EXPLORE THE FUNCTION OF KEL-8 IN OXIDATIVE STRESS RESPONSE AND SEARCH FOR KEL-8 INTERACTING PROTEINS

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ABSTRACT OF THE THESIS

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Previous work in our lab identified KEL-8, a BTB-Kelch superfamily protein, as a substrate receptor for a Cul3 ubiquitin ligase that plays a role in the turnover of GLR-1 AMPA-type glutamate receptors in neurons. *C. elegans kel-8* is similar in sequence to Keap1, which encodes a BTB-Kelch protein that negatively regulates the oxidative stress response pathway, leading us to hypothesize that *kel-8* is also involved in oxidative stress response, repressing the expression of oxidative stress response genes. We have two major questions: what is the role of KEL-8 in oxidative stress and what are the targets of KEL-8. In order to answer the first question, we used the oxidative stress reporter transgene *Pgcs-1::gfp*. The *gcs-1* gene encodes a phase Ⅱ detoxification enzyme, γ－glutamine cysteine synthase, and is expressed in the intestine in response to oxidative stress reagents. I tested the expression level of *Pgcs-1::gfp* in *kel-8(od38)* nonsense mutants and found that the *gcs-1* expression level in *kel-8* is constitutively high compared to wild-type animals. Our preliminary results indicate that KEL-8 functions similarly to Keap1 to repress the

oxidative stress response. However, the repression phenotype is inconsistent. In addition, we did a yeast two-hybrid screen to search for targets or other interacting proteins of KEL-8, and we found 8 candidates. They are ZC434.8, *ric-4*, F32D8.12, *pccb-1*, *ssq-4*, *dim-1*, *aly-3*, and *smo-1*. Further analyses need to be done to determine whether these are biological meaningful interactions.

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Chapter 1. General Introduction

Section A. AMPA-type glutamate receptor

AMPA-type glutamate receptors are important for learning and memory.

Glutamate is one of the major excitatory neurotransmitters in the brain. Ionotropic glutamate receptors are located at the post-synaptic face of synapses, and are involved in synaptic plasticity, which is important in learning and memory (Malenka and Nicoll, 1999; Meldrum, 2000).

There are three types of glutamate receptors according to their different agonists: the N-methyl-D-aspartate (NMDA), alpha amino 3-hydroxy 5-methyl 4-isoxazolepropionic acid (AMPA), and kainate receptors (Dingledine *et al.*, 1999). The AMPA receptors (AMPARs) are tetrameric channels (Mano and Teichberg, 1998). The AMPAR is made up of four subunits in the mammalian system: GluR1-4. Coimmunoprecipitation from CA1/CA2 hippocampal pyramidal neurons showed that the subunit composition of the receptor complex can contain GluR1/GluR2 or GluR2/GluR3 heteromers, with a small fraction of GluR1 homomers (Wenthold *et al.*, 1996). Each subunit is composed of an extracellular N-terminal domain, four highly conserved hydrophobic transmembrane domains (TM1-4), and a cytosolic tail region. The C-terminal cytosolic region of each subunit contains a PDZ-binding domain which can interact with PDZ proteins, many of which are involved in receptor trafficking and synaptic plasticity (Henley, 2003; Malenka,

2003).

Multiple factors regulate AMPA receptor localization to mammalian synapses

Various factors, like phosphorylation, AMPAR-interacting proteins that modulate trafficking of AMPARs, the cytoskeleton, and several signaling pathways could affect the localization of AMPA receptors (Derkach *et al.*, 2007; Greger and Esteban, 2007). The regulation of AMPAR localization is a critical mechanism to modulate neuronal synaptic strength (Bredt and Nicoll, 2003; Malenka, 2003).

GluR1 has two important regulatory phosphorylation sites: serine residue (Ser) 831 and Ser 845. Mice with knock in mutations in these two sites show deficits in long-term potentiation (LTP), long-term depression (LTD), and memory defects in spatial learning tasks (Lee *et al.*, 2003). LTP and LTD are two forms of long enduring synaptic plasticity. LTP induction in the hippocampus is accomplished by applying brief trains of rhythmic high frequency stimulations to excitatory axons that connect to hippocampal neurons (Brown *et al.*, 1990). The result of LTP induction is an increase in the synaptic transmission between two paired neurons. LTD can be induced by pairing the low frequency activity of the climbing fiber and the parallel fiber (Ito, 1989). LTD can also be chemically induced in hippocampal neurons. The result of LTD induction is a decrease in synaptic transmission between pairs of neurons. The phosphorylation and cell

trafficking of AMPARs is thought to underlie many forms of both LTP and LTD.

Phosphorylation of Ser831 in the cytosolic C terminus of GluR1 by calcium/ calmodulin (CaM)-dependent protein kinase II has been shown to significantly increase the single-channel conductance in homomeric GluR1 but not heteromeric GluR1/GluR2 (Derkach *et al.*, 1999; Oh and Derkach, 2005). As GluR1 single-channel conductance increases during LTP (Benke *et al.*, 1998; Luthi *et al.*, 2004) and when CaMKII is constitutively active (Poncer *et al.*, 2002), the CaMKII dependent phosphorylation is thought to contribute to LTP. Phosphorylation of Ser845 by protein kinase A (PKA) increases open probability of GluR1 (Banke *et al.*, 2000) but not GluR1/GluR2, and has been implicated in trafficking of AMPARs (Esteban *et al.*, 2003; Oh and Derkach, 2005; Sun *et al.*, 2005) and in synaptic plasticity (Lee *et al.*, 2000; Esteban *et al.*, 2003; Lee *et al.*, 2003) It has been suggested that CaMKII functions through the trafficking of AMPA receptors. (Rongo, 2002)

Recent findings have revealed a role for small G proteins in the regulation of GluRs. It has been shown that in the hippocampus, the Ras family G proteins, which act through the MAPK ERK, are involved in activity-dependent synaptic incorporation of AMPARs and LTP induction. Ras-related proteins 1 (RAP1)-mediated activation of another MAPK, p38, is required for LTD (Zhu *et al.*, 2002; Zhu *et al.*, 2005). It was also found that another small GTPase, RAB5, was involved in removing AMPARs from synapses during

LTD, but not during constitutive recycling, in a clathrin-dependent manner (Brown *et al.*, 2005). There is still a lot unknown about how AMPA receptors are regulated in vivo.

AMPA-type glutamate receptors in C. elegans.

Our lab studies the trafficking of GLR-1 AMPA-type glutamate receptor subunit in *C. elegans*. The idea of using *C. elegans* as a model system was initiated by Sydney Brenner in the 1960s. *C. elegans* has many advantages as a model system. It has a short life (3-day), small size (1.5-mm-long as adult), and ease of laboratory cultivation. The worm is transparent during the whole life span thus is easy to visualize. Every cell division from fertilized egg to adult worm has been charted, and every point at which a cell adopts a specialized role has been identified. In addition, the complete connectivity of the 302 cells that form the nervous system of the worm has been mapped.

GLR-1 is expressed in the interneurons of *C. elegans*. A mutation in the *glr-1* gene eliminates the nose touch response but not the osmotic response (Hart *et al.*, 1995; Maricq *et al.*, 1995; Zheng *et al.*, 1999). GLR-1 is visualized by chimeric expression of an integrated *glr-1::gfp* fusion gene in living animals (Rongo *et al.*, 1998; Rongo and Kaplan, 1999). GLR-1::GFP is localized postsynaptically in clusters in the nerve ring and ventral nerve cord (Rongo *et al.*, 1998; Rongo and Kaplan, 1999; Burbea *et al.*, 2002).

Genes identified that regulate GLR-1 trafficking

The abundance of GLR-1 in the synapses is regulated by ubiquitination and subsequent endocytosis (Burbea *et al.*, 2002; Juo and Kaplan, 2004). Mutants with decreased ubiquitination have increased amount of GLR-1 at synapses as well as increased synaptic strength, while mutants with increased ubiquitination have decreased abundance of GLR-1 and decreased synaptic strength. These effects can be eliminated by mutations in *unc-11* gene, which encodes a clathrin adaptor protein (AP180) (Burbea *et al.*, 2002; Juo and Kaplan, 2004).

As CaMKII regulates synaptic strength in mammals (Mayford *et al.*, 1995), the CaMKII protein in *C. elegans*, UNC-43, is required for GLR-1 synaptic density and trafficking (Rongo and Kaplan, 1999). There are two mechanisms by which CaMKII regulates GLR-1: regulating transport of GLR-1 from the cell body to neurites and regulating removal at post synaptic sites (Rongo and Kaplan, 1999). The localization of GLR-1 to the clusters in synapses is also dependent on a PDZ-domain containing protein, LIN-10 (Rongo *et al.*, 1998; Rongo and Kaplan, 1999). Mutations in the carboxyl-terminal PDZ domain prevent LIN-10 from regulating GLR-1 localization (Glodowski *et al.*, 2005). While a mutation in the amino terminus does not prevent LIN-10 from functioning to regulate GLR-1 localization in neurons, it prevents LIN-10 from working in the vulval epithelia, where it regulates the trafficking of the EGF receptor LET-23.

RAB-10, a small GTPase required for endocytic recycling (Chen *et al.*, 2006), has been shown to function to regulate GLR-1 localization in synapses as well (Glodowski et al., 2007). Mutants that lack RAB-10 show a similar phenotype with *lin-10* (Glodowski et al., 2007). RAB-10 has been implicated to work in a clathrin-independent manner, while LIN-10 works in a clathrin-dependent way (Glodowski *et al.*, 2007).

RPM-1, the ubiquitin ligase which regulates p38 MAP kinase in presynaptic development (Nakata *et al.*, 2005), is also suggested to regulate GLR-1 trafficking postsynaptically. Mutations in *rpm-1* cause the accumulation of GLR-1 in neurites (Park *et al.*, 2009). Moreover, *rpm-1* mutations enhance the endosomal accumulation of GLR-1 observed in mutants for *lin-10* mutants.

KEL-8 regulates GLR-1 turnover.

Previous work in our lab has found that KEL-8 functions as a substrate receptor for Cullin 3 ubiquitin ligases and is expressed in neurons, where it clusters in ventral nerve cord dendrites (Schaefer and Rongo, 2006). KEL-8 plays a role in the turnover of GLR-1 AMPA-type glutamate receptors in neurons. GLR-1::GFP accumulates at postsynaptic clusters in *kel-8* mutants, and could be rescued by cosmid W02G9 containing the *kel-8* locus or cell autonomously with a *Pglr-1::kel-8* transgene containing wild-type *kel-8* cDNA fused to the *glr-1* promoter (Schaefer and Rongo, 2006). However, we do not

know whether KEL-8 has other targets and other functions other than GLR-1 trafficking.

Section B. Kelch domain proteins and the Ubiquitin Proteasome System

Ubiquitin and Ubiquitination Enzymes

Ubiquitination is used to regulate protein turnover and is involved in regulating various biological functions. Ubiquitination requires participation of three enzymes: ubiquitin-activating enzyme, or E1, ubiquitin-conjugating enzyme, or E2, and ubiquitin-protein ligase, or E3. Ubiquitin first forms a link with E1 and become activated, then gets shuffled to E2, and finally gets transferred to target proteins by E3. Of those enzymes, it is the E3 ligase that recognizes the target protein.

There are about fifty E2 enzymes in mammals, and more than 1000 various E3 proteins that form complexes with specific E2 enzymes. Different E2-E3 complexes recognize distinct signals, thus target various proteins for degradation.

The Proteasome

Ubiquitinated proteins are digested by the 26S proteasome. The proteasome is a multi-protein complex found in the cytosol and nucleus. The proteasome consists of two compartments: the 20S catalytic subunit and the 19S regulatory subunit. The 20S complex consists of four rings of seven subunits that stack to form a barrel structure in

which the substrates are degraded into peptides of seven to nine residues. The 19S complex has two subunits that bind to both ends of 20S complex. The 19S complex binds specifically to polyubiquitin chains and cleaves off ubiquitin molecules. The 19S subunits also unfold the substrates and causes conformational changes of the 20S complex so that substrates can enter the center of 20S proteasome.

Two classes of Ubiquitin Ligases: HECT and RING

Out of 1000 various E3 proteins, mechanistically there are two classes of E3 ubiquitin ligases: HECT domain E3s, which use a covalent mechanism, and RING domain E3s, which do not. HECT domain E3s bind E2s through the conserved HECT domain and transiently binds ubiquitin via a cysteine in this region, while substrate binds to a different region of E3s. Ring domain E3s binds ubiquitin through the ring domain and binds E2s using a different domain (Pickart, 2004).

