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## CHAPTER 5

# Dissection of Genetic Pathways in *C. elegans*

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Abstract

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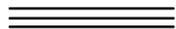
### Abstract

spar0055

With unique genetic and cell biological strengths, *C. elegans* has emerged as a powerful model system for studying many biological processes. These processes are typically regulated by complex genetic networks consisting of genes. Identifying those genes and organizing them into genetic pathways are two major steps toward understanding the mechanisms that regulate biological events. Forward genetic screens with various designs are a traditional approach for identifying candidate genes. The completion of the genome sequencing in *C. elegans* and the advent of high-throughput experimental techniques have led to the development of two additional powerful approaches: functional genomics and systems biology. Genes that are discovered by these approaches can be ordered into interacting pathways through a variety of

strategies, involving genetics, cell biology, biochemistry, and functional genomics, to gain a complete understanding of how gene regulatory networks control a particular biological process. The aim of this review is to provide an overview of the approaches available to identify and construct the genetic pathways using *C. elegans*.

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## I. Introduction

*C. elegans* has emerged as a powerful model system for identifying the genes and genetic pathways that regulate a diverse array of fundamental biological processes. The strengths of *C. elegans* include its invariant cell lineage, simplified cellular landscape, transparency, short life cycle, and hermaphroditic reproduction, which favor rapid genetic analysis. Complementing these traditional attributes, more recent functional genomic technologies, including genome-scale transcriptional and phenotypic profiling as well as physical interaction mapping, have led to more instruments in the *C. elegans* researcher's tool box to uncover the genetic networks that control developmental, behavioral, cell biological, and physiological processes. Highlighting the utility of *C. elegans* is the leading role that this model system has played in elucidating the genetic pathways regulating key biological processes such as cell-fate specification (Greenwald *et al.*, 1983; Sternberg, 2004; Sternberg and Horvitz, 1986), apoptosis (Kimble and Hirsh, 1979; Metzstein *et al.*, 1998; Sulston *et al.*, 1983; Sulston and Horvitz, 1977), RNA interference (RNAi) (Fire *et al.*, 1998), microRNA biology (Reinhart *et al.*, 2000; Simon *et al.*, 2008), axon guidance (Chan *et al.*, 1996; Hao *et al.*, 2001; Walthall and Chalfie, 1988), cell polarity (Goldstein and Hird, 1996; Kemphues *et al.*, 1988), and aging (Friedman and Johnson, 1988; Kimura *et al.*, 1997).

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The goal of this review is to provide a comprehensive overview of the approaches available to *C. elegans* researchers to identify and construct the genetic pathways that control biological events. Often these start with a simple genetic screen to identify the key nonredundant genes that regulate a process of interest. Alternatively, researchers sometimes stumble into a biological process when conducting reverse genetics – knocking out or reducing the function of a gene of interest (often disease-related) and studying the resulting phenotype. Once key genes are identified, reverse genetics and sensitized screening approaches can be used to better focus and identify redundant or modulatory genes. These approaches can often be complemented with functional genomic and systems-level studies to gain a more complete understanding of genetic pathways and the networks that guide these processes. In this review, we first discuss representative designs underlying diverse genetic screens in *C. elegans* with a uniform aim of identifying genes involved in a biological process of interest. We then discuss new techniques being utilized to discover candidate genes, including functional genomic techniques and their integration into systems-level analysis. This is followed by an outline of strategies for constructing pathways with genes identified. Throughout we provide specific examples of screening and genetic pathway construction to illustrate how these techniques are implemented.

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## II. Strategies for Identifying Components in Pathways of Interest

Three general strategies are currently used by *C. elegans* biologists to discover the specific components of pathways: (1) forward genetic screens that encompass a diverse array of screening methods and can be complemented with reverse genetic approaches; (2) functional genomic approaches that utilize genome-wide analysis of transcription, protein–protein interaction, DNA-binding site analysis, and loss-of-function techniques; and (3) systems biology approaches that integrate the use of functional genomic techniques.

sec0015

### A. Forward Genetic Screens

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#### 1. Forward Genetics and Reverse Genetics

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Biological processes are precisely regulated by highly coordinated gene regulatory networks that are comprised of numerous interacting signaling pathways. The basic constituent of signaling pathways are proteins encoded by corresponding genes. Perturbation of these genes could cause deregulation of associated pathways or even the entire network. Deregulation sufficient to disrupt a biological function may result in an observable outcome referred to as a phenotype. The collective status of perturbed genes is called genotype. The biological relationship in which a genotype determines a phenotype is the foundation for dissecting genetic pathways.

