained the translational fusion plasmids *ras-2*p::GFP::*ras-2* (pDM#1222) and *ras-2p*::GFP::*ras-2*G26V (pDM#1223). The latter plasmids were used to generate the transgenic lines that over-express WT RAS-2 (DM7443) and activated RAS-2 (DM7447), respectively. These two lines were used to identify the expression patterns of WT or activated RAS-2 and to evaluate phenotypes caused by overexpressed WT RAS-2 or activated RAS-2.

Table 3.1. Plasmid constructs and transgenic worm lines

Plasmid name	**Plasmid concentration**		**Transgenic line**
	Test plasmid	Marker plasmid pRF4 *[rol-6 (su1006)]*	
*ras-2*p::GFP (pDM#1218)	4ng/µl	86ng/µl	DM7442 - *raEx442[rol-6(su1006)+ras-2p::GFP]*
*ras-2*p::GFP::*ras-2* (pDM#1222)	0.5ng/µl	86ng/µl	DM7443 - *raEx443[rol-6(su1006)+ras-2p::GFP::ras-2]*
*ras-2*p::GFP::*ras-2*G26V (pDM#1223)	0.5ng/µl	86ng/µl	DM7447 - *raEx447[rol-6(su1006)+ras-2p::GFP::ras-2G26V]*

3.2 Expression studies

3.2.1 Localization of the *ras-2*p::GFP transcriptional fusion

To understand the expression patterns of *ras-2* in *C. elegans*, the transgenic line DM7442 that expresses *ras-2*p::GFP transcriptional fusion was used. Monitoring *in vivo* expression of GFP driven by the *ras-2* promoter allows better understanding of the tissue distribution of RAS-2. I observed the DM7442 strain under the fluorescence microscope and found that GFP was localized in the amphid sensory organ, in the proximal myoepithelial sheaths of the gonads, and in the spermatheca in adult hermaphrodites (Figure 3.1A a & e). In adult males, GFP localization is restricted to the amphid sensory organ (Figure 3.1A c). Furthermore, I examined expression in the larval stages L3 and L4 and found that GFP was expressed in the amphid sensory organs

(Figure 3.1A b & d) and in the proximal myoepithelial sheaths of these stages as well (Figure 3.1A f & g). I labelled the amphid neurons of DM7442 with the dye, DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine), and confirmed that GFP positive cells in the head region belong to the amphid sensory apparatus (Figure 3.1B).

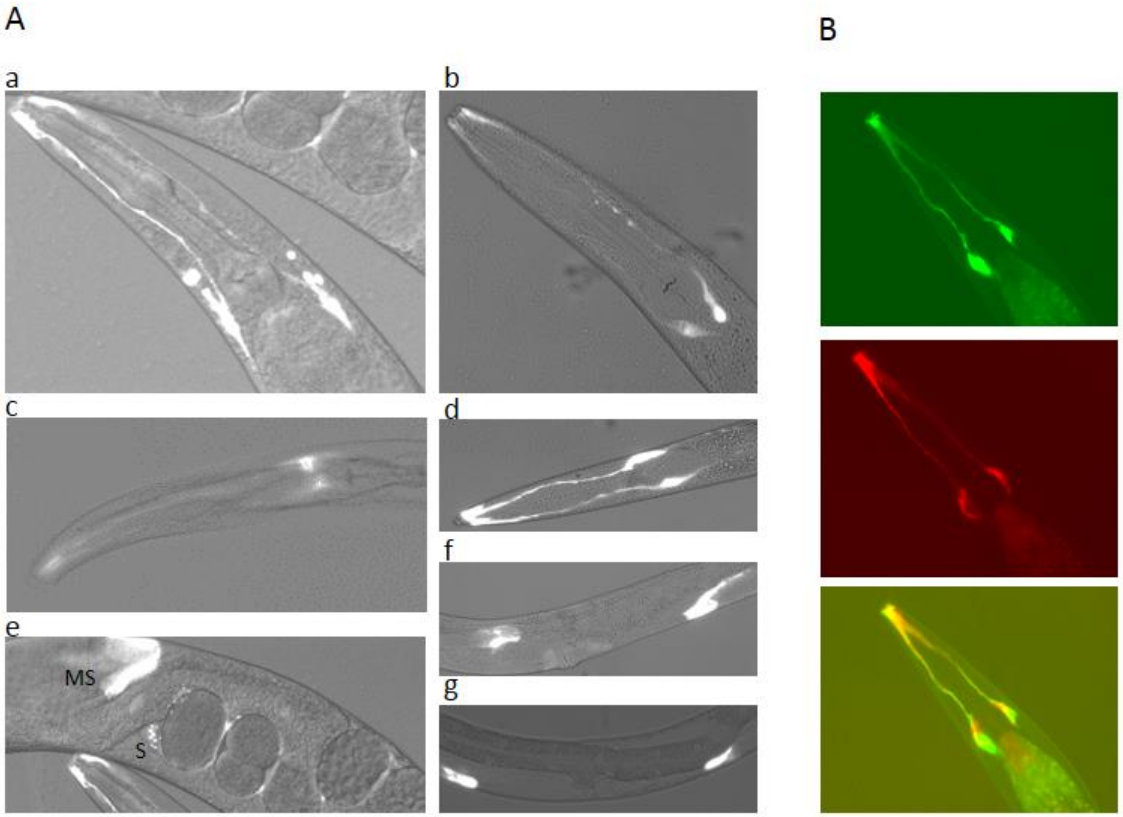

Figure 3.1. Localization of the *ras-2p*::GFP transcriptional fusion in *C. elegans*. **(A)** Differential interference contrast (DIC) micrographs (20X) depict the *ras-2* promoter driven GFP localization in the amphid sensory organ of the adult hermaphrodites (a) and males (c), the larval stages L4 (b) and L3 (d) and in the proximal myoepithelial sheath (MS) and the spermatheca (S) of the adult hermaphrodites (e), and in the proximal myoepithelial sheaths of the L4 (f) and the L3 (g). **(B)** Fluorescence micrographs of the DiI stain of amphids of *ras-2*p::GFP expressing adult hermaphrodites (20X). The *ras-2p*::GFP under GFP channel (Top), DiI stain under the RFP channel (middle) and the merge (bottom).

3.2.2 Expression profiles of WT RAS-2:GFP and RAS-2 G26V:GFP fusion proteins

The transgenic lines DM7443 and DM7447 that carry the *ras-2*p::GFP::*ras-2* and *ras-2p::*GFP*::ras-2*G26V translational fusions respectively were examined for GFP expression. In DM7443, the GFP expression level was very low and was restricted to proximal myoepithelial sheaths, spermatheca, and distal gonad of hermaphrodites, and to the developing gonads of the young adults (Figure 3.2A-D). Microscopic analysis of GFP expression profiles of DM7447, indicated that the expression level of the RAS-2 G26V:GFP fusion protein also was very low and confined to the proximal myoepithelial sheaths of adult hermaphrodites and developing gonads of young adults (Figure 3.2E & F). No obvious phenotypes caused by RAS-2 overexpression were noted in either strain.