BTB domain proteins interact with Cullin RING proteins

Cullin RING ligases are a subgroup of RING ligases that consist of a RING protein that recruits E2s, a Cullin scaffold protein, and a substrate receptor. Sometimes there is also an adaptor between the substrate receptor and Cullin (Willems *et al.*, 2004). There are seven kinds of Cullin ring ligases (CRL1-7), and each family can assemble with various substrate receptors. For example, CRL1 (or SCF) consists of CUL3 bound to an F-box-containing substrate receptor, CRL2 is comprised of CUL2 bound to a

SOCS/BC-box-containing substrate receptor. CRL3s recruit substrates by binding to 'Broad complex, Tramtrack, Bric-a-brac' (BTB) proteins, which are substrate receptors (Petroski and Deshaies, 2005).

The BTB-Kelch family of proteins

The Kelch motif was found as a repeated element in Drosophila Kelch ORF1 protein, typically four to seven repeats form a beta-propeller tertiary structure in Kelch repeat domain (Xue and Cooley, 1993). Drosophila KELCH protein is localized to actin-rich ring canals and regulates the flow of cytoplasm between cells. (Xue and Cooley, 1993; Robinson and Cooley, 1997) The Kelch motif is ancient and evolutionarily widespread. The Kelch motif contains 44-56 amino acids in length. The sequence identity between different Kelch motifs is low, but multiple sequence alignment shows eight conserved residues.(Xue and Cooley, 1993; Bork and Doolittle, 1994) The Kelch motif contains multiple potential protein-protein interaction sites and many specific binding partners have been identified. (Adams *et al.*, 2000)

The localizations of Kelch domain proteins vary from intracellular compartments, cell surface, to the extracellular milieu. As expected from their diverse localization, Kelch domain proteins have various functions.

There are 125 BTB-Kelch proteins in humans and 10 in *C. elegans*. Several BTB-Kelch

proteins have been found to interact with Cul3 (Stogios and Prive, 2004). Most Cullin-RING ligases play a role in cell division (Willems *et al.*, 2004; Petroski and Deshaies, 2005). An exception is KEAP1, a BTB-Kelch protein that binds to NRF2 and regulates oxidative stress response.

Section C. Oxidative stress

Keap1 and Nrf2 are involved in oxidative stress response in vertebrates.

Around 2–3% of the oxygen atoms taken up by the mitochondria are reduced insufficiently to reactive oxygen species (ROS). These ROS include hydrogen peroxide, the superoxide ion, and the hydroxyl radical. ROS can oxidize cell membranes, proteins, and nucleic acids, resulting in cellular damage. Cells remove toxic compounds by phase I and phase II enzymes. The P450 isozyme system is the major agent of phase I metabolism in mammalian cells. Phase I reactions result in the addition of chemically reactive, oxygen-containing functional groups, which allow further metabolism of otherwise inactive compounds. The expression of Phase I enzymes like P450 is induced by a host of foreign chemicals, as well as endogenous factors like growth hormone. Phase I reactions are often required for phase II. Phase II enzymes act on oxidized substrates. Some phase II enzymes are methyltransferases, acetyltransferases, glutathione transferases, uridine 5′-diphosphoglucuronosyl transferases, sulfotransferases, nicotinamide adenine dinucleotide (NAD)- and nicotinamide adenine dinucleotide

phosphate (NADP)-dependent alcohol dehydrogenases, quinone reductases, NADPH diaphorase, azoreductases, transaminases, and hydrolases. However, how oxidative stress is sensed, and how the response pathways are turned on, is not well understood.

In vertebrates, studies on the oxidative stress response pathway have focused on transcriptional regulation. NF-E2-related factor 2 (Nrf2), a member of the Cap'n'Collar family of basic region-leucine zipper (bZIP) transcription factors (Igarashi et al., 1994; Moi et al., 1994), forms a heterodimer with small Maf proteins (Motohashi et al., 1997). The heterodimer binds to a consensus ARE core sequence, and induces expression of phase II detoxification enzyme genes (Itoh *et al.*, 1997; Alam *et al.*, 1999; Nguyen *et al.*, 2000; Wild and Mulcahy, 2000). One theory of the mechanism by which Keap1 regulates Nrf2 is that several key cysteines in Keap1 have been shown to be critical for the repression of Nrf2. The function of individual cysteines has been tested by mutating them to serines. Keap1-C151S has been shown to be a constitutive repressor of Nrf2 dependent reporter gene expression, whereas Keap1-C273S and Keap1-C288S have impaired ability to repress Nrf2 dependent reporter gene expression (Zhang and Hannink, 2003). But whether Keap1 senses ROS signal directly or some other factors act as sensors and regulate Keap1 function is unclear.

SKN-1, an ortholog of Nrf2, works in oxidative stress response in *C. elegans*.

In *C. elegans*, the transcription factor SKN-1 is distantly related to the same bZIP protein

subgroup as Nrf2. SKN-1 acts as a monomer through a basic region (BR) similar to that of bZIP proteins, which are obligate dimmers (Blackwell et al., 1994). SKN-1 was originally found in a screen for genes that are required maternally for formation of pharyngeal tissue(Bowerman et al., 1992), but later it was shown to have a function in triggering oxidative stress response genes (An and Blackwell, 2003; Inoue et al., 2005). The p38 mitogen-activated protein kinase PMK-1 regulates the oxidative stress pathway through the transcription factor SKN-1. In response to oxidative stress, PMK-1 phosphorylates SKN-1, leading SKN-1 to accumulate in intestine nuclei.

SKN-1 regulates the transcription of phase II detoxification genes, including GCS-1.

Translocated SKN-1 triggers the transcription of several phase II detoxification enzymes, including GCS-1, which is γ-glutamine cysteine synthase heavy chain (An and Blackwell, 2003; Inoue et al., 2005). It is yet not clear what other gene transcription SKN-1 triggers, although it has been shown that SKN-1 can bind to the consensus sequence of promoter regions for several phase II genes (An and Blackwell, 2003). It is not known that whether there is an equivalent protein to Keap1 in *C. elegans*. The presence of a Keap1 homolog in *C. elegans* could allow further investigation into the mechanisms of oxidative stress sensation using powerful genetic tools in an intact, in vivo system.

One possible candidate is KEL-8. KEL-8, as previously described, is a BTB-Kelch superfamily protein, which is most similar to human Kelch-like 8 (KHL8), contains six

Kelch repeats and an N term BTB domain (Figure 1). Many BTB proteins act as substrate adaptors and assemble with the E3 ubiquitin ligase CDL-3 (Cullin-dependent ligase) (Deshaies, 1999; Joazeiro and Weissman, 2000; Pintard et al., 2003; Xu et al., 2003). Kelch repeats are protein-protein interaction domains, and Kelch proteins interact with varieties of other proteins such as actin cytoskeleton (Adams et al., 2000). Among about 10 BTB-Kelch proteins in *C. elegans*, KEL-8 has the most similarities with KEAP1. KEL-8 also has many cysteines in its BTB and linker domain like KEAP1.

The regulation of the oxidative stress response might be critical to understand the process of aging. The oxidative damage theory of aging says that an increased oxidative stress response contributes to longevity, whereas a decreased response results in shortened lifespan (Brys et al., 2007). For example, worms with mutations that increase the synthesis of ROS-degrading enzymes live much longer than wild-type nematodes (Vanfleteren and De Vreese, 1996). By contrast, *skn-1* mutant worms, which cannot activate the stress response pathway, have shortened lifespan compared to wild-type worms (An and Blackwell, 2003). The oxidative damage theory of aging posits that ROS and the oxidative stress response regulate aging in a cell autonomous fashion. However, recent studies show that dietary restriction activates *skn-1* in ASI neurons, which promotes cell non-autonomous signaling to the peripheral tissues that increase respiration and lifespan (Bishop and Guarente, 2007). Thus, the role of SKN-1 in aging is likely to be more complex than simply turning on the expression of oxidative stress

response genes to deal with cell-autonomous ROS-induced damage. Further complicating our understanding of oxidative stress and aging, there is evidence suggesting that glucose restriction induces mitochondrial respiration, resulting in short-term stress (ROS), which increases catalase activity, long-term stress resistance and extended lifespan (Schulz *et al.*, 2007). Thus, the quantity of ROS, ROS type, and length of exposure of cells to ROS might decide whether oxidative stress limits or extends lifespan; the exact role of ROS in aging remains controversial.

In addition to the role of oxidative stress in aging, numerous other studies have shown that the insulin/IGF pathway, mitochondrial mechanisms, dietary restriction, SIR2 deacetylase activity, TOR and TUBBY signaling, and telomere length contribution are involved in aging (Kenyon, 2005; Schaffitzel and Hertweck, 2006). Insulin/IGF pathway is the most well studied pathway that regulates aging. In the insulin/IGF pathway, AGE-1 is a homolog of the p110 catalytic subunit of phosphoinositide 3-kinase (PI3K) (Morris et al., 1996), whereas DAF-2 is an insulin/insulin-like growth factor 1 receptor (I/IGF-1R) homolog and functions in the same pathway as AGE-1(Kimura et al., 1997). AGE-1 and DAF-2 work through downstream factors to repress a FOXO transcription factor DAF-16, which activates various response genes (Kenyon et al., 1993). There is also evidence suggesting that AKT-2, a homolog of the serine/threonine kinase Akt/PKB, is also in the I/IGF-1 pathway, downstream of AGE-1. Interestingly, AKT-2 phosphorylates and prevents the translocation of SKN-1 into nuclei (Blackwell lab, 16th

International *C. elegans* meeting); thus, one of the effective outputs of the Insulin/IGF pathway might be to regulate the oxidative stress response.

We hypothesized that KEL-8 might function as the Keap1 homolog in *C. elegans.* Specifically, we reasoned that KEL-8 might repress the activation of the oxidative stress response genes by ubiquitinating one or more components of the signaling pathway that activates response pathway expression. As *kel-8* mutants would be expected to constitutively express the oxidative stress response pathway, we reasoned that these mutants would have lower levels of cellular ROS, perhaps resulting in an extension of their lifespan.

Section D. P38 MAP kinase

The p38 MAPKs play an important role in adaptation, homeostasis, immune response and specialized stress responses.

In addition to Keap1 and Nrf2, the p38 MAP kinases are also involved in oxidative stress response. The p38 MAP Kinases are highly conserved in evolution and have also been shown to regulate cellular responses to various stimulations including mitogens, inflammatory cytokines, and UV irradiation (Davis, 2000; Harper and LoGrasso, 2001). The downstream targets include transcription factors, kinases, and pathways influencing pro-inflammatory cytokines (Harper and LoGrasso, 2001).

The p38 mitogen-activated protein kinase PMK-1 regulates the oxidative stress pathway through the transcription factor SKN-1.

ROS can also activate MAP kinase (MAPK) signaling pathways. For example, in vertebrates oxidative stress can activate MAP kinase kinase kinase 1 (MEKK1), transforming growth factor-β-activated kinase (TAK1), and apoptosis signal-regulating kinase (ASK1), resulting in the induction of ARE reporter genes, which is mediated by an Nrf2-dependent mechanism (Yu *et al.*, 2000). In mammals, there are more than a dozen MAPK genes. The best known are the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK(1–3)) and p38(α, β, γ and δ) families. ASK1 is required for oxidative stress-induced sustained activations of JNK/p38 (Tobiume *et al.*, 2001; Hsieh and Papaconstantinou, 2006). Inhibition of MAP kinase p38 inhibited the arsenic-induced Nrf2 accumulation (Tobiume *et al.*, 2001; Pi *et al.*, 2007). In *C. elegans*, as we just mentioned, the p38 mitogen-activated protein kinase PMK-1 phosphorylates SKN-1 in response to oxidative stress, leading SKN-1 to accumulate in intestine nuclei.

PMK-1 may interact with KEL-8 according to a yeast two-hybrid screen.

As shown by a two yeast hybrid screen by the Vidal lab, PMK-1 could interact with KEL-8 when PMK-1 was fused with bait protein and KEL-8 fused with prey protein (Li *et al.*, 2004), leading us to wonder whether KEL-8 has a role in oxidative stress, or PMK-1 has a role in regulating GLR-1 trafficking.