par0025

Forward genetic approaches (investigation directed from phenotype to genotype) and reverse genetic approaches (investigation directed from genotype to phenotype) are two powerful ways of elucidating the function of genes that regulate a biological process of interest. Forward genetic screens identify genes in an unbiased manner based on phenotypes of mutants. The screens start by searching for a desired phenotype caused by a mutation that is introduced into a gene by mutagens, such as EMS (1-methylsulfonyloxyethane, also known as ethyl methanesulfonate) or ENU (1-ethyl-1-nitrosourea, also known as N-ethyl-N-nitrosourea). Identity of the mutation-harboring genes can be determined by positional cloning or candidate-gene testing. Forward genetic studies in *C. elegans* have made significant contributions to our understanding of a wide range of developmental processes. For example, the forward genetic screens pioneered by Nobel Prize Laureate Robert Horvitz for mutants defective in programmed cell death (PCD) identified the underlying genetic pathways that direct apoptosis, a process conserved among metazoans, including humans (Metzstein *et al.*, 1998).

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Similarly, reverse genetics has provided considerable insights into many biological processes. Reverse genetic approaches begin with a set of genes with known sequences that are of particular interest such as disease-related genes (Ahringer, 1997; Barr *et al.*, 2001; Derry *et al.*, 2001). Genes are inactivated by target-selected approaches such as creation of deletion mutants using chemical mutagens or UV light (Gengyo-Ando and Mitani, 2000; Jansen *et al.*, 1997; Liu *et al.*, 1999),

transposon (Tc1) insertion (Rushforth *et al.*, 1993), and RNAi (Fire *et al.*, 1998). The availability of the complete and well-annotated genome sequence and RNAi libraries currently covering over 94% of the predicted genes in *C. elegans* (Ahringer, 2006) allow investigation of nearly any gene in *C. elegans*. For example, Derry *et al.* (2001) used sequence analysis to identify *cep-1*, the *C. elegans* ortholog of the mammalian p53 tumor suppressor gene (Rubin *et al.*, 2000). By generating a *cep-1* deletion mutant and using RNAi for functional assays, they identified and characterized the roles of CEP-1 in regulating apoptosis, stress response in somatic cells, and chromosome segregation in the germ line. This work laid the foundation for subsequent genetic screens that have added novel insights into how p53 mediates these conserved processes (Fuhrman *et al.*, 2009; Gao *et al.*, 2008; Schumacher *et al.*, 2005; Sendoel *et al.*, 2010).

par0035

As two independent approaches for deciphering gene function, forward and reverse genetics can often complement each other. A notable example comes from studies on the *C. elegans* orthologs of two mammalian polycystin proteins, PKD1 and PKD2, which are defective in human autosomal dominant polycystic kidney disease (ADPKD), one of the most common monogenic human disorders, affecting 1 in 400–1000 individuals (Igarashi and Somlo, 2002). In a forward genetic screen for male mutants defective in the ability to locate the hermaphrodite vulva, Barr and Sternberg (1999) isolated a mutation in *lov-1*. Cloning the *lov-1* gene revealed it to be the ortholog of the human disease gene PKD1, which encodes a large transmembrane receptor-like protein (Harris and Torres, 2009; Xiao and Quarles, 2010). Barr and Sternberg found this gene to be exclusively expressed in male-specific sensory neurons. Loss of *lov-1* function displayed no phenotype in hermaphrodites, which is likely the reason this gene was not previously identified, as male-specific phenotypes, especially behavioral, are not often examined. With this knowledge of *lov-1* in hand, they employed a reverse genetic strategy and generated a deletion mutant of *pkd-2*, the worm ortholog of a second PKD disease gene, which encodes a transient receptor potential channel (Clapham, 2003). Strikingly, they found a similar mating defect in males (Barr *et al.*, 2001). Both *lov-1* and *pkd-2* localize to the ciliated endings of male-specific sensory neurons, the site of sensory mechanotransduction, but they are not required for ciliogenesis. This was important, as this study was the first to indicate that these genes may function in sensory transduction in cilia, a location where mammalian PKD1 and PKD2 genes were later found to reside and mediate mechanosensation (Nauli *et al.*, 2003; Pazour *et al.*, 2002; Yoder *et al.*, 2002). Dysfunction of these genes may cause ADPKD due to the inability of renal epithelial cells to sense fluid flow, which might alter various cell functions, including gene expression, growth, differentiation, and apoptosis (Nauli *et al.*, 2003).

