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Foreword

Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

DSE Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

DSE In conducting research utilizing recombinant DNA technology, the investigators adhered to current guidelines promulgated by the National Institutes of Health.

DSE In the conduct of research utilizing recombinant DNA, the investigators adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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Abstract

ras genes encode small GTPase proteins, which when mutated, have been shown to result in breast cancer as well as cancers in a variety of other tissues. The study of *Caenorhabditis elegans* and other model systems has demonstrated that Ras is part of a conserved Ras/MAPK signaling pathway involved in many aspects of development and cell regulation. The *C. elegans* vulva is induced by an EGF like signal that activates the Ras/MAPK pathway. Constitutively active alleles of *ras* lead to hyperactivity of the signal transduction pathway and result in a multivulva (Muv) phenotype where numerous pseudovulvae are formed from vulval precursor cells (VPCs). By initiating suppressor screens of activated *let-60 ras*, many previously unknown components of this pathway have been identified. One gene, *sur-9*, has been defined by a semi-dominant allele isolated in a screen for temperature sensitive mutations that suppress the *let-60(n1046)* allele. While homozygous, *sur-9(ku258)* can suppress *let-60(n1046)* from 80% Muv to <1% Muv. *sur-9(ku258)/+* also suppresses the *let-60(n1046)* phenotype to 10% Muv. Additional genetic analysis has suggested that *sur-9* acts at a late step in the Ras/MAPK signaling pathway. Animals carrying the *sur-9(ku258)* mutation are also unhealthy, semi-sterile and show defects in other developmental processes. *sur-9(ku258)* has been mapped to LG III and fine mapping is currently being carried out. We hope to report the cloning and elucidation of *sur-9*'s molecular identity which could contribute to our knowledge of a highly conserved and important biological pathway.

Additionally, we have taken a molecular approach to understanding downstream targets of this important biological pathway. A DNA binding site enrichment or SELEX experiment was performed on both LIN-1 and LIN-31, the terminal transcription machinery of the Ras/MAPK pathway. A consensus was obtained for both factors and the genome is currently being analyzed for putative binding sites that could represent regulatory regions for genes activated by the Ras/MAPK pathway. Hopefully such an approach will broaden our knowledge of the downstream events of this pathway.

Background and significance

The Ras family of small GTPases were first identified in mammalian systems as a result of their cellular transformation activity in a number of human carcinomas (8). Additionally, it has been shown that Ras can be activated by a variety of receptors/activators and is thought to act as a core signaling component at the top of a kinase cascade. This cascade ends with the phosphorylation of a member of the mitogen activated protein kinase (MAPK) family of dual specificity serine/threonine kinases. MAPKs can then translocate to the nucleus and alter the activity of various transcription factors (4) (9).

Much of what is known about the Ras/MAPK pathway was elucidated genetically in both worms and flies (10) (2). The *let-60* gene, which encodes RAS was initially identified as a lethal mutation. However, it was not until gain of function mutations were isolated with their very specific vulval defects that *ras* was cloned(11). *C. elegans* vulval induction occurs when an EGF like signal (LIN-3) induces three of six equipotent vulval precursor cells (VPCs) via the Ras pathway to adopt a vulval fate (Figure 1)(12). Constitutive activation of RAS or other components of this pathway leads to over-induction or a multivulva (Muv) phenotype.

Conversely, a loss of function mutation in a member of this pathway leads to under-induction or a vulvaless (Vul) phenotype (Figure 2). The *ras* gain of function mutation, with its obvious Muv phenotype, has since proven useful for genetic dissection of Ras signaling in vulval development(13). Besides identifying many of the core components of the pathway, sensitized genetic suppressor screens on activated *let-60* (*n1046*) have revealed a number of components that have no phenotype on their own but interact directly with core components of the pathway(14) (15) (16). However, until *sur-9*, no suppressor of *let-60* (gf) has been isolated that