Summary

The aim of this thesis is to discuss the role of KEL-8 in oxidative stress response and to find out what the targets of KEL-8 are. Our hypothesis is that KEL-8 functions in oxidative stress response possibly by regulating PMK-1 ubiquitination. Chapter 2 describes the expression of an oxidative stress response reporter gene *Pgcs-1::gfp* in *kel-8* mutants, the lifespan phenotype of *kel-8*, and the expression and localization of PMK-1 in *kel-8* mutants. Chapter 3 describes a yeast two-hybrid screen to find interacting proteins of KEL-8 and 8 possible candidates identified in the screen.

Chapter 2. Explore the Function of KEL-8 in Oxidative Stress Response.

Section A. Abstract

Previous work in our lab showed that KEL-8, a BTB-Kelch superfamily protein, plays a role in the turnover of GLR-1 AMPA-type glutamate receptors in neurons (Schaefer and Rongo, 2006). Nrf2, a transcription factor as previously described, regulates the transcription of oxidative stress response genes and is suppressed by Keap1 in vertebrates. SKN-1 is distantly related to Nrf proteins, and *kel-8* is similar in sequence to Keap1, leading us to hypothesize that *kel-8* is also involved in oxidative stress response and represses the expression of oxidative stress response genes. In order to test this hypothesis, we used the oxidative stress reporter transgene *Pgcs-1::gfp* (An and Blackwell, 2003; Inoue et al., 2005). I tested the expression level of *Pgcs-1::gfp* in *kel-8(od38)* nonsense mutants and found that *Pgcs-1::gfp* expression level in *kel-8* mutants is constitutively high compared to wild-type animals. Our preliminary results indicate that KEL-8 functions similarly to Keap1 to repress the oxidative stress response. However, after further examination, we found that the suppression of oxidative stress response by KEL-8 is not consistent.

Section B. Introduction

Oxidative stress from the production of reactive oxygen species (ROS) is implicated in cardiovascular and neurodegenerative disease (Ames and Shigenaga, 1992; Butterfield

and Kanski, 2001; Golden et al., 2002; Rao and Balachandran, 2002). Oxidative stress has also been implicated in aging, although its specific effect on aging is controversial. Cells respond to oxidative stress by expressing proteins that detoxify ROS and/or repair cellular damage. The machinery for activating this oxidative stress response is not fully understood. In addition, the mechanism for how oxidative stress is sensed still needs to be addressed. Finally, it is important to know whether the factors that sense and respond to oxidative stress in turn influence aging and healthspan.

In vertebrates, the bZIP transcription factor, Nrf2, induces basal and ROS-inducible expression of phase II detoxification enzyme genes, which detoxify ROS and repair cellular damage (Zhang et al., 2004; Zhang et al., 2005). Keap1, an upstream regulator of Nrf2, is a BTB-Kelch protein that functions as a substrate adaptor for a Cul3-dependent E3 ubiquitin ligase complex. Keap1 targets Nrf2 for ubiquitination and subsequent degradation, and oxidative stress inactivates Keap1, allowing for the expression of the phase II enzymes (Zhang et al., 2004; Zhang et al., 2005). In *C. elegans*, the transcription regulator SKN-1, an ortholog of Nrf2, is phosphorylated and activated by PMK-1, a p38 mitogen-activated protein kinase, under oxidative stress. The phosphorylated SKN-1 translocates to the nuclei and activates the transcription of phase II detoxification genes, including GCS-1, the γ-glutamine cysteine synthase heavy chain. (An and Blackwell, 2003; Inoue et al., 2005). While Keap1 might directly sense oxidative stress and ubiquitinate Nrf2 in vertebrates, it is uncertain whether an equivalent E3 ligase senses

oxidative stress and ubiquitinates SKN-1 in *C. elegans*. Interestingly, SKN-1 has also been implicated in aging in *C. elegans*, where it functions in the ASI neuron to regulate lifespan extension in response to dietary restriction.

Section C. Materials and Methods

Strains

Standard methods were used to maintain *C. elegans* (Wood, 1988). Animals were grown at 20ºC on standard NGM plates seeded with OP50 *Escherichia coli* unless stated otherwise. Transgenic strains were generated by microinjecting various plasmids (typically at 50ng/ul) together with either *rol-6dm* (C. Mello) or pJM23 in *lin-15* (n765ts). The transgene *Pgcs-1::gfp* was a gift from T.K. Blackwell (An and Blackwell, 2003; Inoue et al., 2005). Strain *age-1(hx546)* was a gift from Monica Driscoll's lab. Strain *kel-8(od38)* was cloned and mapped previously by our lab (Schaefer and Rongo, 2006).

Molecular Biology

The *Pvha-6::pmk-1::gfp* construct was generated using *pmk-1* cDNA sequence (clone ID: B0218.3 from Open Biosystems). Full-length *pmk-1* cDNA was inserted into pDONR containing *vha-6* promoter and c terminal *GFP* (a gift from Barth Grant lab).

Fluorescent Microscopy

GFP and RFP tagged fluorescent proteins were visualized in nematodes by mounting L4 and young adults on 2% agarose pads with 10mM levamisole at room temperature. Fluorescent images were observed using a Zeiss Axioplan II and either a 100 × or 63 × (1.4NA PlanApo for both) objective, and imaged with an ORCA CCD camera (Hamamstsu, Bridgewater, NJ) using ImagePro version 4.1 (media Cybernetics, Silver Spring, MD) and VayTek version 6.2 software (VayTek, Fairfield, IA). Exposure times were chosen to fill the 12-bit dynamic range without saturation.

Arsenite assays

To determine the induction of *Pgcs-1::gfp* by arsenite stress, animals grown on normal plates were transferred to 1.5-mL test tubes in M9 buffer. Worms were incubated in M9 buffer with or without 5mM sodium arsenite for 30 minutes at room temperature. Worms were then transferred back to normal plates and allowed to recover for 3 hours before examination.

Lifespan Assays

Lifespan assays were conducted at 20ºC and 25ºC. The animals were cultured on standard agar plates. Day of birth was used as the first time point. Animals were moved to a new plate every few days to remove them from contamination of their progenies

during their reproductive period. Animals were considered dead when they ceased to move and stopped responding to prodding (Hsin and Kenyon, 1999). Wild type worms were used as negative control, and *age-1(hx546)* mutants, which have a longer lifespan than wild type animals, were used as positive control.

Section D. Results

***kel-8* mutants show enhanced expression of pGCS-1::GFP from an extrachromosomal array**

To begin to test the role of *kel-8* in the oxidative stress response, we used the oxidative stress reporter transgene *Pgcs-1::gfp* as an indicator of the stress response. The *gcs-1* gene encodes a phase II detoxification enzyme, γ-glutamine cysteine synthase heavy chain. *Pgcs-1::gfp* is constitutively expressed in the pharynx and ASI neurons, and is expressed in intestine in response to oxidative stress inducing agents. This inducible expression in the intestine requires *pmk-1* activity (An and Blackwell, 2003). In previous studies, heat shock, paraquat or sodium arsenite were employed to do oxidative stress assays (An and Blackwell, 2003; Inoue *et al.*, 2005). I tried all of these methods to confirm the expression pattern of *Pgcs-1::gfp* in wild-type worms as well as in *pmk-1* mutants. I found that all of these treatments gave the same results as reported in the previous literature. Of these approaches, I found the treatment of worms with 5M sodium arsenite for 1 hour gave the strongest and most consistent expression of the reporter.

Therefore, I used arsenite treatment for my subsequent oxidative stress assays.

Next, I tested the expression level of *Pgcs-1::gfp* in *kel-8(od38)* nonsense mutants and found that the basal expression level of *Pgcs-1::gfp* in *kel-8* mutants is constitutively high compared to wild type, in which there is little expression in the absence of stress (Figure 2). My results suggest that KEL-8 negatively regulates oxidative stress response under basal conditions.

kel-8 mutants have longer lifespan than wild type worms

Since lifespan is related to stress response (Brys et al., 2007), I tested the lifespan of *kel-8* worms. I synchronized the worms, and picked them to new plates each day or every other day to separate them from their progeny during their reproductive period. Then I recorded the lifespan of each worm. I used wild type worms as negative control, and *age-1* (hx546) mutants, which have a longer lifespan than wild type animals, as a positive control. I observed that *kel-8* mutants had a longer mean lifespan and maximal lifespan than wild type worms at 25ºC (Figure 3). However, *kel-8* mutants appeared to have similar lifespan with wild type worms at 20ºC (data not shown). The lifespan assay for 25ºC was repeated twice and the results of three assays were consistent.

PMK-1 expression level and localization is not changed in _kel-8_ mutants

In a yeast two hybrid screen done by the Vidal lab, PMK-1 clones were identified using

KEL-8 as bait (Li *et al.*, 2004). Interestingly, according to same the yeast two hybrid screen (Li *et al.*, 2004), several oxidative stress response genes such as dehydrogenase and glutathione S-transferase interact with KEL-8 as well. KEL-8 could alternatively directly target the oxidative response enzymes for ubiquitination and probably degradation.

To check whether KEL-8 interacts with PMK-1 in vivo, I made the construct of *pmk-1* cDNA under an intestine-specific promoter with a C terminal GFP tag. I then investigated the expression pattern and level of the construct in wild-type worms and in *kel-8* mutants. I made an integrated line of *Pvha-6::pmk-1::gfp* and crossed it with *kel-8* mutants. I found that the construct was expressed in the nuclei of intestinal cells. The *pmk-1* expression level was not different between *kel-8* mutants and in wild type background. This result suggests that KEL-8 may not directly target PMK-1 for ubiquitination and degradation (Figure 4). Alternatively, the interaction of KEL-8 and PMK-1 could be neuron specific.

kel-8 mutants do not show enhanced expression of integrated pGCS-1::GFP

To test whether KEL-8 regulates the oxidative stress response, I made integrated lines of *Pgcs-1::gfp* by X-ray irradiation or bombardment. I then crossed both lines with *kel-8* mutants. Neither of the integrated lines showed consistent expression of *Pgcs-1::gfp.* In a wild type background, only a small percentage of worms had consistent *Pgcs-1::gfp*

expression in the pharynx, while others did not have any expression of *Pgcs-1::gfp* in the pharynx. Also a very small fraction of worms appeared to have spontaneous *Pgcs-1::gfp* expression in the intestine without any stress. In addition, there is no clear difference between *kel-8* mutant background and wild type background. I also treated worms carrying the *Pgcs-1::gfp* transgene with *kel-8* RNAi and found no significant phenotype (data not shown).

Section E. Discussion

Why does the expression of *Pgcs-1::gfp* differ between the extrachromosomal array and the integrant? The variation of integrated *Pgcs-1::gfp* expression is possibly due to an incomplete promoter. The P*gcs-1*::*gfp* transgene contains 1840 bp upstream of the *gcs-1* start codon. It is possible that certain enhancers upstream of the promoter are missing in the transgene, thus leading to variations in pGCS-1::GFP expression. It also might be because I used *rol-6* as coinjection marker when I observed the phenotype at first and switched to *lin-15* marker when I made the integrated line.

There could be several possible reasons to explain why KEL-8 was found to interact with PMK-1 in the yeast two-hybrid screen done by the Vidal lab, but that PMK-1 expression levels and localization are not changed in *kel-8* mutants. It is possible that KEL-8 targets PMK-1 for ubiquitination but not degradation, which could be tested by a GST pull down assay. We could tag PMK-1 with GST, tag KEL-8 with FLAG, cotransfect them into

COS7 cells, do a GST pull down assay, and detect bound proteins by Western blot using specific antibodies that recognizes KEL-8. It is also possible that KEL-8 does not interact with PMK-1 in vivo because yeast two-hybrid screens can give false positive results. I did a yeast two-hybrid assay to see whether there is interaction between KEL-8 and PMK-1 and found that the interaction is very weak, if any (data shown in chapter 3).

Another question is why *kel-8* mutants have longer lifespan but lack consistent enhanced pGCS-1::GFP expression. One explanation is that KEL-8 regulates lifespan through other oxidative stress response genes. GST-43 (Glutathione S-Transferase) and glutathione transferase zeta-1 encoded by D1053.1 turned out to interact with KEL-8 in the yeast two hybrid screen by Vidal lab (Li *et al.*, 2004). We could construct GFP tagged reporters of these genes and examine their expression pattern in wild type worms and in *kel-8* mutants to test this theory. Alternatively, KEL-8 could have an effect on lifespan that is not through directly regulation of oxidative stress response genes. It is possible that KEL-8 functions in other pathways that regulate lifespan. For example, KEL-8 could work through the Insulin/IGF-1 pathway to regulate lifespan. We could test this by making double mutants of *kel-8* and *age-1* or *daf-2* which functions in Insulin/IGF-1 pathway.