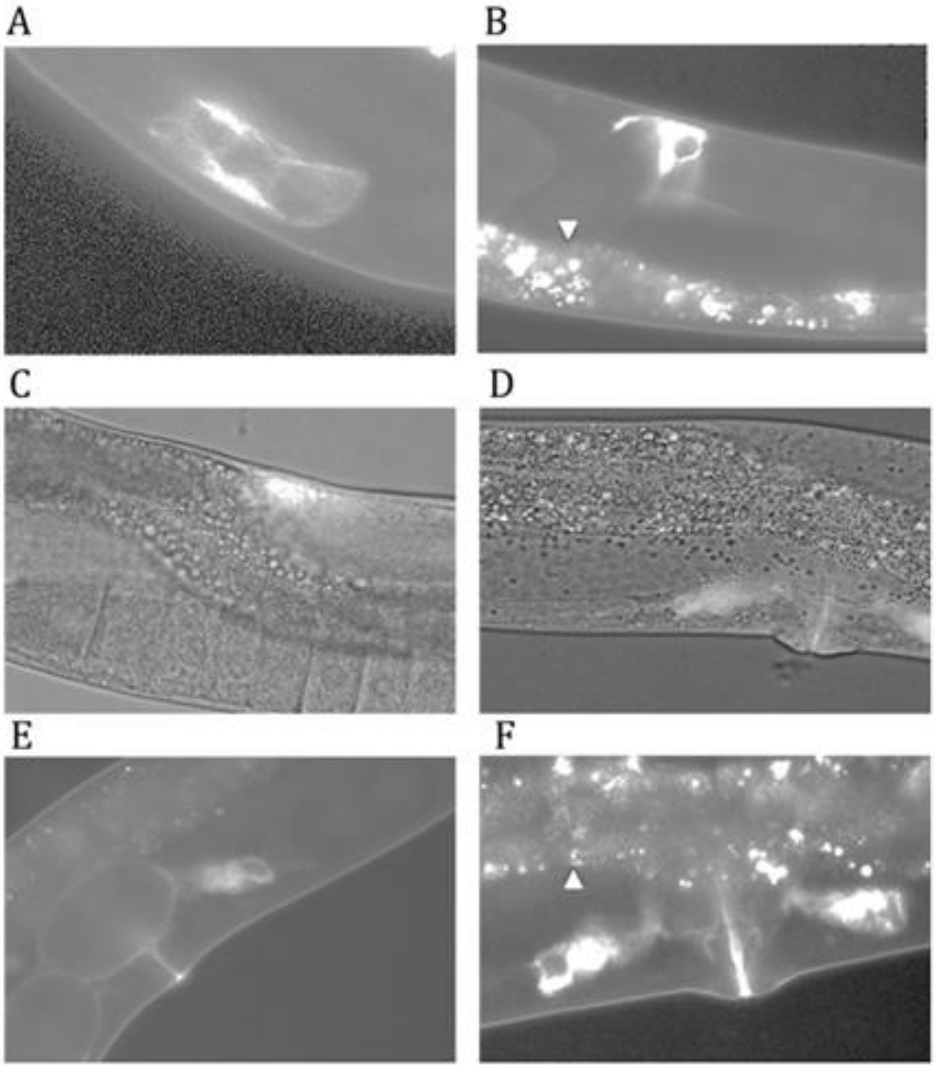

Figure 3.2. Localization of WT RAS-2:GFP and RAS-2 G26V:GFP in *C. elegans***.** WT RAS-2:GFP is localized in proximal myoepithelial sheaths (A) Spermatheca (B) distal gonad (C) of the adult hermaphrodites and in the developing gonad (D) of the young adult. RAS-2 G26V:GFP is localized in proximal myoepithelial sheaths (E) of adults and developing gonads (F) of young adults. The white arrowheads in B and F indicate auto-fluorescence caused by gut granules. A, B, E and F are fluorescence micrographs (40X) and C and D are DIC micrographs (40X).

3.2.3 Gene expression of *ras-2* in different cells, tissues, or developmental stages of *C. elegans*

The British Columbia *C. elegans* gene expression consortium at the British Columbia Genome Science Centre maintains the *C. elegans resources* database (http://elegans.bcgsc.ca/home/) that provides information about gene expression in *C. elegans*, including serial analysis of gene expression (SAGE), promoter::GFP fusions and RNA expression profiles in various different tissues and cells, and developmental stages. I screened *C. elegans resources* database's multiSAGE library and microarray data generated by Affymetrix GeneChip™ (http://elegans.bcgsc.ca/perl/affymetrix/index) to identify the types of cells and tissues, and the developmental stages of *C. elegans* where *ras-2* was found to be expressed. I obtained a list of cells and developmental stages that *ras-2* is expressed (Table 3.2). *ras-2* expression was reported at all developmental stages analyzed, including embryo, all larval stages, and in young adults. Gene expression was also reported for neuronal cells and tissues. Thus, the *ras-2* gene expression data overlapped considerably with the GFP fusion expression data in the transgenic lines.

Table 3.2. Cells and developmental stages of *C. elegans* where *ras-2* is expressed. YES/NO-denotes *ras-2* is detected/not detected by the gene profiling method used. N/A- denotes not available data for the gene profiling method used.

Cells/developmental stages	**Detected by SAGE?**	**Detected by Affymetrix gene chip?**
Embryo	YES	YES
L1	YES	YES
L2	YES	YES
L3	YES	YES
L4	YES	YES
Young adults	NO	YES
Young adults in liquid culture	YES	N/A
Starved L1	YES	YES
Two-weeks old dauer larvae	YES	N/A
Oocyte	YES	YES
FACS sorted muscle cells	YES	N/A
FACS sorted hypodermal cells	YES	N/A
FACS sorted pan-neural cells	YES	N/A
FACS sorted pharynx cells	YES	N/A
FACS sorted AFD neurons	YES	N/A
FACS sorted pharyngeal marginal cells	YES	N/A
FACS sorted ASER neurons	YES	N/A
FACS sorted pharyngeal gland cells	YES	N/A

3.3 Analysis of chemosensation

Given the high levels of expression of RAS-2 in the amphid sensory organ, we tested chemosensory responses in worms lacking *ras-2*.

3.3.1 *ras-2* null worms are not defective in chemoattraction to diacetyl (butane-2, 3-dione)

C. elegans has a highly developed chemosensory system, which mediates chemotaxis to the chemosensory cues associated with food, danger or other animals (Bargmann et al., 1990). The sensory response to volatile and non-volatile chemicals is mediated by three chemosensory sensilla namely amphid, phasmid and inner-labial sensilla (Vowels & Thomas, 1994). In order to determine whether *ras-2* null worms have any defects in chemosensation, I performed a chemotaxis assay. I used diacetyl (butane-2, 3-dione), a chemical detected by amphid neuron AWA and known to attract WT *C. elegans*, and compared *ras-2* null worms to WT and mutant *odr-10*, a strain that lacks receptor for diacetyl. I obtained the chemotaxis index (CI) for each strain. The chemotaxis response to diacetyl of *ras-2* null worms was very similar to that of WT worms. There was no statistically significant difference between the CI of WT and *ras-2* null worms (WT 0.82 +/- 0.033; *ras-2* 0.90 +/- 0.026) (Figure 3.3). Thus, *ras-2* null worms are not defective in chemosensation of diacetyl. The CI for the negative control *odr-10* was significantly different from that of WT and *ras-2* worms (WT 0.82 +/- 0.033; *ras-2* 0.90 +/- 0.026; *odr-10* 0.18 +/- 0.019) (Figure 3.3).

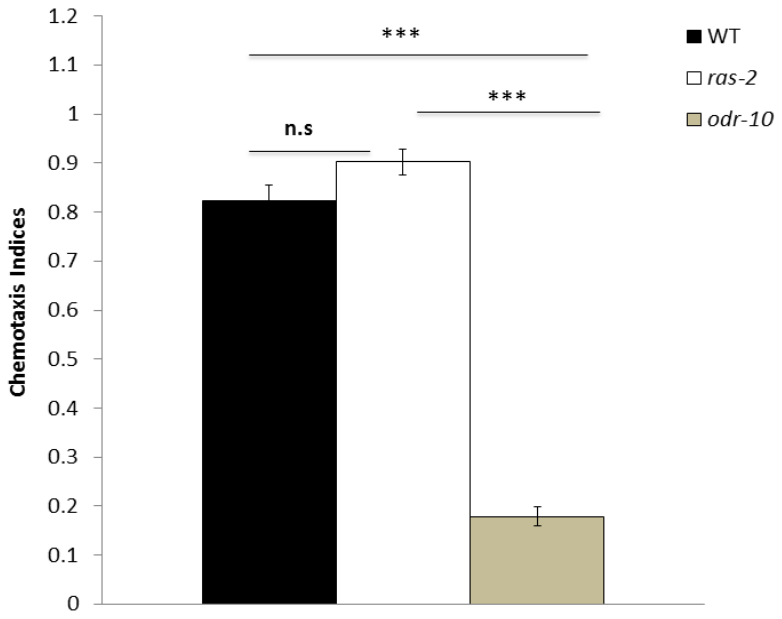

Figure 3.3. Chemotaxis indices for WT, *ras-2* and *odr-10* for butane-2, 3-dione. The difference between CI of WT and *ras-2* null worms is non-significant (n.s). There is a statistically significant difference between CI of WT and *odr-10* (P = < 0.0001) and CI of *ras-2* and *odr-10* (P = < 0.0001) (n = 4 plates with 50-150 worms per plate for each strain). All values graphed as mean +/- SEM. P values were obtained from unpaired Student's t test.

3.3.2 *ras-2* null worms are not defective in avoiding high osmotic glycerol (8M)

WT *C. elegans* are repelled by high concentrations of glycerol, sodium chloride, sorbitol, sodium acetate, ammonium acetate, fructose and glucose (Culotti & Russell, 1978). To avoid these non-volatile repellents *C. elegans* require their amphid neurons to be exposed to the chemicals (Bargmann et al., 1990). In order to investigate whether *ras-2* null worms are defective in avoiding high osmotic repellents, I performed the glycerol ring assay with 8M glycerol. I used the WT N2 strain as a positive control and *osm-9*, which lacks TRP ion channels that mediate sensory transduction in many amphid neurons, as a negative control. Animals that cross the glycerol ring were considered as

non-avoiders and animals that did not cross the barrier were considered as avoiders of high osmotic glycerol solution. Number of avoiders and non-avoiders were recorded every 2 minutes over 10 minutes of period for each strain and the fraction of non-avoiders for each strain was calculated. The fractions of non-avoiders of WT worms and *ras-2* null worms were not significantly different from one another, but both were significantly different from *osm-9* at 2 minutes (WT 1.54% +/- 1.07; *ras-2* 0.69% +/- 0.69; *osm-9* 15.01% +/- 4.87), 4 minutes (WT 2.00% +/- 1.11; *ras-2* 1.20% +/- 1.03; *osm-9* 28.21% +/- 7.88), 6 minutes (WT 2.31% +/- 1.19; *ras-2* 3.16% +/- 1.72; *osm-9* 39.81% +/- 8.00), 8 minutes (WT 4.19% +/- 1.68; *ras-2* 3.99 % +/- 2.42; *osm-9* 58.48 % +/- 7.77) and at 10 minutes (WT 8.43% +/- 2.49; *ras-2* 5.49% +/- 3.02; *osm-9* 74.30 % +/- 7.99) (Figure 3.4). This suggest that *ras-2* null worms behave like WT worms in avoiding high osmotic glycerol and do not have defects sensing high osmotic glycerol like *osm-9* mutants.