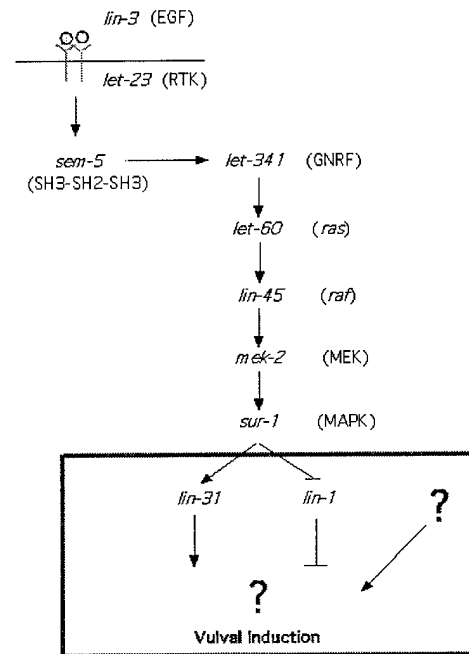


Figure 1. The genetic pathway regulating vulval induction. Various positive and negative regulators of this pathway have been left out for simplicity. The backbone of this pathway, the Ras/MAPK module, is a highly conserved kinase cascade capable of phosphorylating the transcription factors LIN-1 and LIN-31. Transcriptional targets of this signaling pathway are not yet known.

could also suppress either of the transcription factors downstream of MAPK, *lin-1* or *lin-31*, discussed below.

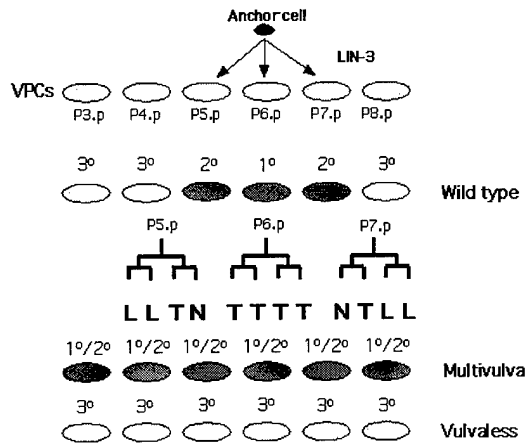


Figure 2. The six vulval precursor cells P(3-8).p are equivalent and capable of adopting a 1°, 2° or non-vulval 3° fates. When P(5-7).p are induced to adopt 1° or 2° vulval fates by the LIN-3 signal, they undergo three rounds of cell division to generate 8 or 7 vulval cells respectively. In Vul animals less than three VPCs adopt a vulval fate. In Muv animals more than three VPCs adopt vulval fates.

Ras activation can have drastically different outcomes in different cell types⁽²⁾ (3) (17). In the worm, ras activity is required for a number of processes: excretory duct cell fate determination, male tail formation, sex myoblast migration, germ cell development, P12 specification and of course vulval development⁽¹⁸⁾ (13). Due to the generality of the core Ras/MAPK pathway, various tissue specific regulatory mechanisms for the pathway have evolved

to control the proper outcome of activation⁽¹⁷⁾ (3). One level at which tissue specificity of the Ras pathway can be achieved is at the level of transcription. Both ubiquitous Ras pathway responsive transcription factors such as the ETS family members, as well as cell type specific factors such as *lin-31* in the *C. elegans* vulva or MITF in humans are thought to work together to produce a specific response⁽³⁾. Defining that response and the genes that ultimately lead to transformation or a specific cellular response has been a difficult task. Numerous studies have been done looking at genome wide changes in transcription as a result of Ras activation⁽¹⁹⁾ (20) (21). However, making sense of and organizing this data into a precise picture has been very difficult. Few people have done genetics to look for transcriptional targets of the Ras signaling pathway in multicellular organisms, which if successful could lead to a better picture of Ras transcriptional outcome and the hierarchical nature of those targets⁽²²⁾.

Proper vulval induction requires the normal function of several transcription factors. Two of these transcription factors, *lin-1* ETS family member and *lin-31* HNF3/forkhead like family member, have been shown to be downstream of MAPK both genetically and biochemically. Loss of function (lf) *lin-1* alleles have been isolated that result in a 100% Muv phenotype, much like *let-60* (gf) alleles, indicating a negative role for *lin-1* in vulval induction. Additionally, mutations have been isolated in the DNA binding domain suggesting that DNA binding is

important for proper *lin-1* function ⁽⁷⁾. The vulval specific *lin-31* gene's null phenotype (null alleles have been isolated that map to the DNA binding domain) is 77% Muv; however, cell fate decision appears to be completely deregulated and a lateral signaling pathway, regulated by a Notch-like receptor (LIN-12), no longer functions properly. There is also a small percentage (15%) of animals that appear vulvaless ⁽⁶⁾. This implies that the *lin-31* (lf) allele alone is not enough to mimic constitutive RAS activation like *lin-1* (lf). Thus, since it is not the ability to execute the three fates that is disrupted but how and where they are executed, it appears that the role of *lin-31* is largely regulatory and more positive than *lin-1*.