It has also been suggested that increased ROS and other stresses might trigger a positive response and lead to reduced net stress levels and possibly extended lifespan (Johnson *et*

al., 2002; Sinclair, 2005). Since KEL-8 is an E3 ligase, the stress level of *kel-8* mutants could be higher than that of wild type, which might leads to overexpression of detoxification genes and longevity.

Section F. Figures

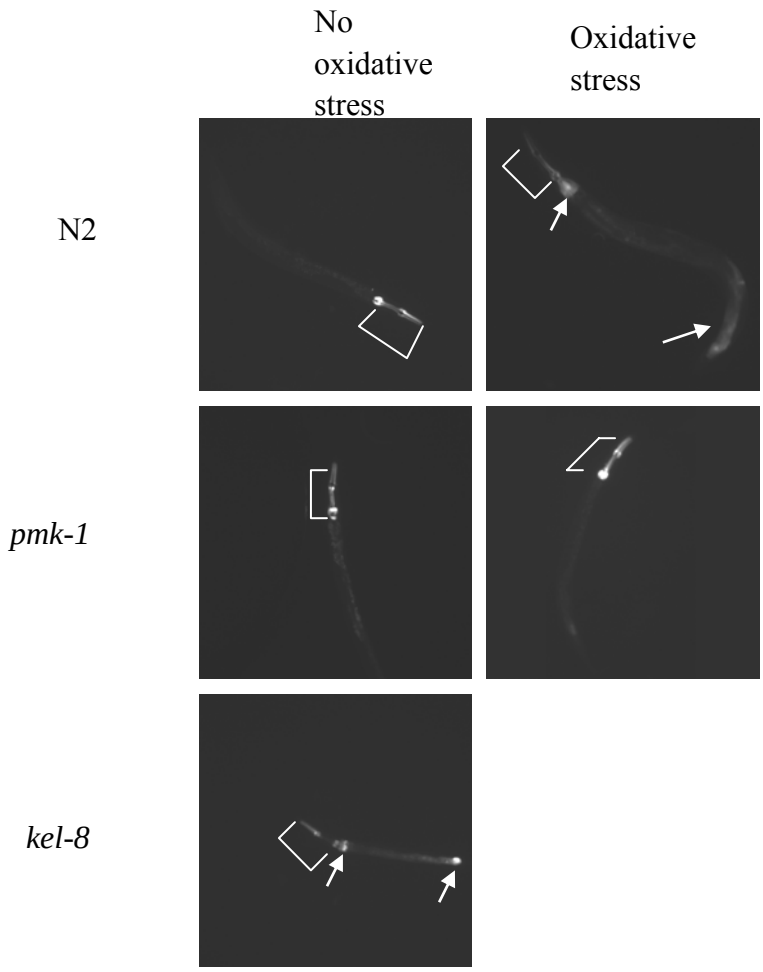

No oxidative stress
Oxidative stress
N2
pmk-1
kel-8

Figure 1. The expression level of pGCS-1::GFP in the intestine of *kel-8* is constitutively high. PGCS-1::GFP is continuously expressed in the pharynx (bars). In wild type worms, PGCS-1::GFP expression is barely detectable in the intestine without oxidative stress while it is expressed after stress (arrows). In *pmk-1* mutant worms, PGCS-1::GFP expression level is still low, if any, in the intestine even after oxidative stress. In *kel-8* mutant worms, PGCS-1::GFP level is high even without oxidative stress.

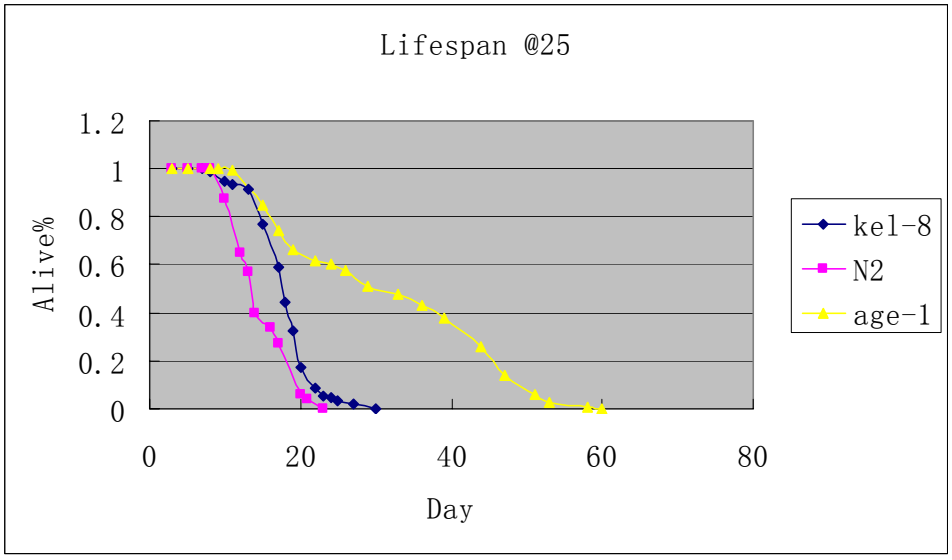

Lifespan @25
1.2
1
0.8
0.6
0.4
0.2
0
Alive%
0
20
40
60
80
Day
kel-8
N2
age-1

Figure 2. Lifespan of N2, *kel-8* and *age-1* at 25℃. *Kel-8* mutant worms have a longer lifespan than N2. Since many factors can influence the lifespan of worms, the assay was repeated twice to confirm.

odIs47 *kel-8*; *odIs47*

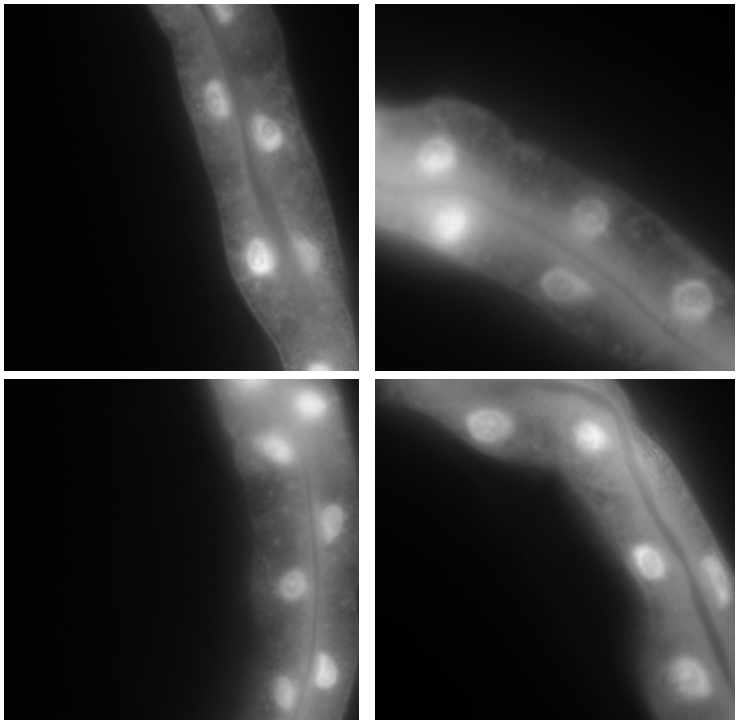


Figure 3. Expression pattern of integrated *Pvha-6::pmk-1::gfp* (*odIs47*) in both wild type and *kel-8* mutant background. The left two panels are *Pvha-6::pmk-1::gfp* (*odIs47*)**,** and the right two panels are *kel-8*, *Pvha-6::pmk-1::gfp* (*odIs47*). The expression level and localization of *Pvha-6::pmk-1::gfp* shows no difference between wild type worms and *kel-8* mutant background.

Chapter 3. Search for KEL-8 Interacting Proteins by Yeast Two-Hybrid Screening.

Section A. Abstract

In order to better understand the mechanism by which KEL-8 regulates GLR-1 turnover and explore other functions of KEL-8, we conducted a yeast two-hybrid screen to search for KEL-8 interacting proteins. From the screen 8 candidates were found. They are ZC434.8, *ric-4*, F32D8.12, *pccb-1*, *ssq-4*, *dim-1*, *aly-3*, and *smo-1*. Further analysis will need to be done to determine whether there is a biological meaning for these interactions.

Section B. Introduction

The yeast two-hybrid system is a powerful tool to detect protein-protein interactions and to look for novel interactors of a protein of interest. The target protein is fused to a DNA-binding domain that localizes it to the regulatory region of a reporter gene as bait, while the binding partner is fused with a DNA-activating domain as prey. When these two target proteins interact in the yeast cell nucleus, they bring two halves of a transcriptional activator together, which then triggers the expression of the reporter gene. The reporter gene is often one that will permit growth on selective media. Bait and prey fusion proteins are generated by standard cloning techniques. In screening cases, a single bait protein of interest is used to fish for interacting partners among a large collection of prey from a library generated by ligating DNA encoding the activation domain of a

transcriptional activator to a large mixture of DNA fragments from a cDNA library (Figure 4).

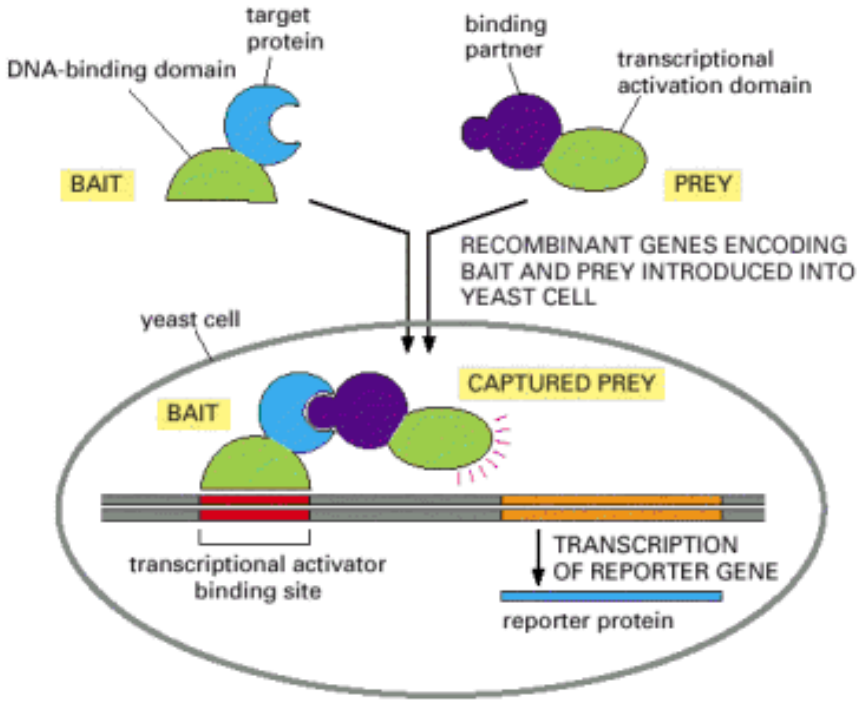


Figure 4. Illustration of yeast two-hybrid system. The target protein is fused to a DNA-binding domain that localizes it to the regulatory region of a reporter gene as bait, while the binding partner is fused with a DNA-activating domain as prey. When these two target proteins interact in the yeast cell nucleus, they bring two halves of a transcriptional activator together, which then triggers the expression of the reporter gene

We used DuPLEX-A™ system in our screen, which is a LexA-based version of the yeast two hybrid system originally developed by Fields and Song (Fields and Song, 1989). The DuPLEX-A system was a more versatile and accurate yeast two-hybrid system (Gyuris *et al.*, 1993).

The yeast two-hybrid system of Fields and Song uses the DNA binding domain and activation domain of a yeast transcriptional activator protein GAL4 to generate the bait and prey protein, respectively. The DuPLEX-A™ system utilizes DNA binding protein LexA from Escherichia coli and the acid blob activation domain of B42. Neither one of them is able to activate transcription of the reporter. When brought together, they can trigger reporter gene expression (Gyuris *et al.*, 1993).

The advantages of DuPLEX-A™ system over other yeast two-hybrid system are as follows:

- It reduces false positives, as prokaryotic (LexA and B42) rather than eukaryotic proteins are used.
- It is able to fish out potentially toxic prey proteins, as their expression is galactose-inducible.
- It is easy to detect whether a particular bait protein will enter the yeast nucleus and bind LexA operators.
- There are reporters with varying sensitivities available so that baits which

self-activate can still be assayed by using a less sensitive reporter.