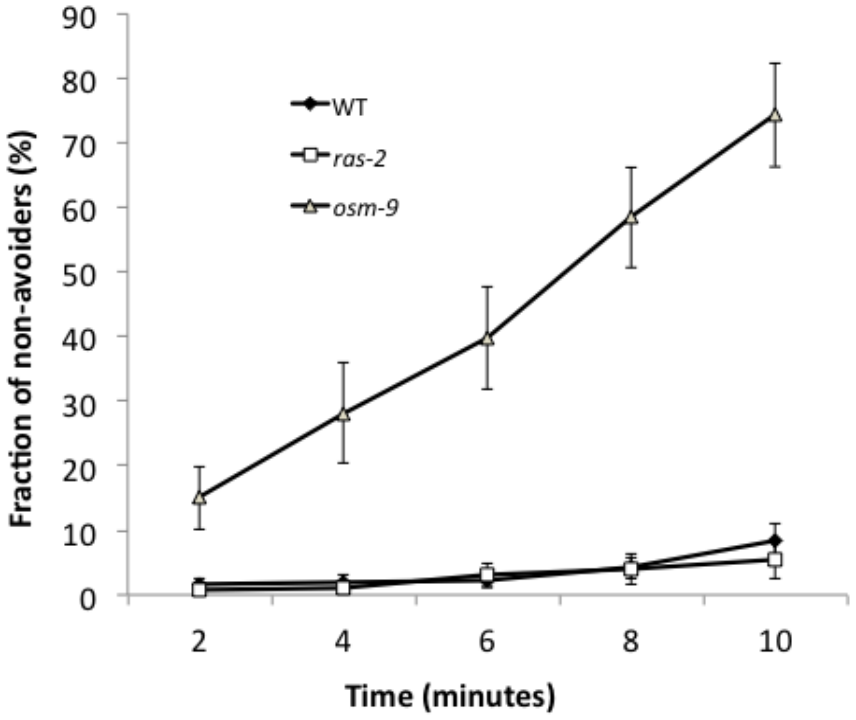

Figure 3.4. The fractions of non-avoiders for WT, *ras-2* and *osm-9* over a 10 minute period. The fraction of non-avoiders for *ras-2* worms is not significantly different from WT worms at 2 minutes (P > 0.05), 4 minutes (P > 0.05), 6 minutes (P > 0.05), 8 minutes (P > 0.05) and at 10 minutes (P > 0.05) n= (WT 11 plates; *ras-2* 10 plates). All values graphed as mean +/- SEM. P values were obtained from unpaired Student's t test.

3.3.3 *ras-2* null worms are smaller than age-synchronized WT worms

Defects in sensory perception in *C. elegans* have been reported to result in abnormalities in growth of the animal. Moreover, regulation of body size through sensory perception required amphid neurons (Fujiwara et al., 2002). To determine the body size of the *ras-2* null worms, I used the multi worm tracker, which can determine the body length, body width and body area of multiple worms at the same time. Excitingly, I found that *ras-2* null adult worms (96 hours old) are significantly smaller than age-synchronized WT worms in body area (WT 0.148 +/- 0.0012; *ras-2* 0.110 +/-

0.0017), body length (WT 1.126 +/- 0.0050; *ras-2* 0.932 +/- 0.0065) and body width (WT 0.11 +/- 0.0014; *ras-2* 0.096 +/- 0.0012) (Figure 3.5A). The *ras-2* null worms were 82.78%, 84.53% and 74.39% in body length, body width and body area of WT worms, respectively. In order to determine the body size at different stages of the life cycle, I constructed growth curves for *ras-2* null and WT worms. I measured the body length of the worms from the tip of the head to the tip of the tail of the animal in different time points. Overall, I found that *ras-2* null worms are smaller in body length than WT worms, starting at 32 hours of age (Figure 3.5B). Specifically, the differences are significant at 32 hours (WT 0.44 +/- 0.011; *ras-2* (0.39 +/- 0.012), 56 hours (0.95 +/- 0.018; *ras-2* 0.85 +/- 0.021), 72 hours (WT 1.10 +/- 0.031; *ras-2* 1.02 +/- 0.017), 80 hours (WT 1.18 +/- 0.027; *ras-2* 1.06 +/- 0.026), 88 hours (WT 1.24 +/- 0.016; *ras-2* 1.13 +/- 0.018), and 96 hours (WT 1.33 +/- 0.018; *ras-2* 1.14 +/- 0.035) of age. Although not statistically significant, *ras-2* null worms showed smaller body size than WT worms at 40 hours (WT 0.50 +/- 0.010; *ras-2* 0.47 +/- 0.013) 48 hours (WT 0.67 +/- 0.024; *ras-2* 0.63 +/- 0.020) and 64 hours (WT 1.00 +/- 0.023; *ras-2* 0.96 +/- 0.023) of age.

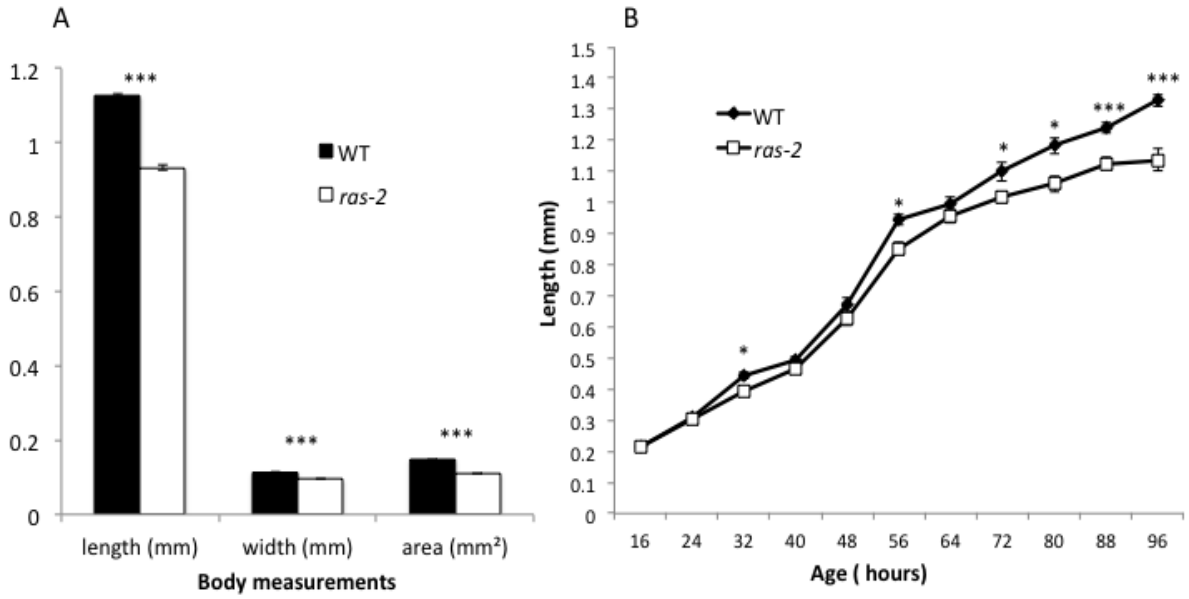

Figure 3.5. *ras-2* null worms are smaller than age-synchronized WT worms**. (A)** Body measurements determined by the multi worm tracker indicate *ras-2* null worms are significantly smaller than WT worms in body length (P = <0.0001), width (P = <0.0001) and body area (P = <0.0001) (n = 6 plates with 50-60 worms per plate for each strain). All values are graphed as mean +/- SEM. P values were obtained from unpaired Student's t test. **(B)** Body length measurements at different ages indicate *ras-2* null worms are smaller than WT in body length starting from 32 hours of age. The difference in the body length is significant at 32 hours (P = 0.006), 56 (P = 0.003), 72 (P = 0.02), 80 (P = 0.004), 88 (P = 0.0001) and 96 (P = 0.0003) hours (n = 9-11 worms for each strain at each time point). All values graphed as mean +/- SEM. P values were obtained from unpaired Student's t test.

3.4 Phenotypic and behavioral analyses of *ras-2* worms

3.4.1. Phenotypic analyses of *ras-2* worms

Apart from the smaller size, we did not observe any gross anatomical abnormalities in *ras-2* worms. This included abnormalities in the appearance of the vulva. Since vulval development is known to be regulated by LET-60 signaling (reviewed in Sundaram, 2006), we concluded that the absence of RAS-2 is inconsequential in this case.

3.4.2 The *ras-2* null worms show faster forward movements than age synchronized WT worms

Next, we observed *ras-2* worms using the Multi Worm Tracker. *C. elegans* moves on a food lawn in an agar plate by generating sinusoidal waves and propagating the waves along the length of its body (Croll, 1975). Posteriorly propagated waves make the animal move forward and anteriorly propagated waves make it move backwards. We analyzed locomotion data captured by the MWT during the last minute of a five-minute session. Analysis of base line phenotypes related to locomotion indicated that the base line forward moving speed of *ras-2* null worms on a standard food lawn on agar was significantly higher than that of WT worms (WT 0.116 mm/s +/- 0.0035; *ras-2* 0.137 mm/s +/- 0.0070) (Figure 3.6).