sec0025

## 2. Direct Simple Screen

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Dissecting genetic pathways involved in a biological process usually starts from identifying functionally nonredundant components of those pathways. To search for these key players, direct simple screens are often used. In this type of screen, mutants

with desired phenotypes are isolated by direct inspection of descendants of mutagenized or RNAi-treated worms. For example, the very first direct simple screen using EMS as a mutagen in *C. elegans* was performed by Sydney Brenner with particular interests in mutants defective in coordinated movement (Brenner, 1974). He identified mutations in 77 genes affecting movement. Notably, one of these genes, *unc-6*, was later shown to be the ortholog of the vertebrate netrin gene, encoding an important extracellular cue directing axon outgrowth and broadly conserved across the animal kingdom (Harris *et al.*, 1996; Hedgecock *et al.*, 1990; Ishii *et al.*, 1992; Kennedy *et al.*, 1994; Lauderdale *et al.*, 1997; Mitchell *et al.*, 1996; Serafini *et al.*, 1994).

sec0030 3. Forward Screens with the Aid of the Green Fluorescence Protein (GFP)

par0045

In contrast to phenotypes observed in direct simple screens, many phenotypical changes, particularly cellular biological ones, are invisible at the behavioral and light microscope levels. For some phenotypes, this limitation can be overcome with fluorescent proteins. Fluorescent markers, in particular, green fluorescence protein (GFP) from the jellyfish *Aequoria victoria* (Chalfie *et al.*, 1994), have facilitated screens in many biological processes, including axon guidance (Zallen *et al.*, 1998), vesicle transportation (Grant and Hirsh, 1999; Grant *et al.*, 2001; Sato *et al.*, 2008), and synapse formation (Liao *et al.*, 2004; Shen and Bargmann, 2003; Zhen *et al.*, 2000). In these screens, GFP is utilized as a visual indicator of phenotypic alterations for identification of mutants, as it allows selective visualization of normally invisible proteins, subcellular structures, specific cells or tissues, and even gene transcription status by placing the GFP open reading frame downstream of genes' *cis*-regulatory regions. In these types of screens, worms that are engineered to transgenically express GFP are mutagenized and examined for changes in GFP expression levels or patterns. For instance, to investigate the mechanisms underlying left–right functional asymmetry of chemoreceptor gene expression between two morphologically symmetrical neurons, ASE left (ASEL) and ASE right (ASER), Chang *et al.* (2003) performed a screen on transgenic worms with GFP expression in either ASEL or ASER under the control of the cell-specific *cis*-regulatory regions. They identified mutations that lost left–right functional asymmetry by isolating mutants that symmetrically expressed GFP in both cells or neither. From this screen, they uncovered several microRNAs and transcription factors that formed a complex regulatory cascade directing left–right asymmetrical chemoreceptor gene expression, thus shedding light on chemosensory neuron differentiation (Chang *et al.*, 2004; Johnston and Hobert, 2003).

par0050

The manual isolation of mutants using fluorescence markers is often laborious, as it requires visual inspection of a large number of mutagenized worms at the microscopic level. Recently, an automated worm sorter (Complex Object Parametric Analysis and Sorter, COPAS), which is a flow cytometry machine used to sort worms based on their optical sizes, density, changes in color, and fluorescence intensity (Doitsidou *et al.*, 2008), has been developed to facilitate isolation of mutants. For

example, to study genes involved in executing the dopaminergic cell fate, Doitsidou *et al.* (2008) devised a worm-sorter-based screen using a transgenic strain with all dopaminergic neurons exclusively labeled by a cell type-specific GFP reporter. As a failure to execute dopaminergic cell differentiation can result in fewer GFP positive neurons, mutants with reduced GFP fluorescence were sought. To control for the variability in fluorescence intensities among individual worms, they introduced a broadly expressed red fluorescence protein (RFP) reporter into the transgenic strain as an internal reference for GFP/RFP ratiometric measurements. The worm sorter was accordingly set to detect a reduced GFP/RFP ratio. This screening strategy was highly sensitive as it allowed the identification of mutants lacking GFP expression in only one or two of the eight dopaminergic neurons. In comparison with a manual screen performed in parallel, the worm-sorter-based screen displayed a higher efficiency in isolating mutants, as ~50,000 individual worms per hour were screened – in contrast to ~1000 manually screened with a microscope. The automated screen identified 22 mutants over a few days, whereas the manual screen isolated 10 mutants over a few months.