- It is easy to do a co-immunoprecipitation assay of bait and potential positives since HA tag is fused downstream to B42.

Section C. Materials and Methods

Strains

The yeast strain EGY48 has the upstream activating sequences of the chromosomal Leu2 gene replaced with LexA operators. EGY48 contains 6 LexA operators that direct transcription of the Leu2 gene, which makes it one of the most sensitive strains for transcription activation. So EGY48 is a basic strain used to detect interactions.

Escherichia coli strain KC8, which lacks *trp* gene, is used to select for prey plasmids from mixture of prey, bait and reporter plasmids.

Molecular Biology

Reporter plasmid pSH18-34, which contains a LexA operator-LacZ fusion gene, is used to detect protein-protein interaction as well. It contains 8 LexA operators, which make it one of the most sensitive indicator plasmids for transcriptional activation.

Full-length KEL-8 bait plasmid was constructed by putting *kel-8* full-length cDNA sequences downstream of Lex-A sequences in plasmid pEG202 (called pEG202-kel-8 or

bait plasmid in following text). Bait plasmids containing the BTB domain of KEL-8 were constructed by subcloning amino acid 99-215 of KEL-8 into plasmid pEG202 through the LR reaction. Bait plasmids containing the BTB and BACK domain of KEL-8 were constructed by subcloning amino acid 99-369 of KEL-8 into plasmid pEG202 through LR reaction. Bait plasmids containing the Kelch domain of KEL-8 were constructed by subcloning amino acid 371-656 of KEL-8 into plasmid pEG202 through LR reaction. Prey protein was constructed by putting KEL-8 or PMK-1 full-length cDNA sequences downstream of B42 sequences in plasmid pJG4-5.

Growth Assay

To test whether yeast strains can grow on Gal/CM-Leu media, growth assays were performed either by serial dilution or by a single streak. In serial dilution assay, a single colony of yeast strain was picked using a sterile loop or wooden stick into 0.5mL of sterile distilled water. Then it was vortexed and three serial 1:10 dilution were made. Afterwards we put 100ul of original solution and the dilutions onto Gal/CM +Leu plate, Gal/CM -Leu plate and Glu/CM –Leu plate. The plates were incubated for 2-3 days at 30ºC.

Beta-galactosidase assay

Beta-galactosidase assay was done either using filter paper soaked in the buffer that

contains Xgal or directly adding Xgal to the plates. To do a beta-galactosidase assay using filter paper, we first streaked a Glu/CM master plate with test colonies, positive controls and negative controls. Then we placed a filter paper on the yeast plate, allowing it to become wet through. The filter paper was removed from plate, dried for 5 min, chilled in liquid nitrogen for 5 times. Afterwards, the paper was soaked in Z buffer containing 1mg/ml Xgal and incubated in 30ºC. The filter paper was checked after 30 min and only the positive control colonies turn blue.

Section D. Results

Assays for testing bait autoactivation

It is important that the bait protein cannot activate the Leu2 gene or the LexA gene by itself. Bait vector pEG202-kel-8, which contains KEL-8 full-length sequence and the LexA gene, was transformed into the EGY48 strain along with the pSH18-34 reporter. Bait autoactivation was tested by –Leu growth assay and LacZ assay.

1. Growth assay

A single colony of EGY48 containing the bait plasmid and pSH-18-34 was picked and serial dilutions were made (materials and methods section). Afterwards we put a fraction of original solution and the dilutions onto Gal/CM -Ura-His plate and onto Gal/CM

-Ura-His-Leu plate. The plates were incubated for 2-3 days at 30ºC.

There was no growth on –Leu plate for EGY48 containing bait plasmid and pSH18-34, while the positive control had strong growth and the negative control had no growth on –Leu media (Figure 5). The positive control was EGY48 containing the pSH18-34 reporter and the pSH17-4 plasmid, which has LexA fused to the GAL4 activation domain. The negative control was EGY48 containing pSH18-34 reporter and pRFHM1[e,f] plasmid, which had LexA fused to the homeodomain of bicoid to produce nonactivating fusion.

2. Beta-galactosidase assay

The ability of bait protein to activate LacZ gene in the reporter plasmid by itself was tested by the beta-galactosidase assay. We streaked a Glu/CM-Ura-His master plate with test colonies, positive controls and negative controls. Then we did the beta-galactosidase assay using filter paper (Materials and Methods section). The filter paper was checked after incubated at 30ºC for 30 min and the positive control colonies turned blue while the negative control and our test colonies were white. (Figure 6)

The results of growth assay and beta-galactosidase assay were consistent and both of them indicated there was no self-activation of bait protein alone. Thus we could proceed to the next step.

Repression assay for testing whether the bait protein can enter the nucleus

A repression assay was used to test whether the bait protein can enter the yeast nucleus. EGY48 strain was transformed with bait plasmid and pJK101 (test), pRFHM1 and pJK101 (positive control), or pJK101 alone (negative control). Plasmid pJK101 contains the upstream activating sequences (UAS) from the GAL1 gene followed by LexA operators upstream of the LacZ coding sequence. So yeast containing pJK101 will have significant beta-galactosidase activity when grown on Gal/CM media because of binding of endogenous of yeast GAL4 to the GAL_{UAS}.

I did both the filter paper assay and the plate assay in which Xgal is put directly in the Gal/CM media. Unfortunately neither of the two assays was able to distinguish test from negative control. The possible reason is that the activation of LacZ by pJK101 is so significant that mild repression is not easily detectable. This has been observed for other genes analyzed in our lab.

Testing the ability of KEL-8 dimerization

As the repression assay did not work, we wanted to find another way to test whether bait protein (pEG202-kel-8) could enter the yeast nucleus. Furthermore, we wanted to test whether KEL-8 is folded appropriately in yeast. One way was to test the interaction of KEL-8 with a binding partner that is already known. Since we know that BTB-Kelch

proteins form dimers through their BTB domain, we put full length KEL-8 sequences into the prey plasmid and tested the interaction of KEL-8 bait and KEL-8 prey.

EGY48 strain containing bait protein and pSH18-34 reporter was further transformed with KEL-8 prey plasmid. Interaction was tested by growth assay on GAL/CM-Ura-His-Trp-Leu plate and beta-galactosidase assay. Test colonies can grow on –Leu media (Figure 8) and turn blue in beta-galactosidase assay (data not shown), suggesting KEL-8 can interact with itself. So we are confident that KEL-8 is folded properly and can enter the nucleus, which makes a yeast two-hybrid screen using KEL-8 bait protein possible.

Test whether KEL-8 interacts with PMK-1 in yeast two-hybrid system

In a yeast two-hybrid screen performed by the Vidal lab, PMK-1 could interact with KEL-8 when PMK-1 was fused with bait protein and KEL-8 fused with prey protein (Li *et al.*, 2004). I tried to see whether the result could be repeated in our lab. I constructed the prey plasmid with full length *pmk-1* cDNA. Then I co transformed yeast strain EGY48 containing reporter plasmid pSH18-34 with bait plasmid pEG202-kel-8 and the *pmk-1* prey plasmid. I performed growth assay and found there is weak interaction, if any, between PMK-1 and KEL-8 (Figure 8).

Twelve candidates that can interact with KEL-8 are identified from a yeast two hybrid screen.

A yeast two-hybrid screen using KEL-8 bait protein was performed. EGY48 strain containing bait protein and pSH18-34 reporter was transformed with pJG4-5-based plasmid library and interaction was detected by both –Leu growth assay and beta-galactosidase assay. About 15ug of pJG4-5-based plasmid library was transformed and $0.8*10^5$ transformants were obtained. These transformants were mixed, harvested, frozen and replated, and 5 times of original transformants were plated on GAL/CM-Ura-His-Trp-Leu. 115 colonies which originally grow on GAL/CM-Ura-His-Trp-Leu were retested by –Leu growth assay and beta-galactosidase assay. Most of the 115 colonies still showed interaction with KEL-8 by these two assays (Figure 9-13).

Mixed plasmids (bait plasmid, reporter plasmid and prey plasmid) were extracted from 58 out of the 115 colonies. The plasmids were then transformed into *Escherichia coli* strain KC8 to select for prey plasmid alone by growing bacteria on –trp minimal media. Prey plasmid was extracted from KC8 cells and digested with EcoRI and XhoI. Plasmids with different digest patterns were sequenced.

Twelve candidates that could interact with KEL-8 were identified from the screen, and

eight of them were confirmed by retransforming plasmids to EGY48 and retesting the interaction. They are ZC434.8, *ric-4*, F32D8.12, *pccb-1*, *ssq-4*, *dim-1*, *aly-3*, and *smo-1*. ZC434.8 encodes an arginine kinase. RIC-4 is a member of SNAP-25 family. F32D8.12 encodes a protein that has an FAD binding domain. PCCB-1 is a Propionyl Coenzyme Carboxylase Beta subunit. SSQ-4 is a member of class Q sperm-specific family. DIM-1 is a novel protein containing three immunoglobulin-like repeats in its carboxyl terminus. ALY-3 encodes an RRM motif-containing protein orthologous to human THOC4 (OMIM:604171), and paralogous to ALY-1 and ALY-2; by orthology, ALY-3 is thought to promote recruitment of mRNA export factor to mRNAs. SMO-1 encodes the *C. elegans* ortholog of SUMO, a small ubiquitin-like moiety that, when attached to protein substrates, regulates their subcellular localization and activity

Find out which domains of KEL-8 interact with the positives

EGY48 containing reporter plasmid pSH-18-34 was transformed with bait plasmids, which have KEL-8 domain sequences (BTB alone, BTB and back, Kelch domain). The strains were tested for bait autoactivation and there was no autoactivation (Figure 14). Repression assay was also done to show that the bait plasmid can enter the yeast nucleus. Afterwards, these three strains (EGY48 containing reporter plasmid pSH-18-34 and different domains of KEL-8) were then transformed with prey plasmids, which showed interaction with full length KEL-8. We found that the BTB domain is likely to interact with F32D8.12 and ALY-3, yet neither BTB and BACK domains, nor KELCH domain

shows interaction with any prey proteins (Figure 15).

Section D. Discussion

Yeast two-hybrid is a powerful tool to detect protein-protein interaction in vivo, yet it has a problem of false positives. The most common types of false positives are heat shock proteins, ribosomal proteins, ferritin and ubiquitin. Other false positives are cytochrome oxidase, mitochondrial proteins, proteasome subunits, tRNA synthase, collagen-related proteins, zinc finger proteins, vimentin, inorganic pyrophosphatase, PCNA, lamins, elongation factors, and cytoskeletal proteins (Guo *et al.*, 2008). So the functional analysis of KEL-8 interacting proteins needs to be done to determine whether there are biological meanings of the interactions. ALY-3 and SSQ-4 might be false positives since BTB-kelch proteins have rarely been known to regulate mRNA export or sperm-specific family. ZC434.8 and PCCB-1, which encode an arginine kinase and a carboxylase, might also be false positives because kinases and carboxylase are common false positives. But some kinases and carboxylses are true interactors for certain proteins, so it might still be worthwhile to do functional analysis on ZC434.8 and PCCB-1. SMO-1, a small ubiquitin-like moiety ortholog, could be a binding partner of KEL-8, yet it will not provide much insight into how KEL-8 works to regulate GLR-1 trafficking and possibly lifespan. RIC-4 is expressed in neurons, so maybe it does interact with KEL-8. There is not much known about the function of F32D8.12, but it is a strong interactor of KEL-8 and might be worth looking into.