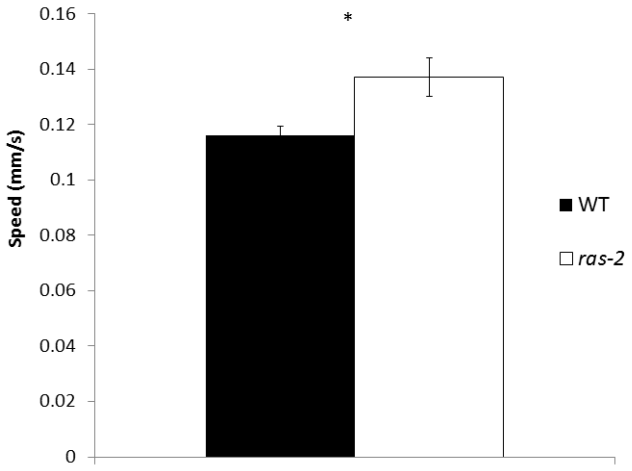

Figure 3.6. Forward moving speed**.** The *ras-2* null worms are significantly faster in forward movements than age-synchronized WT worms (P = 0.02) (n=6 plates with 50-60 worms per plate each strain). All values graphed as mean +/- SEM. P values were obtained from unpaired Student's t test.

3.4.3 Short-term habituation of *ras-2* null worms

C. elegans are capable of learning about their surroundings and modify their behavior in response to environmental changes. Habituation is one of the simplest forms of learning and defined as a gradual decrease in the response to repeated stimuli. Habituation to mechanosensory stimuli in *C. elegans* is well studied and documented (Rankin et al., 1990). When the worms in a petri dish receive a mechanical stimulus as a tap to the side of that dish, they respond by crawling backwards. I tested whether there are differences between WT and *ras-2* null worms in response to the tap stimuli and evaluated the level of habituation of *ras-2 null* worms. To do this, I analyzed two measures, proportion

reversing and the reversal distance in response to 30 tap stimuli delivered at 10 s inter-stimulus intervals (ISI).

Proportion reversing- The *ras-2* worms were as likely as WT worms to respond to the tap stimuli (Figure 3.7A). A two-way ANOVA on the proportion of worms reversing as response to the tap stimuli revealed an insignificant effect of strain ($F(1, 10) = 0.5062$, $P > 0.05$), a significant main effect of stimuli ($F(29, 290) = 28.12$, $P < 0.0001$) and insignificant interaction effect ($F(29, 290) = 1.353$, $P > 0.05$). The significant main effect of stimuli on proportion reversing indicates that both strains habituated to the tap stimuli. I investigated the differences in fractions of worms between two strains that responded to initial and final tap stimuli by using an unpaired Student's t-test. The fractions of *ras-2* worms reversing to respond to the initial and final tap stimuli are statistically indistinguishable from that of WT worms (initial response WT 0.091 +/- 0.061 *ras-2*; 0.858 +/- 0.041; final response WT 0.422 +/- 0.109; *ras-2* 0.468 +/- 0.126).

Reversal distance- Next, I quantified the magnitude of response to the tap stimuli by measuring the distance that worm move backwards during the reversal. Because *ras-2* worms are smaller in body length, I standardized the reversal distances to the average adult (96 hours old) body length of each strain. The reversal magnitudes of *ras-2* worms in response to a tap were greater than that of WT worms. A two-way ANOVA on the reversal distances revealed a significant main effect of strain ($F(1, 10) = 41.67$, $P < 0.0001$), significant main effect of stimuli ($F(29, 290) = 19.81$, $P < 0.0001$) and no significant interaction between stimuli and strain ($F(29, 290) = 1.161$, $P = 0.265$). The significant main effect of stimuli indicates that both strains habituated to the tap

stimuli. I investigated the differences further by comparing the reversal distance of each strain to each stimuli using an unpaired Student's t-test. This analysis demonstrated that there is a statistically significant difference in a reversal distances of *ras-2* and WT at 1st, 2nd, 3rd, 4th, 7th, 9th, 13th, 14th, 16th, 19th, 20th, 21st, 24th, 25th and the 26th tap stimuli ($P < 0.05$) (Figure 3.7B). Considering that *ras-2* worms normally travel at a speed that is approximately 18% faster than that of WT worms at 96 hours of age (see Figure 3.6), it is not surprising to see slightly higher values for reversal distance for most of the later tap stimuli. However, the faster speed alone could not account for the great differences (> 18%) seen at the first 3-4 taps (Figure 3.7B). Thus, worms lacking RAS-2 travel greater distances backwards in response to a novel adverse stimulus, despite their smaller body size. We concluded that although the learning/habituation response appeared normal overall, *ras-2* worms exhibit higher levels of symptoms resembling anxiety.

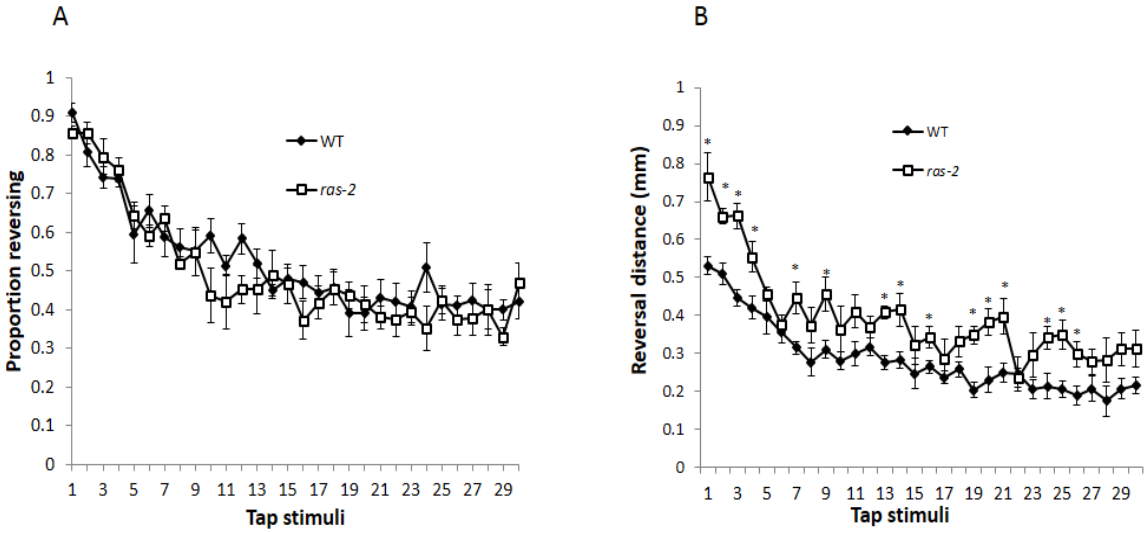

Figure 3.7. Tap habituation curves of 96 hours old *ras-2* null and WT worms in response to series of 30 taps**.** (A) The fractions of *ras-2* worms reversing to respond to the initial and final tap stimuli are statistically indistinguishable from that of WT worms (initial response $P > 0.05$; final response $P > 0.05$) (n = 6 plates of worms with 50-60 worms per plate). (B) There is a statistically significant difference in a reversal distances of *ras-2* and WT at 1st, 2nd, 3rd, 4th, 7th, 9th, 13th, 14th, 16th, 19th, 20th, 21st, 24th, 25th and the 26th tap stimuli ($P < 0.05$) (n = 6 plates of worms with 50-60 worms per plate). All values are graphed as mean +/- SEM. P values were obtained from unpaired Student's t test.

3.5 Evaluation of reproductive efficiency of *ras-2* null worms and mating behavior

3.5.1 Reproductive efficiency in hermaphrodites

C. elegans hermaphrodites are self-fertilized and produce a large number of oocytes and a limited number of sperm. The self-fertilizing machinery is nearly 100% efficient and can result in about 300 self-progeny throughout the reproductive life. Mating with a male can sire additional offspring; the number of cross-progeny can be over 1000. The oocyte maturation, ovulation, fertilization and egg-laying events occur in the somatic gonad of the hermaphrodite, which is composed of several different tissues: distal tip cells, gonadal sheath cells, spermatheca, spermatheca-uterine valve and the uterus. Any defects in theses tissues may result in reduction of reproductive efficiency or reduced progeny size. Because RAS-2 is strongly expressed in proximal myoepithelial sheath cells and spermatheca (Figure 3.1), it may play a role in the functions of these tissues. Therefore, I hypothesized that lack of *ras-2* may result in reduced reproductive efficiency in hermaphrodites.

I observed the egg-laying ability of *ras-2* null worms and determined the total number of progeny produced by *ras-2* hermaphrodites. My observations indicate that *ras-2* worms are as efficient as WT hermaphrodites in producing self-progeny. No significant difference was found in total number of progeny produced by *ras-2* or WT hermaphrodites (WT 358 +/- 40; *ras-2* 330 +/- 50) (Figure 3.8). I also determined the brood size of the two transgenic strains, DM7443 (*raEx443 [rol-6 (su1006)+ras-2p::GFP::ras-2]*) and DM7447 (*raEx447 [rol-6 (su1006)+ras-2p::GFP::ras-2G26V]*) which

produce WT and activated RAS-2 respectively. I found that overexpression of WT RAS-2 or activated RAS-2 does not affect the brood size (*raEx443 [rol-6 (su1006)+ras-2p::GFP::ras-2]* 377 +/- 8; *raEx447 [rol-6 (su1006)+ras-2p::GFP::ras-2G26V]* 305 +/- 30) (Figure 3.8). These findings suggest that *ras-2* does not have a significant effect on brood size of the hermaphrodites.