sec0035 4. Enhancer Screens

par0055

It is estimated that only ~30% of the approximately 20,000 genes encoded in the *C. elegans* genome show a visible, lethal, or sterile phenotype after loss or reduction in function (Hodgkin and Herman, 1998; Johnsen and Baillie, 1991). The majority of genes are phenotypically silent upon loss of function under laboratory conditions. Sometimes, it is because the function of these genes can be compensated either by homologous genes with high structural and functional similarity or through buffering of regulatory networks via nonhomologous genes acting in related pathways (Hartman *et al.*, 2001; Wagner, 2000). These genes, which are thought to constitute a large part of most genetic pathways, are unlikely to be recovered in direct simple screens. Identifying these genes necessitates the loss of two or more genes simultaneously. One screening strategy to accomplish this task is the use of an enhancer screen, which is usually conducted on a starting strain with a defined phenotypic defect caused by a mutation in a single gene. Any gene whose functional disruption can enhance the defects of the starting perturbation is referred to as an enhancer of the starting mutant.

par0060

Two types of genetic interactions are possible between enhancers and a starting gene: (1) synergistic enhancement in which the combined severity is more than the sum of both single mutant phenotypes; (2) additive enhancement in which the severity of the combined defects equals to the sum of the individual defects. The type of genetic enhancement can be informative for constructing genetic pathways (see also discussion in Part III).

par0065

To devise an enhancer screen, genetic nature, particularly dosage effects (loss of function or reduction of function) of starting alleles used for enhancer screens should be taken into careful consideration because the dosage nature of the starting alleles affects the types of enhancer genes that can be identified.

## sec0040 Enhancer Screens Using Null Alleles

par0070

Enhancer screens using null alleles (mutations causing complete loss of function in the corresponding genes) as starting mutations are a widely used and effective way to isolate functionally redundant genes involved in the same biological process. This approach was used to study the negative regulation of a Receptor Tyrosine Kinase – Ras GTPase – Mitogen Activated Protein Kinase signaling cascade (RTK/Ras/MAPK) mediated by LET-23, an epidermal growth factor receptor (EGFR) like-tyrosine kinase, in vulval induction in *C. elegans* (Fig. 1). Activity of LET-23 is dampened by several functionally redundant negative regulators. A mutation in any individual regulator is phenotypically silent with regard to vulval induction but a combination of any two of them displays a hyperinduced-vulval phenotype due to increased activity of LET-23 (Sternberg *et al.*, 1994). To identify new LET-23 negative regulators that might be masked by this redundancy, Hopper *et al.* (2000) conducted an enhancer screen for the hyperinduced-vulval phenotype in the background of a null allele of *sl-1* (an ortholog of the Cbl family of ubiquitin ligases), a known negative regulator of LET-23, showing no phenotype on its own (Hopper *et al.*, 2000). This screen identified a novel negative regulator, *ark-1*, which encodes an ortholog of the Ack-related nonreceptor tyrosine kinase. This gene was later found to be a target of LIN-12/Notch lateral signaling and mediates the interaction between LET-23 signaling and LIN-12 pathway during their cooperative regulation of vulval cell-fate specification (Yoo *et al.*, 2004).

par0075

Although an enhancer screen using a null allele is powerful in identifying functionally redundant genes, it has limitations. As a null allele does not produce a protein to regulate downstream components or be influenced by gene products acting upstream, the linearity of the signaling relay is interrupted and the activity of the corresponding pathway is lost. Additional mutations in genes that act either upstream or downstream would not further enhance the initial phenotype of the null allele. Thus, an enhancer screen using a null allele is ineffective in identifying components that act upstream or downstream of the pathway where the starting null allele resides.

## sec0045 Enhancer Screens Using Hypomorphic Alleles

par0080

A hypomorphic mutation causes partial loss of gene function, which leads to a reduction in the activity of the encoded gene product in the signaling pathway in which this gene is involved. This reduction can be enhanced in various situations (Fig. 2): (1) loss or reduction in function of a gene that encodes a physical interacting partner of this hypomorphic allele; (2) loss or reduction in function of another gene acting in the same or the parallel pathways that functionally compensate for each other. Thus, an enhancer screen using a hypomorphic mutation can identify a broad range of interactors that function in either the same physical complex or the same pathway (upstream or downstream), as well as genes that act in redundant pathways. For example, in an enhancer screen using a hypomorphic allele of *lin-45*, a critical