Section F. Figures

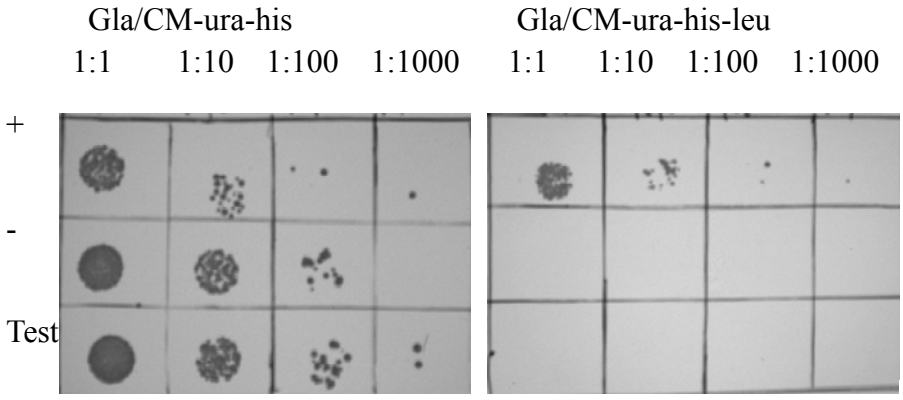


Figure 5. Growth assay. There is no growth on –Leu plate for EGY48 containing bait plasmid and pSH-18-34 (test), while the positive control (+) has strong growth and negative control (-) has no growth on –Leu media (right panel). All of the colonies grow on Gal-Ura-His media (left panel). The positive control is EGY48 containing pSH-18-34 reporter and pSH17-4 plasmid, which has LexA fused to the GAL4 activation domain. The negative control is EGY48 containing pSH-18-34 reporter and Prfhm1^{e,f} plasmid, which has LexA, fused to the homeodomain of bicoid to produce nonactivating fusion.

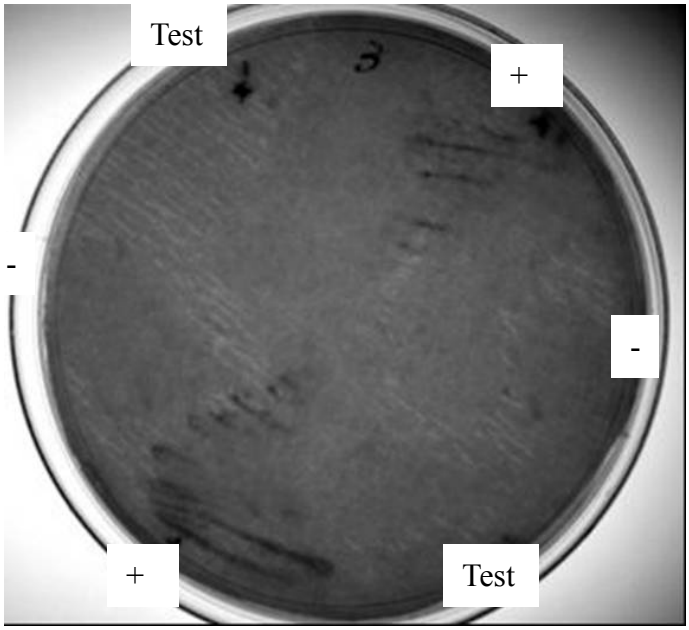

Test
+
-
-
+
Test

Figure 6. Beta-galactosidase assay. Only positive control colonies (+) turned blue after the filter paper was incubated in 30ºC for 30min, while the negative control (-) and test colonies which are EGY48 containing bait plasmid and pSH-18-34 (-) did not turn blue. The positive control is EGY48 containing pSH-18-34 reporter and pSH17-4 plasmid which has LexA fused to GAL4 activation domain. The negative control is EGY48 containing pSH-18-34 reporter and pRFHM1[e,f] plasmid, which has LexA fused to the homeodomain of bicoid to produce nonactivating fusion.

GAL/CM-ura-his-trp-glu

1:1 1:10 1:100 1:1000

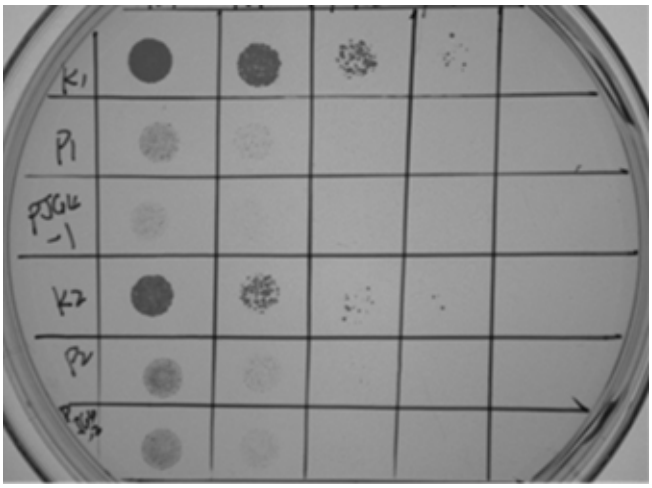


Figure 8. Growth assay to test the interaction between KEL-8 and PMK-1, as well as KEL-8 and KEL-8. Yeast strain EGY48 containing bait plasmid and pSH-18-34 was transformed with bait plasmid containing *kel-8*. The yeast strain was then further transformed with prey plasmid containing either *kel-8* or *pmk-1* separately. The yeast strain with *kel-8* in both bait and prey plasmids showed growth on –Leu media, and were considered as a positive control (+). The yeast strain with the reporter plasmid, *kel-8* in bait plasmid and empty prey vector appeared to have no growth on –Leu media and was considered as negative control (-). The yeast strain with the reporter plasmid, *kel-8* in bait plasmid and *pmk-1* in prey plasmid showed little, if any, growth on –Leu media (test).

GAL/CM-ura-his-trp-glu GLU/CM-ura-his-trp-glu

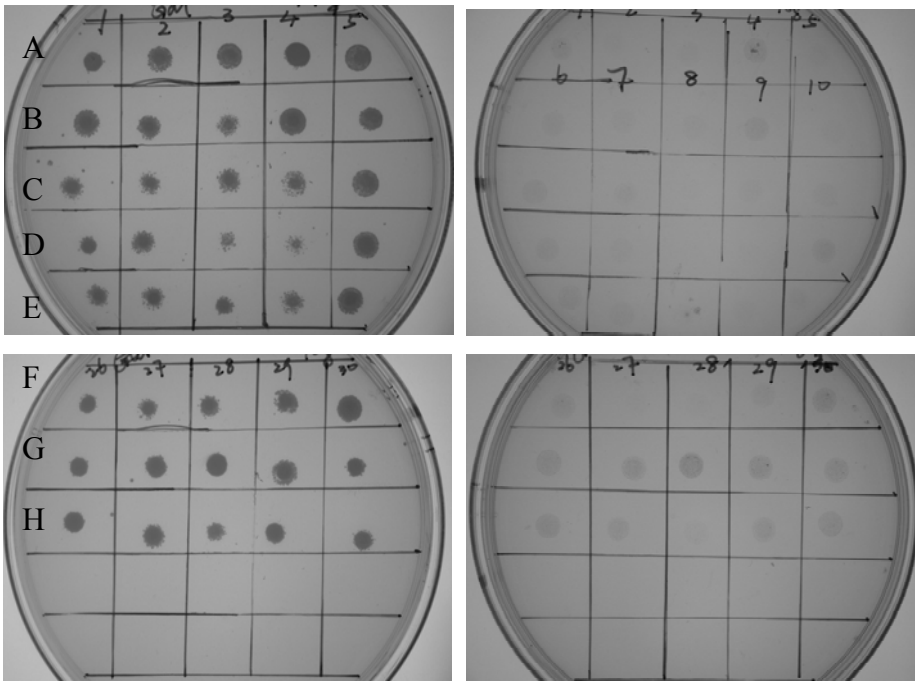


Figure 7. Colonies no 1-40 that grow on original GAL/CM-ura-his-trp-glu are retested for growth on GAL/CM-ura-his-trp-glu and GLU/CM-ura-his-trp-glu plates. All of them show growth on GAL/CM-ura-his-trp-glu but not on GLU/CM-ura-his-trp-glu media. Row A are colonies 1-5 (from left to right), row B are colonies 6-10, row C are colonies 11-15, row D are colonies 16-20, row E are colonies 21-25, row F are colonies 26-30, row G are colonies 31-35, row H are colonies 36-40.

GAL/CM-ura-his-trp+Xgal GLU/CM-ura-his-trp+Xgal

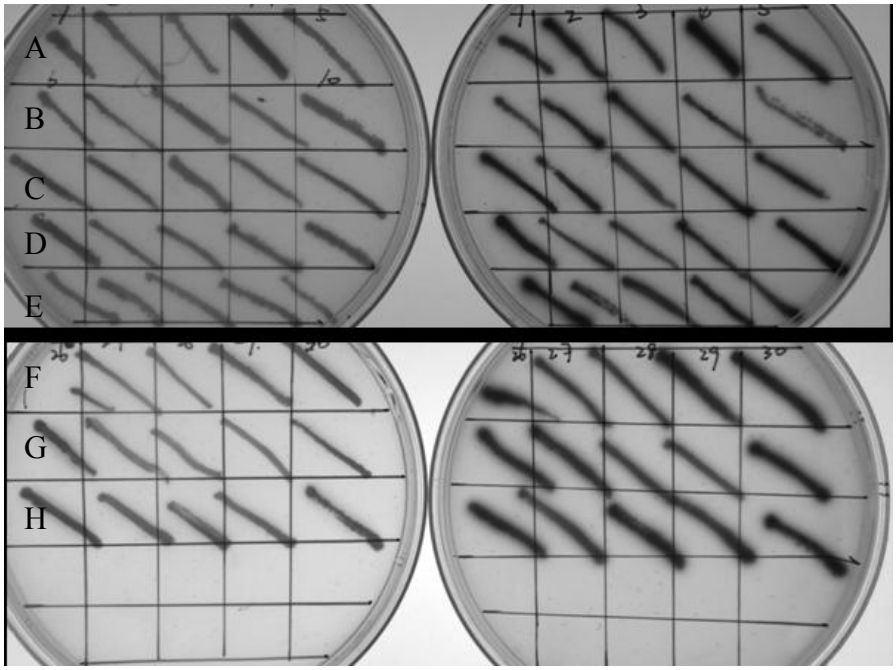


Figure 9. Colonies number 1-40 that grew on original GAL/CM-Ura-His-Trp-Glu were retested for beta-galactosidase assay on GAL/CM-Ura-His-Trp with Xgal and GLU/CM-Ura-His-Trp with Xgal plates. All the colonies are blue on GAL media and white on GLU media. Row A are colonies 1-5 (from left to right), row B are colonies 6-10, row C are colonies 11-15, row D are colonies 16-20, row E are colonies 21-25, row F are colonies 26-30, row G are colonies 31-35, row H are colonies 36-40.

GAL/CM-ura-his-trp-glu GLU/CM-ura-his-trp-glu

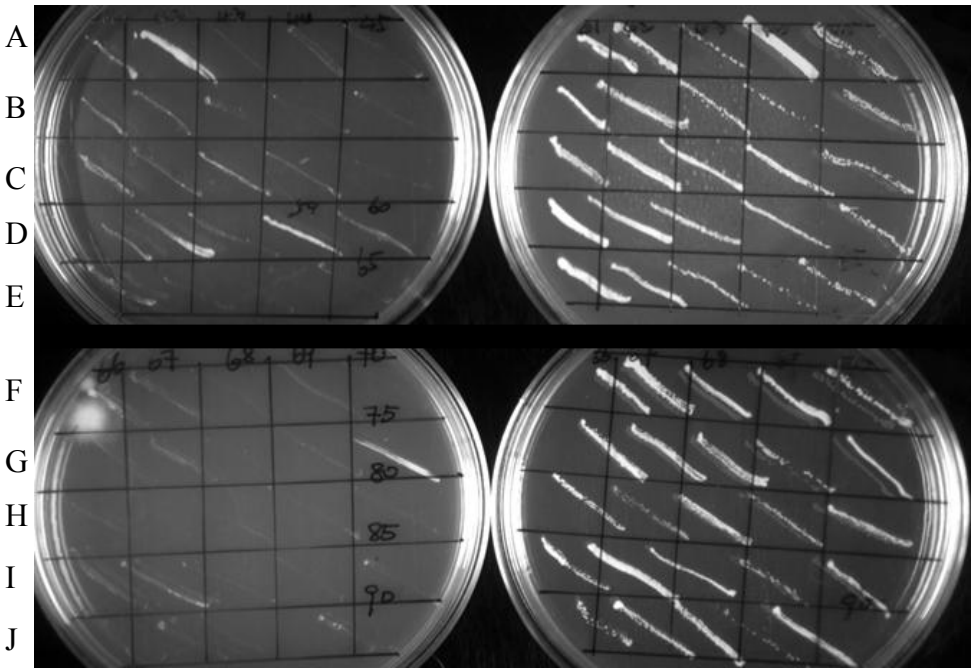


Figure 10. Colonies number 41-90 that grew on original GAL/CM-Ura-His-Trp-Glu were retested for growth on GAL/CM-Ura-His-Trp-Glu and GLU/CM-Ura-His-Trp-Glu plates. All of them show growth on GAL/CM-Ura-His-Trp-Glu but not on GLU/CM-Ura-His-Trp-Glu media. Row A are colonies 41-45 (from left to right), row B are colonies 46-50, row C are colonies 51-55, row D are colonies 56-60, row E are colonies 61-65, row F are colonies 66-70, row G are colonies 71-75, row H are colonies 76-80, row I are colonies 81-85, row J are colonies 85-90.