Biochemical work has been done on LIN-1 and LIN-31 that has shown the two proteins form a heterodimeric complex that inhibits vulval induction. Upon activation, MPK-1 phosphorylates both members of the complex and vulval induction proceeds ⁽²³⁾. While the requirement of this complex could explain the Muv phenotype created by *lin-1* (lf), it does not fully explain the phenotype created by *lin-31* (lf). *Lin-31*'s Muv penetrance is lower than that of *lin-1* suggesting that the Muv phenotype seen in a *lin-31* null is not entirely due to a failure in forming a repressive complex with LIN-1. A *lin-31* suppressor screen might not only identify genes required for vulval induction that are repressed by a LIN-1/LIN-31 heterodimeric complex, but also other regulatory factors that LIN-31 interacts with or their transcriptional targets.

Identification of any of the above factors and the elucidation of the molecular identity and function of *sur-9* could clarify the complex regulatory web controlling vulval induction and how it proceeds. Preliminary data towards this end and future experimental design are outlined in the following sections.

Methods

sur-9 Generation and Characterization

The methods used to isolate and characterize *sur-9* are similar to the methods used in previous annual reports. A genetic suppressor screen was conducted to identify signaling components that lie downstream of an activated *let-60 ras* allele. Standard and widely used genetic and *C.elegans* methods were employed to map the *sur-9* mutation to its present location on chromosome III

LIN-31 SELEX

LIN-31 was fused in frame to an N-terminal 6 His tag vector and transformed into *E.coli*. The protein was then expressed and purified on Ni agarose resin to greater than 90% purity. 10 rounds of SELEX (Selective Enrichment of Ligands by Exponential amplification) were performed using 2 separate 50N random SELEX libraries. Protein bound ligands were identified by EMSA (electrophoretic mobility shift assays). 22 SELEX clones from the final enriched pool of ligands were sequenced and aligned to determine the high affinity consensus for LIN-31.

Results

sur9.

A screen was conducted by Min Han and Kelly Grant to isolate genetic suppressors of an activated RAS phenotype. A worm strain containing the *let-60 (n1046)* gain of function mutation was exposed to the mutagen ethyl methanesulfonate (EMS) and suppressors of the multivulva or Muv phenotype were screened for in the second generation. A suppressor linked to chromosome III was given the allele designation *ku258* and termed *sur-9* (suppressor of activated RAS). Complementation tests were carried out by Kelly Grant using alleles of other genes on chromosome III known to suppress *let-60(n1046)* and it was determined that *ku258* does not correspond to any previously characterized suppressor.

I examined the *ku258* allele for defects in a wild type background. Varying percentages of *ku258* animals displayed an egg laying defective (Egl) phenotype, were sterile and had reduced brood sizes. None of the *ku258* worms were completely vulvaless; however, approximately 5% of the worms had an induction defect in the presumptive primary VPC, P6.p. Often, this under-induction resulted in half the number of P6.p cells being present in the mature vulva compared to a wild type animal. The sterility of the *ku258* animals is most likely caused by gonadal defects. Some *ku258* worms have shortened or missing gonadal arms. These defects are currently being analyzed further to determine in more detail what is happening. Table 1 quantifies some of the defects associated with *ku258* animals.

Genotype	Brood size	Sterility	Egl
N2	approx. 300	0%	0%
sur-9(ku258)	62	20%	28%
let-60 (n1046)	150	<5%	<1%
sur-9(ku258); let-60(n1046)	20	44%	29%

Table 1. Defects associated with *ku258*.