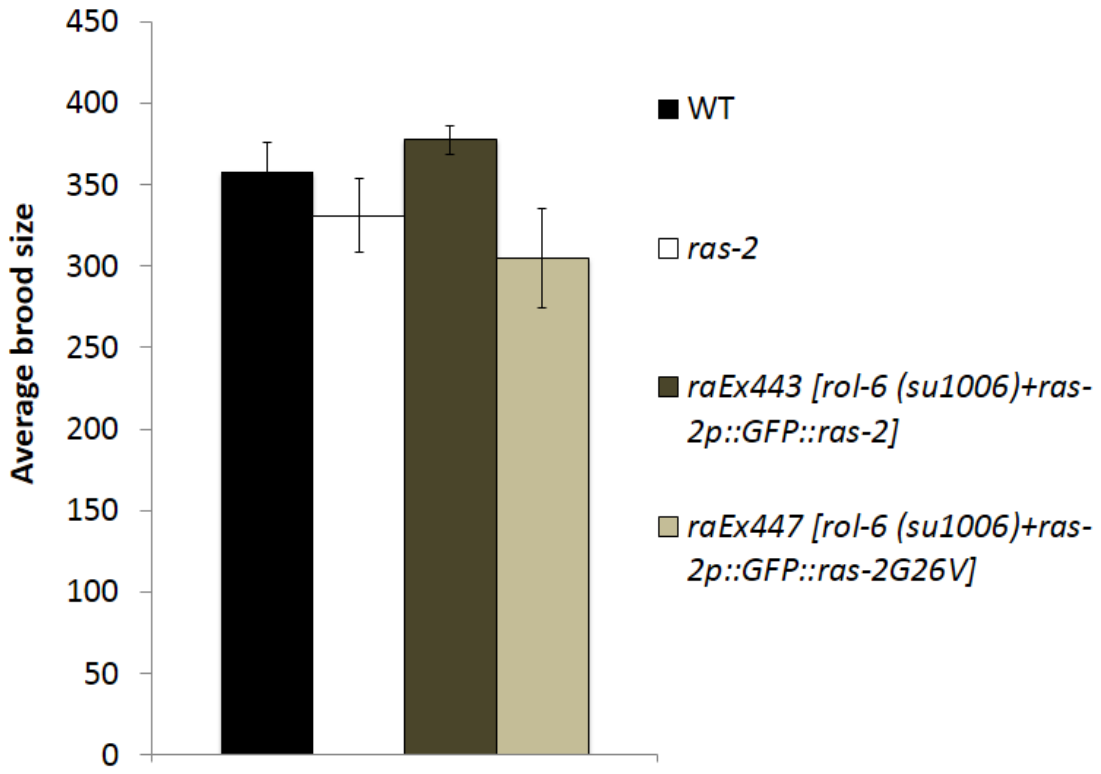

Figure 3.8. Reproductive efficiencies of *ras-2* null hermaphrodites**.** The average brood size of the *ras-2* (P > 0.05, n = 5), (*raEx443 [rol-6 (su1006)+ras-2p::GFP::ras-2]* (P > 0.05, n = 10), or *[rol-6 (su1006)+ras-2p::GFP::ras-2G26V]* (P > 0.05, n = 5), is statistically indistinguishable from WT hermaphrodites (P values were obtained from unpaired Student's t test and all values are graphed as mean +/- SEM).

3.5.2 Reproductive efficiency in male worms

Mating behavior is a complex social behavior in *C. elegans* in which the male performs an active sensory and motor behavior while the hermaphrodite plays a passive role. Hermaphrodites provide sensory signals (pheromones) before mating to attract males. Males from general sensory mutants such as *osm-5* and *osm-6* are unable to respond to the sensory signals from hermaphrodites in mating assays (Simon & Sternberg, 2002). Therefore, males with sensory defects may have impaired mating ability resulting in reduced mating efficiency.

First, I investigated the reproductive efficiency in *ras-2* males. I determined the number of cross-progeny that a *ras-2* male can sire by mating with a *dpy-5* hermaphrodite. I found that of those males that did mate and produced more than 15% cross-progeny of total offspring, there was no significant difference between the mating efficiencies of *ras-2* and WT males (WT 86.96 +/- 3.3 ; *ras-2* 82 +/- 10.1) (Figure 3.9A). However, the frequency of *ras-2* males that produced greater than 15% cross-progeny was lower than that of wild-type males (WT: 64%; *ras-2*: 36%) (Figure 3.9B). There were males that did not produce cross-progenies in both group (Figure 3.9C).

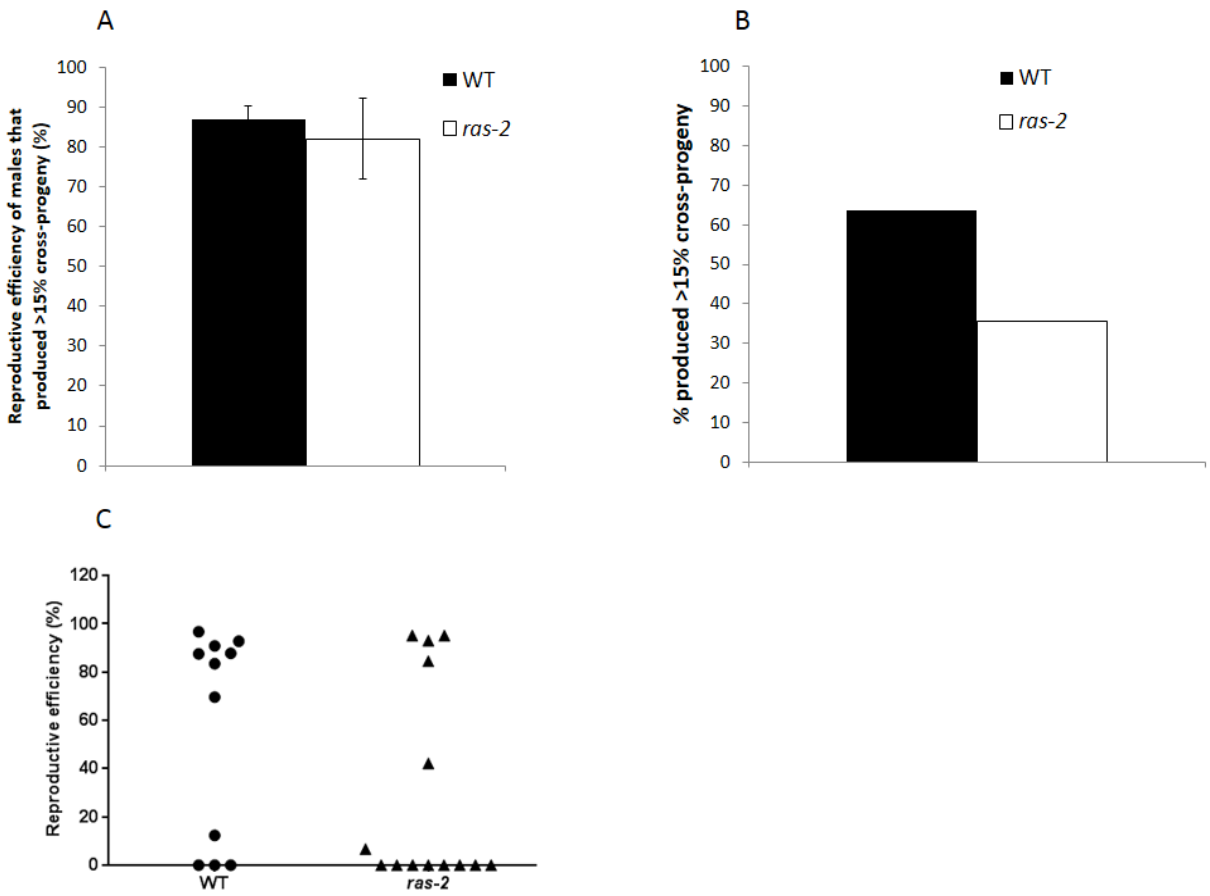

Figure 3.9. Reproductive efficiencies of *ras-2* null males**.** (A) The reproductive efficiency of *ras-2* males that produced > 15% cross-progeny is not statistically significant from WT males (P > 0.05, n = WT 7; *ras-2* 5) (P values were obtained from unpaired Student's t test and all values are graphed as mean +/- SEM). (B) The frequency of *ras-2* males that produced > 15% cross progeny is non-significant from WT males (P > 0.05, n = WT 11; *ras-2* 14). P value was obtained from Chi squared test. (C) The reproductive efficiencies of all the males used in the assay (n = WT 11; *ras-2* 14)

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3.5.3 Scoring of male mating behaviors

Because I noted that *ras-2* males were less likely to mate with a hermaphrodite (see Figure 3.9), I studied the mating behavior of *ras-2* males in more detail, by observing the major sub-behaviors of mating. The key stages in worm mating behavior are: hermaphrodite recognition and contact response, backing, turning, vulva location and

copulation (Liu & Sternberg, 1995). My findings indicate that *ras-2* males are able to recognize hermaphrodites and perform contact response at the same frequency as wild-type males. I determined the percentage of males that performed contact behavior and found that 90% of *ras-2* and WT males are able to perform this behavior (Figure 3.10A). I also determined the average time it took for a male to contact the hermaphrodite. These times varied greatly and there was no significant difference between WT and *ras-2* (WT 261.76s +/- 43.26; *ras-2* 290.7s +/- 46.53) (Figure 3.10B). Furthermore, I determined the frequency of performing consecutive steps in the mating behavior including backing, turning and vulva locating of males who committed response behavior. Although statistically non-significant, *ras-2* worms were more successful in performing above-mentioned steps than WT males (backing; WT 94.4%; *ras-2* 100%; turning; WT 77.8%; *ras-2* 94.4%; locating vulva; WT 77.8%; *ras-2* 94.4%) (Figure 3.10C). Every *ras-2* male who performed the response contact was able to continue to backing behavior. WT males were less likely than *ras-2* males to perform consecutive steps in mating behavior.