GAL/CM-ura-his-trp+Xgal GLU/CM-ura-his-trp+Xgal

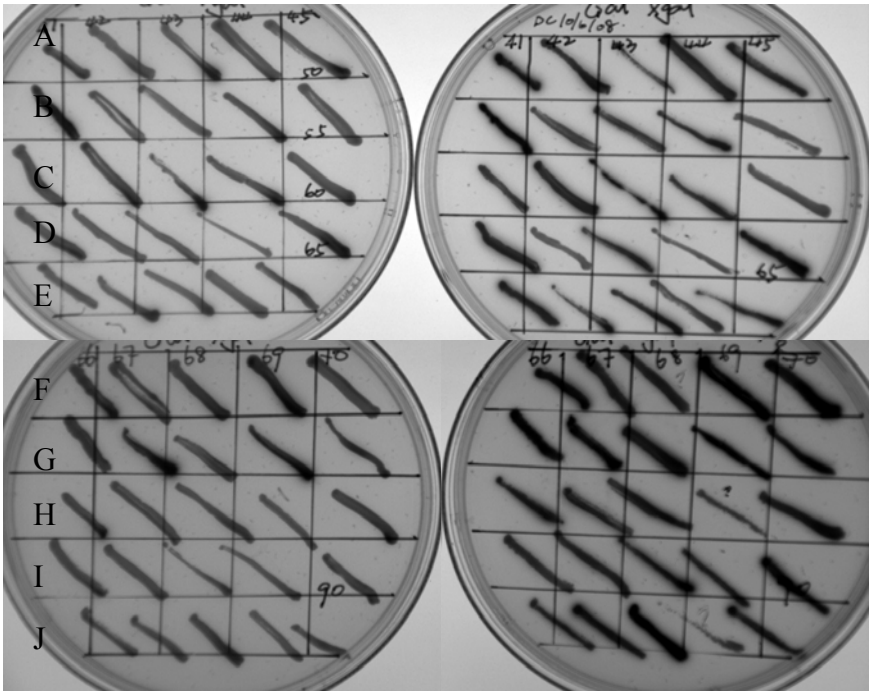


Figure 11. Colonies number 41-90 that grew on original GAL/CM-Ura-His-Trp-Glu were retested for beta-galactosidase assay on GAL/CM-Ura-His-Trp with Xgal and GLU/CM-Ura-His-Trp with Xgal plates. All the colonies are blue on GAL media and white on GLU media. Row A are colonies 41-45 (from left to right), row B are colonies 46-50, row C are colonies 51-55, row D are colonies 56-60, row E are colonies 61-65, row F are colonies 66-70, row G are colonies 71-75, row H are colonies 76-80, row I are colonies 81-85, row J are colonies 85-90.

GAL/CM-ura-his-trp-glu GLU/CM-ura-his-trp-glu

A

B

C

D

E

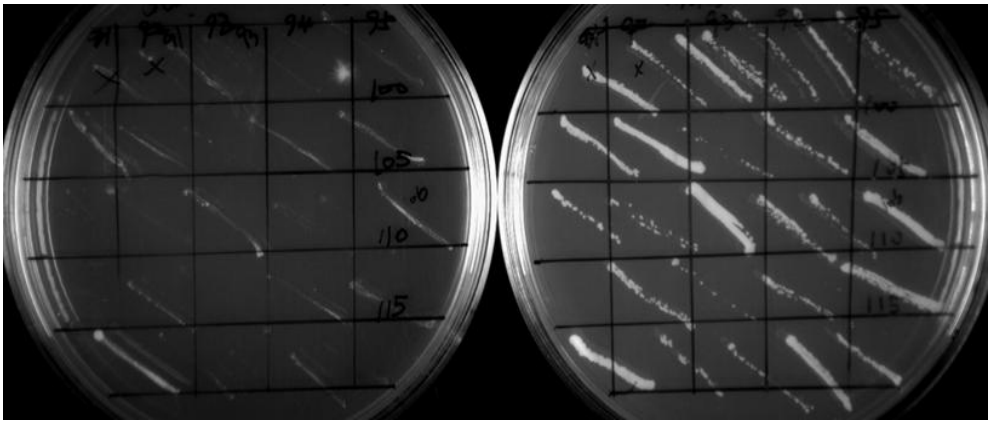


Figure 12. Colonies number 91-115 that grew on original GAL/CM-Ura-His-Trp-Glu were retested for growth on GAL/CM-Ura-His-Trp-Glu and GLU/CM-Ura-His-Trp-Glu plates. All of them show growth on GAL/CM-Ura-His-Trp-Glu but not on GLU/CM-Ura-His-Trp-Glu media. Row A are colonies 91-95 (from left to right), row B are colonies 96-100, row C are colonies 101-105, row D are colonies 106-110, row E are colonies 111-115.

GAL/CM-ura-his-trp + Xgal GLU/CM-ura-his-trp + Xgal

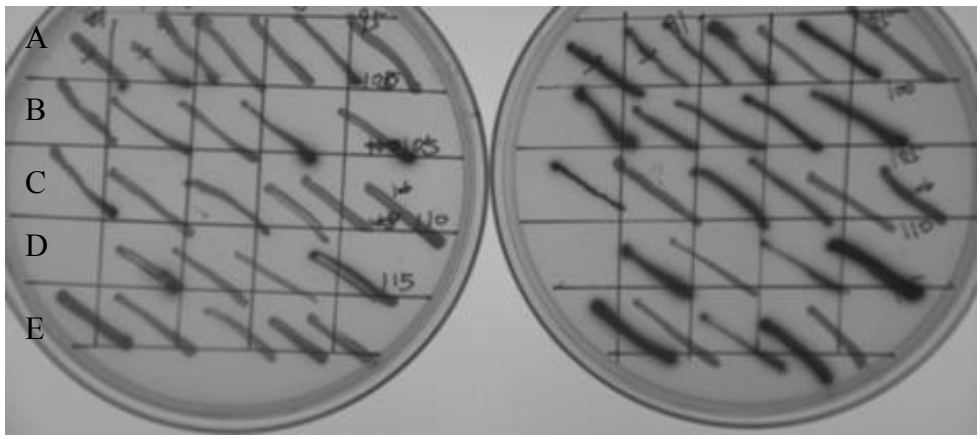


Figure 13. Colonies number 91-115 that grew on original GAL/CM-Ura-His-Trp-Glu were retested for beta-galactosidase assay on GAL/CM-Ura-His-Trp with Xgal and GLU/CM-Ura-His-Trp with Xgal plates. All the colonies are blue on GAL media and white on GLU media. Row A are colonies 91-95 (from left to right), row B are colonies 96-100, row C are colonies 101-105, row D are colonies 106-110, row E are colonies 111-115.

Gal-ura-his-trp-leu

1:1 1:10 1:100 1:1000

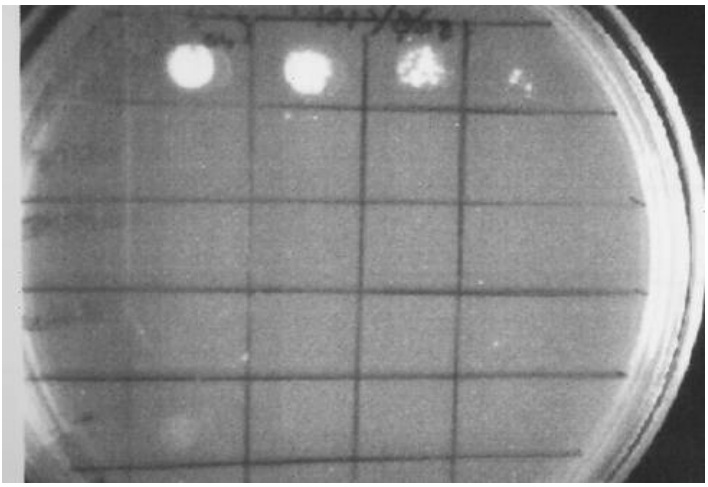
+

BTB

BTB&
Back

kelch

-



Gal-ura-his-trp+Xgal

1:1 1:10 1:100 1:1000

+

BTB

BTB&
Back

kelch

-

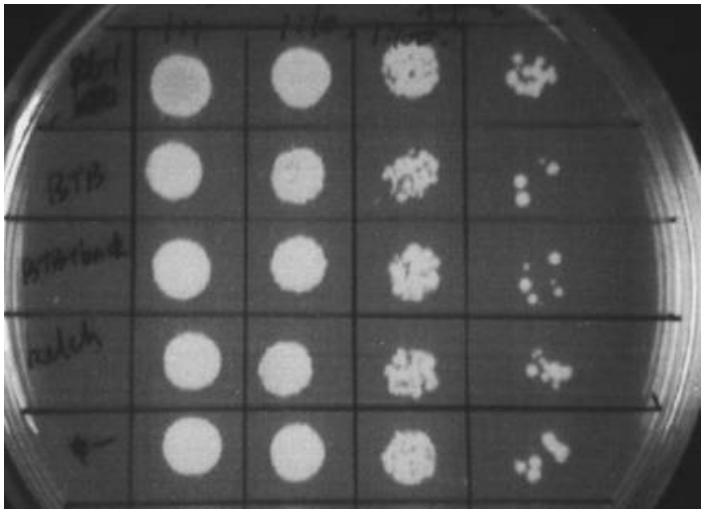


Figure 14. Autoactivation test for bait protein containing different domains of kel-8 (BTB, BTB and BACK, KELCH). Positive control shows growth in Gal-Ura-His-Trp-Leu plate while neither negative control appears to have no growth. EGY48 strain with reporter pSH18-34 and BTB domain of KEL-8 (BTB in the figure) or BTB and Back (BTB & Back in the figure) or kelch domain (Kelch in the figure) did not appear to have any growth, either. For beta-galatosidase assay, only positive control appears to have blue color. So growth assay and beta-galactosidase assay both indicate there is no autoactivation for any of the three baits.

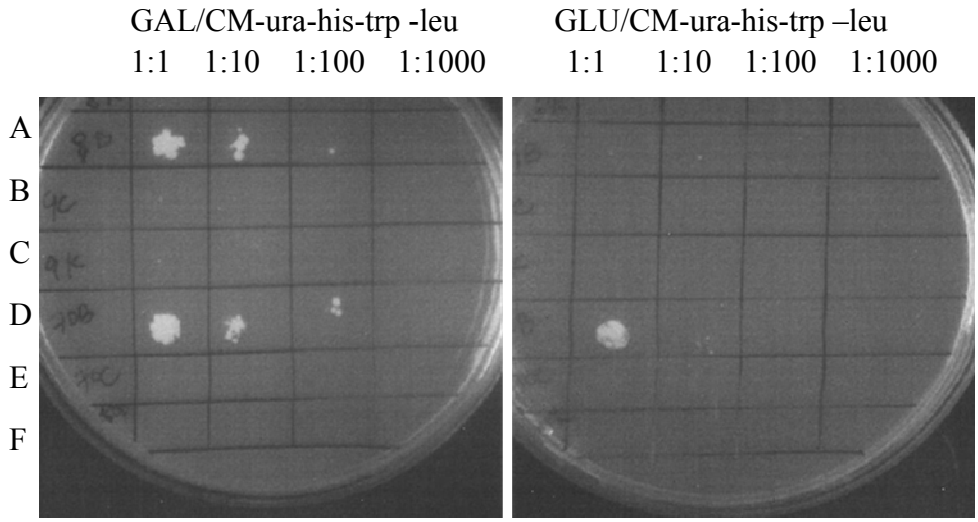

GAL/CM-ura-his-trp -leu
1:1 1:10 1:100 1:1000
GLU/CM-ura-his-trp –leu
1:1 1:10 1:100 1:1000
A
B
C
D
E
F

Figure 15. Testing which domains of KEL-8 interact with prey proteins. Three yeast strains (EGY48 containing reporter plasmid pSH-18-34 and three different domains of KEL-8) are transformed with prey plasmids which show interaction with full length kel-8. Row A shows interaction between BTB domain of KEL-8 and F32D8.12, row B shows interaction between BTB and back domain of KEL-8 and F32D8.12, row C shows interaction between Kelch domain of KEL-8 and F32D8.12, row D shows interaction between BTB domain of KEL-8 and ALY-3, row E shows interaction between BTB and back domain of KEL-8 and ALY-3, row F shows interaction between Kelch domain of KEL-8 and ALY-3. It turns out BTB domain is likely to interact with F32D8.12 and ALY-3, yet neither BTB and BACK domain or KELCH domain shows interaction with F32D8.12 and ALY-3.