To get a better idea of where *sur-9* acts in the ras pathway, I performed epistasis analysis with several other components of vulval induction. *lin-15* causes a Muv phenotype similar to *let-60* (*n1046*) and is thought to act genetically upstream of *let-60* at the level of the LET-23 receptor tyrosine kinase. As expected, *sur-9* was able to suppress this Muv phenotype. Interestingly, when epistasis analysis was performed with *lin-1* (*e1275*), *sur-9* was also able to suppress the Muv phenotype caused by a lesion in this transcription factor. Thus, *sur-9* might act late in the Ras signaling pathway at the level of or after the transcriptional machinery. As mentioned earlier, *sur-9* was isolated due to its ability to suppress *let-60* (*n1046*); however, during the course of quantifying this, it was also determined that *sur-9* is a dominant suppressor. When examined more closely, it was clear *ku258* displays a semi-dominant phenotype on its own as well. The dominant nature of this allele was also observed with respect to *lin-1* suppression. A summary of *sur-9* epistasis/suppression is listed in Table 2.

<i>Genotype</i>	% <i>Muv</i>
N2	0
<i>sur-9</i> (<i>ku258</i>)	0
<i>let-60</i> (<i>n1046</i>)	80
<i>sur-9</i> (<i>ku258</i>); <i>let-60</i> (<i>n1046</i>)	1.6
<i>lin-15</i> (<i>n765</i>)	95
<i>sur-9</i> (<i>ku258</i>); <i>lin-15</i> (<i>n765</i>)	8.4
<i>lin-1</i> (<i>e1275</i>)	80
<i>sur-9</i> (<i>ku258</i>); <i>lin-1</i> (<i>e1275</i>)	2.5

Table2. Epistasis/suppression with *ku258*

Using three point mapping, I was able to map *sur-9* to successively finer locations on chromosome III. The last round of mapping utilized the markers *ced-7* and *unc-69* which are 1.5 map units apart. Recombinant data between these two markers has been obtained and *sur-9* was shown to be bounded by them. Although this is a relatively small region, the dominant nature of the *sur-9* allele necessitates a very precise mapped location of *sur-9* since a traditional cosmid rescue strategy has been attempted and did not work. Mapping is currently under way to determine which side of *sqv-3* the *ku258* allele falls on.

lin-31 Suppressor Screen

The *lin-31* suppressor screen is still in the preparatory stages. An allele with a high penetrance of the Muv phenotype was obtained and is currently being crossed into a *lin-8* Syn Muv gene background to increase the percentage to near 100% and allow an F1 clonal screen to

be conducted. Leilani Miller (Santa Clara University, CA) has conducted a *lin-31* suppressor screen using the *lin-31 (n1053)* allele and was able to isolate a number of mutants that dramatically increase the percentage of vulvaless *lin-31(n1053)* animals from 15% to around 80% (24). She is currently mapping two of these candidates. It is not likely that these mutations represent loss of function or dominant negative alleles of Ras pathway members since these double mutants have already been made and do not change the percentage of *lin-31(n1053)* vul animals by more than 5-10%⁽⁶⁾.

Although the *lin-31* suppressor screen is in the preliminary stages, some molecular work has been carried out that could facilitate the cloning and analysis of mutants isolated in the screen. I performed SELEX (systematic evolution of ligands by exponential enrichment) (25) with the 112 amino acid winged helix DNA binding domain of LIN-31 to determine a high affinity consensus sequence for this transcription factor. Using an initial starting pool of oligonucleotides with a 50N random region, 10 rounds of electrophoretic mobility shift assay (EMSA) based SELEX were performed to obtain a final mixed population of oligonucleotides with a Kd of approximately 20nM. Sequence was obtained from 22 of these oligonucleotides and a consensus was determined (Figure 3).

Additionally, a postdoctoral fellow in the lab, Wade Johnson, has also obtained a high affinity, *in vitro* consensus sequence for the LIN-1 transcription factor. Together, this data should be a convenient tool to examine the possibility of activation by either transcription factor alone or regulation by a transcriptionally repressive heterodimeric complex.

Summary

Although no papers have yet been generated from the data discussed in this report, two papers have been published from previous years of funding from this grant and were discussed in past annual reports. It is however highly likely that as this work progresses, it will lead to publications a more complete understanding of how the Ras/MAPK pathway operates. This in turn will allow for analysis of the mechanisms of cellular transformations such as breast cancers and someday to therapies for such cancers.

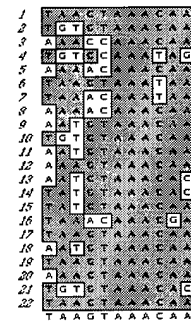


Figure 3. Alignment of DNA binding sites derived from LIN-31 SELEX procedure. The consensus is T A A G T A A A C A A.

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