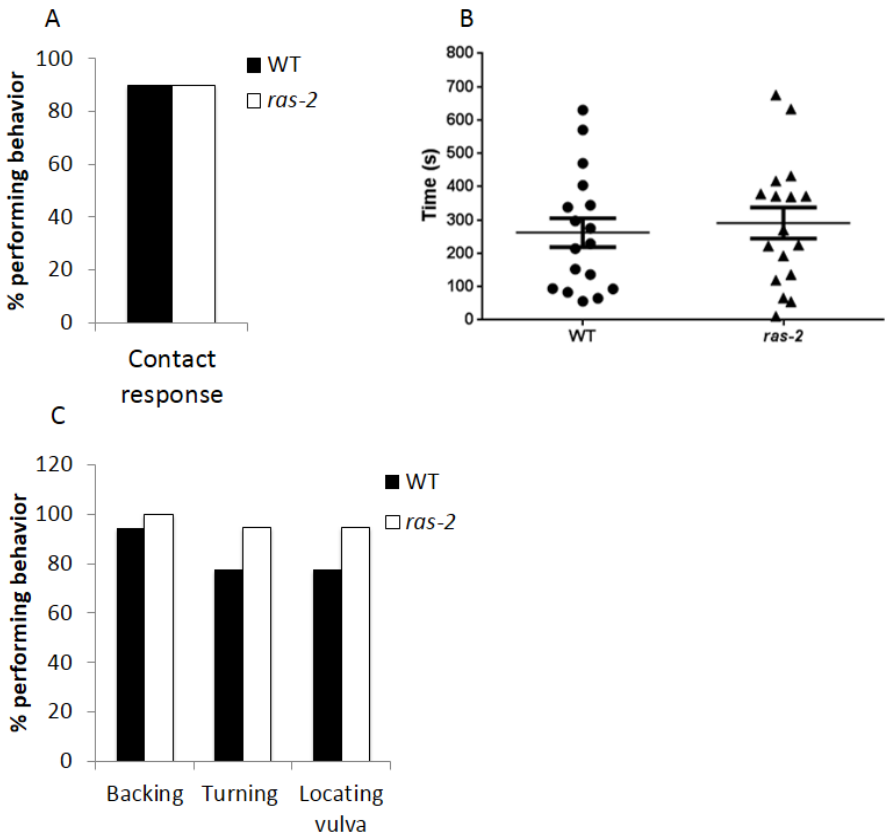

Figure 3.10. Scores of male mating behavior**.** (A) The percentage of males that performed contact response behavior in mating was similar in WT and *ras-2* males (n = 20 for both WT and *ras-2*). (B) The average time taken by *ras-2* males to recognize a hermaphrodite and perform a contact response is not statistically different from WT worms (P > 0.05, n = 20 for both WT and *ras-2*). P values were obtained from an unpaired Student's t test and values are graphed as mean +/- SEM. (C) The frequency of *ras-2* males performing consecutive steps in mating behavior was slightly higher but statistically indistinguishable from WT males (backing; P > 0.05, turning; P > 0.05, locating vulva; P > 0.05, n = 19 for both WT and *ras-2*) P values were obtained by Chi squared test.

3.6 Scoring of vulval phenotypes caused by activated (*gain-of-function*) *let-60* or activated (*gain-of-function*) *let-23* mutations on a *ras-2* null background

Since Ras signaling has been highly implicated in vulval induction in *C. elegans*, mutations in the components of the pathway result in altered vulval phenotypes. For instance, the *gain-of- function (gf)* mutations of *let-23* and *let-60*, both of which activate the Ras/ERK signaling pathway, cause a multivulva (Muv) phenotype (reviewed in Sundaram, 2006). Some suppressors of the Muv phenotype have been identified. One of these is *sur-8* (*soc-2*) (Sieburth et al., 1998). Because Shoc-2 (SUR-8) and M-Ras (RAS-2) were reported to interact in mammals, we hypothesized that *ras-2* may also be involved in regulation of the Ras/ERK signaling pathway in *C. elegans*, and that lack of *ras-2*, like *sur-8* (*soc-2*), may suppress the Muv phenotype caused by *let-60 (gf)* and *let-23 (gf)*.

In order to test this hypothesis I scored the different vulval phenotypes in a cross between *ras-2* null and *let-60 (gf)* mutants (*let-60 (n1046) iv; ras-2 (ok682) iii*) and *let-60 (gf)* mutants (*let-60 (n1046)*) as the control strain (Figure 3.11A). I observed that Muv occurred with the same frequency in the presence or absence of *ras-2* (61.06% with *ras-2*, 61.39% without *ras-2*). However, *let-60* (*gf*) exhibited higher frequencies of protruding vulva (Pvul) in the absence of *ras-2* (26.05% vs. 18.39% in the presence of *ras-2*). The higher frequency of Pvul led to a decrease in the occurrence of normal, or wild-type, vulvae in the absence of *ras-2* (4.02% vs. 13.58% with *ras-2*). Both *let-60* (*gf)* mutants (with or without *ras-2*) also showed Pvul in combination with Muv at low frequency, but there was no significant difference between these two groups (6.95% in the presence of *ras-2*, 8.52% in the absence of *ras-2*).

Similar to *let-60 (gf)*, I also tested whether *ras-2* may be essential in pathological signal transduction caused by *let-23 (gf)*. I examined the cross between *ras-2* null and *let-23 (gf)* mutants (*let-23 (sa62) ii; ras-2 (ok682) iii)* for suppression of Muv. I observed that there was no suppression of the Muv phenotype caused by *let-23 (gf)* on a *ras-2* null background. The frequency of Muv in the presence of *ras-2* was 85.61%, and 81.61% in the absence of *ras-2* (Fig. 3.11B). Other vulval phenotypes observed in let-23 worms with or without *ras-2* were also very similar in frequency (with/without *ras-2*): Pvul - 0.78%/2.05%; Muv with Pvul - 2.21%/2.35%; wild-type vulva - 11.37%/13.97%.

These findings were unexpected and suggest that even though *ras-2* did not suppress the Muv phenotype caused by *let-60 (gf)* or *let-23 (gf)* mutants, it may modulate other signaling pathways that contribute to different developmental abnormalities such as Pvul.

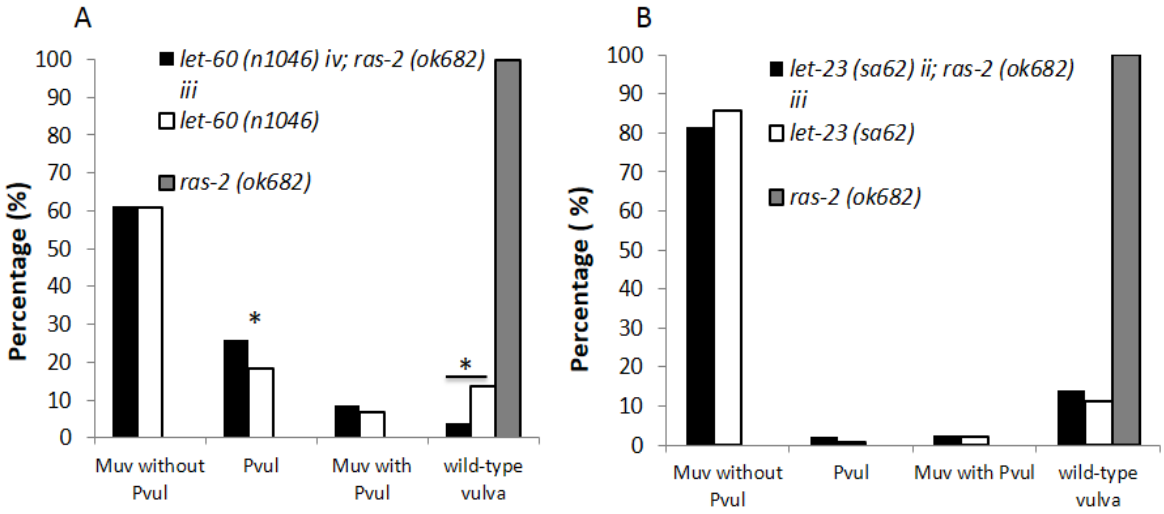

Figure 3.11. Different Vulval phenotypes caused by *let-60 (gf)* and *let-23 (gf*) mutants on a *ras-2* null background**.** (A) The frequency of Pvul and the frequency WT vulva in (*let-60 (n1046) iv; ras-2 (ok682) iii)* are statistically different from (*let-60 (n1046))* mutants ($P < 0.05$ n = *let-60 (n1046) iv; ras-2 (ok682) iii* 1044; *let-60 (n1046)* 1207) P values were obtained from Chi squared test. The differences in Muv without Pvul and the Muv with Pvul are statistically non-significant between the two strains. (B) There were no significant differences in any of the vulval phenotypes of *let-23 (sa62) ii; ras-2 (ok682) iii* and *let-23 (sa62)*.