Appendix

Section A. Investigate whether SEL-12, a presenilin ortholog, plays a role in GLR-1 trafficking.

Introduction

SEL-12 and HOP-1 are homologs of presenilin1, which is a Catalytic subunit of γ-secretase. The mutations of presinilin1 contribute to Alzheimer's disease (Wittenburg *et al.*, 2000; Miller *et al.*, 2006; Tu *et al.*, 2006). Previous work in our lab showed that in *sel-12* mutant worms, GLR-1 receptor puncta number in the ventral nerve cord are reduced in number (E. Nothstein, personal communication). We reasoned that thought SEL-12 might have an effect on GLR-1 trafficking. It is also known that the PTB domain of X11 (a homolog of LIN-10) binds to APP, and the PDZ domains of these proteins bind to presenilin-1. In addition, Alcadein (a homolog of CASY-1 in worms) can form a tripartite complex with X11 and APP and prevents the processing of APP (Miller *et al.*, 2006). So we were also interested to know whether *casy-1* mutant strains have any nervous system defects.

Materials and Methods

Strains

Standard methods were used to maintain *C. elegans* (Wood, 1988). Animals were grown at 20ºC on standard NGM plates seeded with OP50 *Escherichia coli* unless stated otherwise. Transgenic strains were generated by microinjecting various plasmids (typically at 50ng/ul) together with either *rol-6dm* (C. Mello). Strains *hop-1*(ar179), *sel-12*(ar131), *sel-12*(ty11), *sel-12*(ar171) *unc-1*(e538), *glp-1*(or178), *glp*(e2141), *lin-12*(n137n460), *casy-1*(tm718), *unc-1*(e538), *nuIs25[glr-1::gfp]* were used in this study.

Molecular Biology

Constructs *sel-12::gfp*, *gfp::sel-12*, *hop-1::gfp*, *gfp::hop-1* were made by subcloning cDNA sequence of *sel-12* or *hop-1* into PV6 plasmids containing N terminus or C terminus GFP tags.

Results

To explore these questions, I crossed *sel-12* and *hop-1* mutant strains to *nuIs25* (*glr-1::gfp* integrated strain) to check the phenotype our lab previously observed. I found that the decrease of GLR-1 puncta number in the nerve cord was not very significant (data not shown), but found that *sel-12* mutant worms had abnormal axon protrusions in several neuron cell bodies (Figure 15). This finding indicates that *sel-12* affects neuron cell body morphology.

To explore this result further, I attempted to observe the subcellular localization of presenillins in worm neurons. I made four constructs with *sel-12* and *hop-1* cDNA tagged with GFP at either the N terminus or the C terminus. I injected the constructs into wild-type worms and found that the localization of the GFP tagged proteins is characteristic of proteins localized in the ER (Figure 16). In addition, it has been indicated that presinilin is involved in calcium homeostasis (Arispe *et al.*, 1993; Begley *et al.*, 1999; Tu *et al.*, 2006). Thus, we suspect that SEL-12 and HOP-1 are localized in the ER and perhaps function as a calcium channel.

To test whether *casy-1* mutant worms have any nervous system defects, I crossed *casy-1* with *nuIs25* but did not see any defects. It has been shown that SEL-12 facilitates the function of the Notch receptor (LIN-12 protein). I crossed *nuIs25* to several alleles of notch receptor mutants, but again did not see any phenotype. As Mints can interact with presenilins, we wondered whether LIN-10 might be regulating the trafficking of SEL-12 or HOP-1. I injected SEL-12 and HOP-1 tagged with GFP constructs into *lin-10* worms, but *lin-10* did not seem to influence the localization of SEL-12 and HOP-1 (data not shown).

Figure

sel-12(ar171) unc-1(e538); nuIs25 *nuIs25*

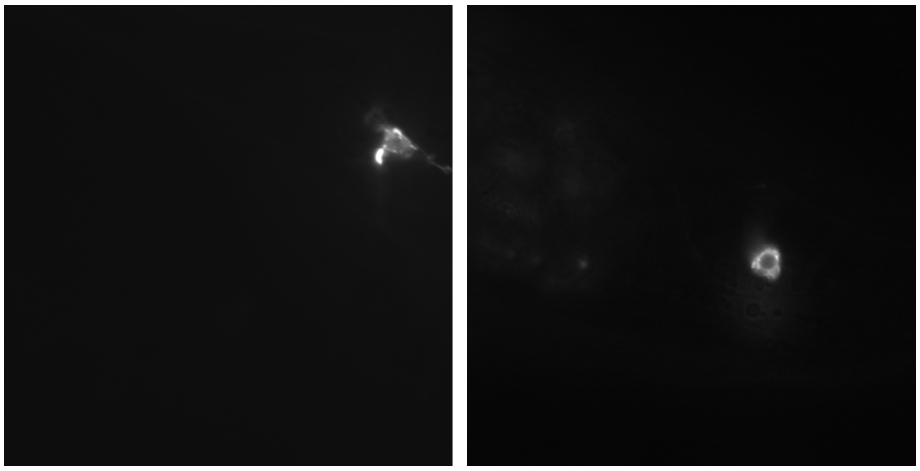


Figure 16. *sel-12* mutant worms have abnormal axon protrusions in several neuron cell bodies. *sel-12(ar171) unc-1(e538)* mutant strain was crossed with *nuIs25* and showed abnormal axon protrusions in several neuron cell bodies compared to *nuIs25* worms.

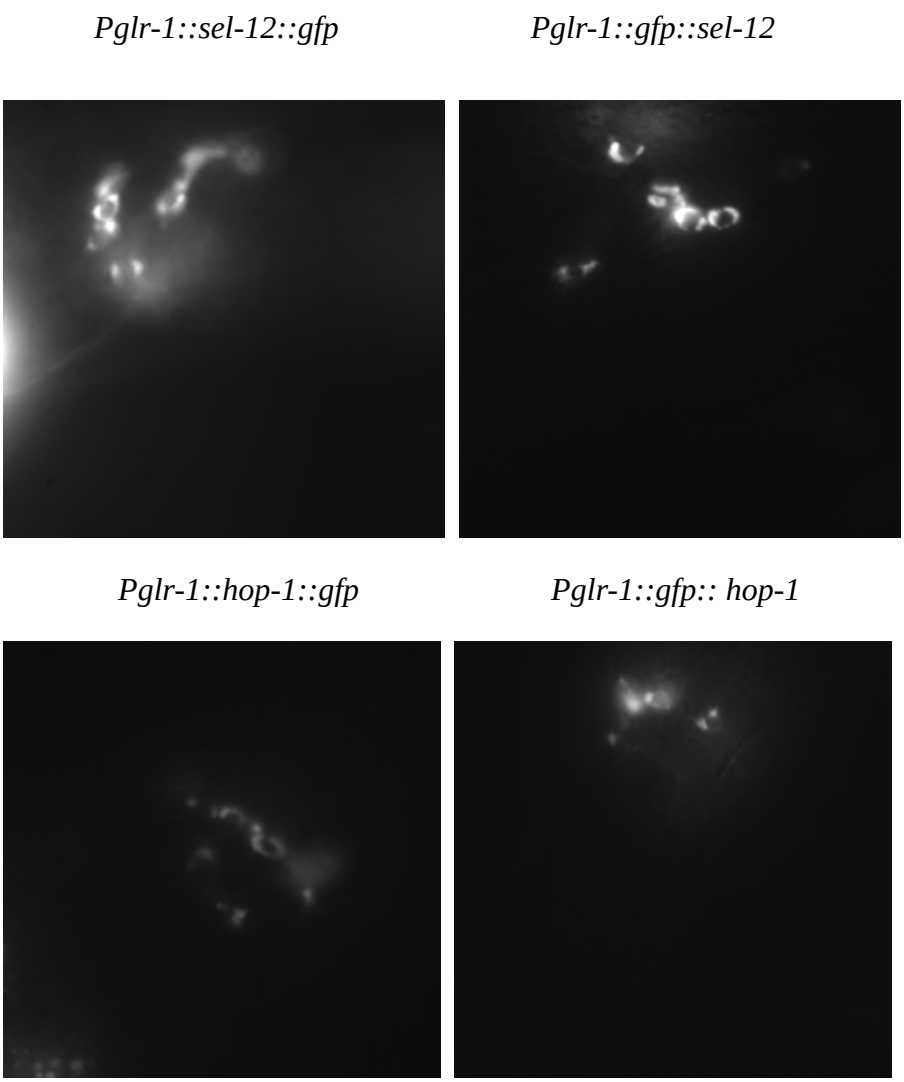


Figure 17. HOP-1 and SEL-12 are probably localized in the ER. Four constructs *Pglr-1::sel-12::gfp*, *Pglr-1::gfp::sel-12, Pglr-1::hop-1::gfp, Pglr-1::gfp:: hop-1* were injected into wild type N2 worms and localization of them were examined. According to the expression pattern, the four constructs are probably all localized in the ER.

Discussion

The fact that SEL-12 and HOP-1 are probably localized in the ER, and that presinilin is

involved in calcium homeostasis makes is possible that SEL-12 and HOP-1 function as a calcium channel. To explore this further, we could coinject GFP tagged SEL-12 or HOP-1 constructs with ER markers to check whether SEL-12 and HOP-1 are truly localized in the ER. We could also investigate the effect of SEL-12 and HOP-1 on proteins that encodes calcium channels. UNC-2 encodes a calcium channel alpha subunit (Schafer and Kenyon, 1995; Mathews *et al.*, 2003) and UNC-43 encodes the type II calcium/calmodulin-dependent protein kinase (CaMKII) ortholog (Rongo and Kaplan, 1999). Indeed, mutations in these calcium signaling factors result in neurite outgrowth from the neuron cell bodies that resembles that observed in the *sel-12* mutants. Thus, SEL-12 might contribute additional calcium signaling to this pathway, explaining the morphology defects observed in *sel-12* mutants. We can check the expression pattern and level of UNC-2 and UNC-43 in *sel-12* or *hop-1* mutant background to see if SEL-12 or HOP-1 has an effect on calcium channel signaling.

Section B. Make a dominant negative version of UNC-104, a kinesin-like motor protein homologue.

Introduction

Motor proteins never came out of the screen looking for genes that affect GLR-1 trafficking, which is surprising because it is reasonable to assume that motor proteins are involve in GLR-1 trafficking. One of the possible explanations is that mutations in motor proteins are lethal. Most kinesin proteins contain an N terminal catalytic pocket

responsible movement empowered by hydrolysis of ATP, and a C terminal binding domain that interacts with cargo (Setou *et al.*, 2002; Miki *et al.*, 2005). Thus, dominant negative versions of kinesin proteins can be made by removing the N terminal motor domain (Miki *et al.*, 2005). Here we test the approach of making dominant negative kinesins in *C. elegans*.

Materials and Methods

Strains

Strain *odIs1[snb-1::gfp]* was used in this study.

Molecular Biology

Dominant negative UNC-104 with RFP tag was made by replacing the N terminal motor domain in yk1063a8 with RFP tag.

Results and Discussion

One way to find out which motor protein is related to GLR-1 trafficking is to make dominant negative versions of each of the 21 kinesin proteins in *C. elegans*. In order to test whether the dominant negative version works or not, we started by making the dominant negative version of UNC-104, a kinesin-like motor protein homologue which is known to be required for anterograde axonal transport of synaptic vesicles as well as

differentiation of pre- and postsynaptic domains at inhibitory neuromuscular junctions. UNC-104 is known to regulate the trafficking of SNB-1, a synaptic vesicle-associated protein termed synaptobrevin.

To test this approach, I made a dominant negative version of UNC-104 by replacing the N terminal motor domain with RFP tag. I injected the construct into *odIs1[snb-1::gfp]* and checked SNB-1 localization. I found that the phenotype of worms with dominant negative UNC-104 resembles *unc-104* mutants in *odIs1* background, but at a low penetrance (data not shown). Alternative strategies for dominant negatives should be considered.

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