CHAPTER 4: DISCUSSION

M-Ras/RAS-2 is highly conserved throughout evolution and both nematodes and mammals have orthologs, which share about 50% amino acid identity. Even though there are published data on the functions and significance of M-Ras in mammals, M-Ras/RAS-2 in *C. elegans* has not been studied yet.

4.1 The *ras-2* expression profiles in *C. elegans*

In order to gain insight of function or role of a certain gene in an organism, it is very important to understand its detailed expression pattern. This can be achieved on a gene transcript level using gene expression profiling techniques such as RNA microarrays and *in situ* mRNA localization. On a protein level, the protein can be detected histochemically using antibodies, if available, or indirectly through the use of transgenic animals that carry the promoter of the gene of interest fused to a reporter such as green fluorescence protein (GFP) (Chalfie et al., 1994). This promoter::GFP fusion technique has been used in large scale genome-wide studies in *C. elegans* (McKay et al., 2003). Generating transgenic animals with promoter::reporter fusions allows better understanding of the temporal and tissue specific expression patterns of the promoter::reporter constructs, which reflects the transcriptional regulation of the genes *in vivo*. Since no antibodies are currently available for RAS-2, we chose the promoter::reporter gene approach to reach the goal of identifying *ras-2* expression patterns in *C. elegans*.

I generated a transgenic worm line that expresses GFP driven by endogenous *ras-2* promoter. Strikingly, I found GFP expression in several different cells and tissues of *C. elegans* including the amphid sensory organs in hermaphrodites and males, and the proximal myoepithelial sheaths and spermatheca of adult hermaphrodites. The L3 and L4 larvae also exhibited a similar pattern in the amphid neurons (Figure 3.1A). GFP expression was strong and the expression pattern was similar between individuals in the stable transgenic line. The expression profile of the *ras-2*p::GFP transcriptional fusion was comparable with the expression data collected from SAGE libraries and the mRNA expression data provided by Affymetrix GeneChip™. The *ras-2* SAGE tags were detected in FACS sorted AFD and ASER amphid neurons (Table 3.2). *ras-2* expression was not limited to the amphid neurons; SAGE data indicates that tags were also detected in FACS sorted pan-neural cells. Even though there is no obvious GFP expression in neurons other than amphids in transgenic animals, there may be non-detectable levels of GFP in the other neurons. Alternatively, *ras-2* could be specific to neurons and supportive cells in the amphid. *C. elegans* have a pair of bilaterally located amphid sensilla in the head region that function as the major sensory organ of the animal. Each amphid is a collection of 12 sensory neurons and supportive cells; a sheath cell and a socket cell. In order to confirm the specific cell type in which the GFP was detected, further analysis is needed. The expression of GFP was also strong and stable in the proximal myoepithelial sheath and in the spermatheca of the adult hermaphrodites. There are no available data on *ras-2* expression in these structures generated by SAGE tags or Affymetrix GeneChip™ gene chip.

Since the promoter::GFP fusion was successfully expressed in worms, I used the promoter::GFP::open reading frame (ORF) fusion strategy to express WT or activated RAS-2 to obtain more precise information on RAS-2 protein localization. Unfortunately, GFP fluorescence of the RAS-2:GFP fusion proteins was very low and varied among individuals in the line. I found that protein localization of both WT and activated RAS-2 was limited to the gonads of hermaphrodites (Figure 3.2A-F). This discrepancy between transcriptional and translational fusions may be due to the intrinsic instability of the chimeric protein in the transgenic line carrying the translational fusions. Intrinsic instability of the chimeric protein may result in less bright translational fusion products. Fusion of GFP can alter the protein structure and can disrupt the function of the protein (Boulin et al., 2006). Another explanation would be that RAS-2 overexpression is not well tolerated by some cell types, and that gene expression may be suppressed by, for example, methylation. No obvious phenotypes caused by overexpression of the RAS-2:GFP fusion proteins were detected in either strain, which may be due to the very low expression levels.

4.2 Characterization of *ras-2* null worms

The *ras-2*p::GFP expression patterns, SAGE and the Affymetrix GeneChip™ data of *ras*-2 expression were the basis for the subsequent phenotypical and behavioral analyses of *ras-2 null* worms. The expression profiles of *ras-2* suggested that *ras-2* may be implicated in sensory perception mediated through the amphid sensory organ, and functions associated with tissues and cells of the gonad of *C. elegans*. Therefore, *ras-2*

null worms may be defective in functions of amphid sensory organ and gonads, and experiments were carried out to determine whether this was the case.

4.2.1 Role of *ras-2* in the amphid sensory organ

The most remarkable and interesting phenotype I found during my study on *ras-2* null worms is the body size variant. The *ras-2* worms are significantly smaller than WT worms in body length, body width and body area. At the early stages of development, until 32 hours of age, *ras-2* worms are not different in body length from WT worms. The smaller body length became apparent after 32 hours of age (Figure 3.5).

Perception of sensory cues from the surrounding environment can alter the development of an organism. Female mice exposed to sexual pheromones show accelerated growth due to induced endocrine changes (McLellan et al., 1998). Worms that are raised in isolation are smaller in body size, less responsive to mechanosensory stimulation and slower in the onset of laying eggs than age-matched group-grown worms due to decreased sensory stimulation from the environment (Rose et al., 2005). More importantly, Fujiwara et al. reported (2002) that regulation of the body size of *C. elegans* is mediated by sensory input through amphid neurons. Mutants with defects in sensation due to cilium structure abnormalities, such as *che-2*, *che-3* and *osm-6* exhibit a smaller body size than wild-type animals from the L4 stage to late adulthood. And this is not due to an inability to locate food. Not only cilium structure mutants but also mutants that are defective in sensation due to impaired sensory signal transduction such as *tax-2* and *tax-4* exhibit smaller body sizes than WT animals (Fujiwara et al.,

2002). These findings suggest that a proper sensory perception is required for a normal body growth in *C. elegans*. Therefore, I conclude that *ras-2* worms may be defective in perceiving environmental cues that can stimulate body growth. Such cues could include pheromones.

Interestingly, the other findings in this study indicate that *ras-2* worms are not defective in sensory perception in general. Chemosensation of diacetyl (butane-2, 3-dione) and osmosensation of high concentrations of glycerol were normal in *ras-2* worms (Figure 3.3 & 3.4). Although *ras-2* worms were not defective in sensing the chemicals tested, there may be abnormalities in perceiving some other environmental cues such as cues generated by conspecifics. The *daf-11* and *daf-21* mutants that are defective of amphid neurons, exhibit no defects in avoiding of certain non-volatile repellents but are defective in chemoattraction to certain chemicals or in response to dauer-inducing pheromones (Vowels & Thomas, 1994). This is due to the specific functions of the individual sensory neurons in the amphid. Thus, it may be important to elucidate the cell-specific expression of *ras-2* in amphid neurons.

Another discovery we made using the MWT was that *ras-2* worms show faster forward movements than WT worms. *C. elegans* moves on a food lawn in an agar plate by generating and propagating sinusoidal waves along the length of its body (Croll, 1975). Posteriorly propagated waves allow forward movement, whereas anteriorly propagated waves result in backward movement. These waves are generated by successive contraction and relaxation of body wall muscles and are coordinated by the neural circuitry responsible for locomotion. There are several parameters that define the

forward speed of worm, such as amplitude of the sinusoidal body bend and the rate of bend propagation (Zhao et al., 2003). The smaller body size of the *ras-2* worms may alter these parameters, causing faster forward movements than WT worms. It would be interesting to know which of these parameters causes the changes in the speed of *ras-2* worms. Any abnormality that alters function of the musculature and the neural circuitry responsible for locomotion may reflect altered forward speed of the worms. Altered forward speed in mutants lacking sensory neurons such as PVD and FLP has been reported (Albeg et al., 2011).

4.2.2 Role of *ras-2* in learning and memory

C. elegans is a good model to understand the mechanisms involved in learning and memory. Habituation is one of the simplest forms of learning and defined as a gradual decrease in the response to repeated stimuli. The availability of sophisticated tools like the MWT allows us to identify novel genes that are implicated in learning and memory in *C. elegans*.

During this study I evaluated the ability of *ras-2* worms to habituate to the mechanical stimuli, which tests both mechanosensation and behavioral plasticity. In higher species such as mice and chickens, M-Ras has been implicated in synaptic plasticity in the hippocampus and the hypothalamus (Robles et al., 2003; Labunskay & Meiri, 2006). I measured the level of habituation of *ras-2* worms by two parameters, the reversal probability and the reversal distance as the response to tap stimuli. I found that *ras-2* worms habituate to tap stimuli as well as WT worms do. The decreasing reversal

probability and decreasing reversal distance with the mechanical stimuli indicate that *ras-2* worms have no defects in learning and short term memorizing (Figure 3.7). This is consistent with the findings that indicate M-Ras null mice are not defective in memory-related functions evaluated by fear conditioning and Morris water maze assays (Nunez Rodriguez et al., 2006).

However, the reversal distance of *ras-2* worms in response to each tap was greater than that of WT worms at nearly all of the 30 taps administered. Given that *ras-2* null worms are only about 82% the size of WT worms, but travel at speeds that are about 18% faster under normal conditions, these differences may be insignificant at most of the 30 tap responses. However, the reversal distance at the first few taps was much greater than 18%. This may be suggestive of a higher fear-like response in *ras-2* worms than WT worms. Interestingly, we have also noted higher levels of anxiety in M-Ras$^{-/-}$ mice that undergo open field or dark/light box tests (unpublished observations).

4.2.3 Role of *ras-2* in reproduction and mating behavior

RAS-2 expression was also found to be high in the gonads, suggesting that it could play a role in functions of this organ. Thus, I evaluated the reproductive efficiency of both *ras-2* hermaphrodites and males. I observed that *ras-2* hermaphrodites do not show a statistically significant difference in reproductive efficiency from WT worms (Figure 3.8). In addition, over-expression of WT or activated RAS-2 in hermaphrodites did not affect the brood size (Figure 3.8). Likewise, of those males that mated with a hermaphrodite, the reproductive efficiency was not significantly different. However, the frequency of

ras-2 males that sired cross progeny was only about half that of WT males (Figure 3.9). These observations suggested that while *ras-2* males do not have anatomical defects in tissues involved in successful reproduction, their mating behavior could have been different from that of WT males. However, there may be defects in sensory perception that, in turn, is required for optimal mating behavior.

During the mating process, hermaphrodite recognition is the initial step and males are attracted to chemical cues secreted by hermaphrodites (Simon & Sternberg, 2002). These chemical cues have been identified as a blend of dauer-inducing ascaroides (Srinivasan et al., 2008) sensed by the ASK and CEM sensory neurons (Macosko et al., 2009). However, my findings indicate that *ras-2* worms are as successful as WT males in mate finding (Figure 3.10A & B). This observation suggests that *ras-2* males are not defective in detecting pheromones secreted by hermaphrodites in mating. Even though *ras-2* males were successful at locating the hermaphrodite and were able to initiate mating behavior, failures in subsequent steps may lead to reduced fertility. Surprisingly, I found that - even though statistically insignificant - the frequency of *ras-2* males performing sub-behaviors in mating was actually greater than that of WT males (Figure 3.10C). Therefore, I conclude that the lower frequency of *ras-2* males that were willing to mate with a hermaphrodite was likely not due to a sensory defect or inability to perform proper mating behavior. Male M-Ras$^{-/-}$ mice exhibit similar abnormalities in mating behavior. We observed that the time it took to mount a female was significantly shorter for M-Ras$^{-/-}$ males (unpublished data). We are currently investigating whether differences in motivation could account for this phenotype.

4.3 Role of *ras-2* in development of vulval phenotypes caused by *let-60 (gf)* or *let-23 (gf)*

During the post-embryonic development of *C. elegans* hermaphrodites there are 6 vulval precursor cells (VPCs) each of which has the potential to undergo vulval formation or hypodermal cell generation. During the vulval induction at L3 stage, an inductive signal LIN-3 (EGF) from the anchor cell of the gonad initiates the induction of three VPCs to undergo vulva formation. LIN-3 is the ligand for LET-23 (EGFR) and upon binding, it activates the RTK/Ras/ERK pathway in VPCs. The other three VPCs generate the hypodermal cells. The three VPCs that undergo vulval formation give rise to 22 vulval cells which migrate towards the anchor cell and subsequently invaginate to form the vulva (reviewed in Sternberg, 2005).

Since Ras signaling is involved in the vulval development of *C. elegans*, mutations in components of Ras signaling pathway cause vulval defects. For instance, *C. elegans* p21 Ras, *let-60 loss-of-function (lf)* mutants lack a vulva (Vulvaless or Vul phenotype) whereas the *gain-of-function (gf)* mutants exhibit the multivulva (Muv) phenotype (reviewed in Sundaram, 2006). Therefore, the vulval development of the *C. elegans* is a powerful tool to identify and characterize the genes associated with Ras-mediated signal transduction pathways.

The gene *sur-8* (also known as *soc-2*) was identified as a positive regulator of Ras-mediated signal transduction during vulval development in *C. elegans* (Sieburth et al., 1998). The *loss-of-function* mutation of *sur-8* strongly suppresses the Muv phenotype caused by the *let-60 (gf)* mutation. Shoc-2, the mammalian ortholog of *sur-8*, was found

to be a specific effector of the activated M-Ras but not of activated p21 Ras, and its function was essential for the activation of ERK pathway by growth factors (Rodriguez-Viciana et al., 2006). Furthermore, Sieburth et al. (1998) reported that expression of human Shoc-2 rescued the suppression of the Muv phenotype by *sur-8 (lf)* mutation. Since both Shoc-2 and M-Ras are conserved in *C. elegans*, we believe that the specific affinity of mammalian Shoc-2 for activated M-Ras is conserved in *C. elegans*. Thus we hypothesized that lack of *ras-2* would result in the same phenotype caused by *sur-8 (lf)* in *let-60 (gf)* mutants, suppressing the Muv phenotype.

Surprisingly, we found that removing functional *ras-2* from either *let-60 (gf)* or *let-23 (gf)* did not suppress the frequency of the Muv phenotype. However, I noticed protruding vulvas (Pvul) in *let-60 (gf)* that had not been reported before, and there was a significant increase in Pvul in the absence of *ras-2*. This suggests that RAS-2, unlike SUR-8, may not modulate LET-60 signal transduction and *C. elegans* vulval development. Even though Shoc-2 is a specific effector of activated M-Ras in mammals, the interaction of their orthologs in *C. elegans* may be different. In support of this hypothesis, SUR-8 was reported to interact with LET-60 in *C. elegans* (Sieburth et al., 1998). This is contradictory to the findings of Rodriguez-Viciana et al. in human cells, where no interaction of Shoc-2 with p21 Ras was observed (Rodriguez-Viciana et al., 2006).

Protruding vulva phenotype in *C. elegans* can arise due to defects in vulval morphogenesis. During *C. elegans* vulval morphogenesis, cell shape changes and movements are regulated by Rac GTPases. The double *loss-of-function* mutants of *C. elegans rac*-like genes *ced-10* and *mig-2* resulted in the Pvul phenotype (Lundquist et

al., 2001). This is due to aberrant cell migration during the vulval morphogenesis. Mammalian Rac proteins are involved in cytoskeleton organization and the cell migration. M-Ras is also known to participate in reorganization of actin cytoskeleton (Matsumoto et al., 1997). In mammalian cells, activated classical Ras leads to activation of Rho GTPase through the Raf/MEK/ERK pathway (Chen et al., 2003). Activated Rho is able to inhibit Rac proteins through the activation of Rac GAP and ARHGAP22 (Sanz-Moreno et al, 2008). The Pvul phenotype caused by the *let-60 (gf)* mutation can be explained as a result of aberrant vulval cell migration due to inhibition of Rac by activated Rho proteins. Lack of *ras-2* may impair the proper organization of the actin cytoskeleton in vulval cells and may contribute to inappropriate cell migration caused by inactivated *rac* proteins. Furthermore, activated M-Ras activates Rac-1 in mammalian cells (Grill & Schrader, 2002). Thus, it is conceivable that the lack of *ras-2* may enhance the Pvul phenotype in *let-60 (gf)* mutants via inhibition of Rac signaling. A mutation in human Shoc-2 which causes N-myristoylation and brings it permanently to the cell membrane resulted in protruding vulva phenotype when expressed in *C. elegans* (Cordeddu et al., 2009).

4.4 Summary and future directions

We show here for the first time the expression patterns of *ras-2* in *C. elegans* and the findings of phenotypic characterization of *ras-2* null worms. We report here for the first time that *ras-2* null worms are smaller in body size and exhibit faster movements than wild-type worms. Furthermore, our analyses demonstrated that the *ras-2* null

background did not suppress the multivulval phenotype caused by activated *let-60*. However, activated *let-60* in a *ras-2* null background increased the frequency of a protruding vulva phenotype.

The findings in this thesis outlined some of the possible directions for further investigation of the function of M-Ras/RAS-2. It is important to understand the mechanisms underlying the novel phenotypes that we found in *ras-2* null worms. One strategy to begin these follow up studies would be to rescue the phenotypes due to loss of *ras-2,* through exogenous expression of WT RAS-2 in *ras-2* null worms. This would allow us to confirm that these phenotypes are solely due to lack of *ras-2*. It would be interesting to know the underlying signaling pathways that cause these physiological outcomes in *ras-2* null worms. One approach for this could be investigation of the mutants that show the same phenotypes as *ras-2* worms and identify the signaling defects in those mutants. Revealing the signaling cascades in *C. elegans* that RAS-2 might impinge on could provide a framework for future experiments on mammalian M-Ras.

It would also be interesting to understand the pheromone mediated sensory perception and the social interaction of *ras-2* null worms in detail. Determination of the effectiveness of *ras-2* null worms to enter dauer formation in response to ascaroides will provide a better insight of pheromone sensation.

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