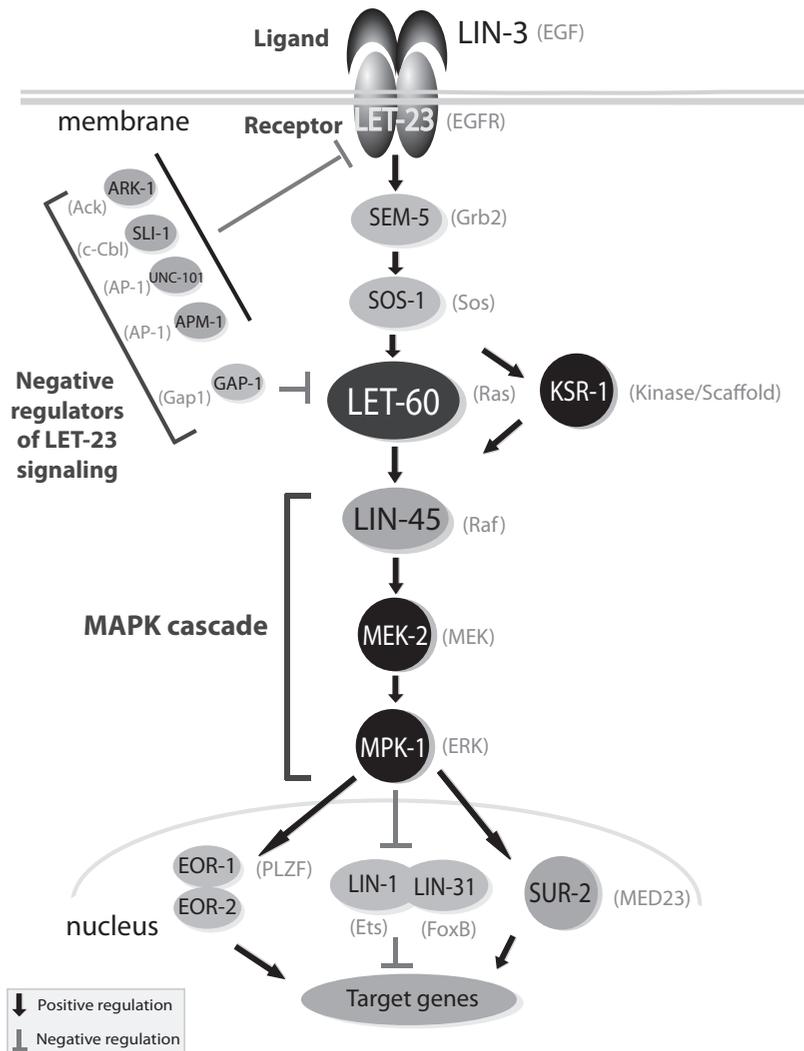


fig0005

**Fig. 1** The RTK/Ras/MAPK signaling pathway in vulval induction in *C. elegans*. The ligand, its receptor, and core signal transducers are indicated. LIN-3 encodes the worm epidermal growth factor (EGF) ligand and is secreted from the gonadal anchor cell. It binds to its receptor LET-23 on the vulval precursor cells which then dimerizes and undergoes autophosphorylation (Aroian *et al.*, 1990; Hill and Sternberg, 1992). SEM-5, an adaptor protein, binds phosphorylated LET-23 (Clark *et al.*, 1992) and recruits SOS-1 (Chang *et al.*, 2000), a guanine nucleotide exchange factor (GEF) that activates LET-60, a Ras GTPase. KSR-1, a putative scaffold protein, is required for robust activation of LET-60 downstream signaling (Kornfeld *et al.*, 1995b; Sundaram and Han, 1995). LET-60 activates a mitogen-activated protein kinase (MAPK) cascade, including LIN-45 (Raf), MEK-2 (MAPK kinase), and MPK-1 (MAPK) (Chong *et al.*, 2003; Han *et al.*, 1993; Kornfeld *et al.*, 1995a; Lackner *et al.*, 1994; Sternberg *et al.*, 1993; Wu *et al.*, 1995; Wu and Han, 1994). Activated MPK-1 inhibits LIN-1, a transcription factor forming a complex with LIN-31. Upon LIN-1 inhibition, LIN-31 is released to promote the acquisition of vulval cell fates (Tan *et al.*, 1998). MPK-1 also indirectly activates SUR-2, a mediator protein that positively regulates vulval cell fate (Singh and Han,

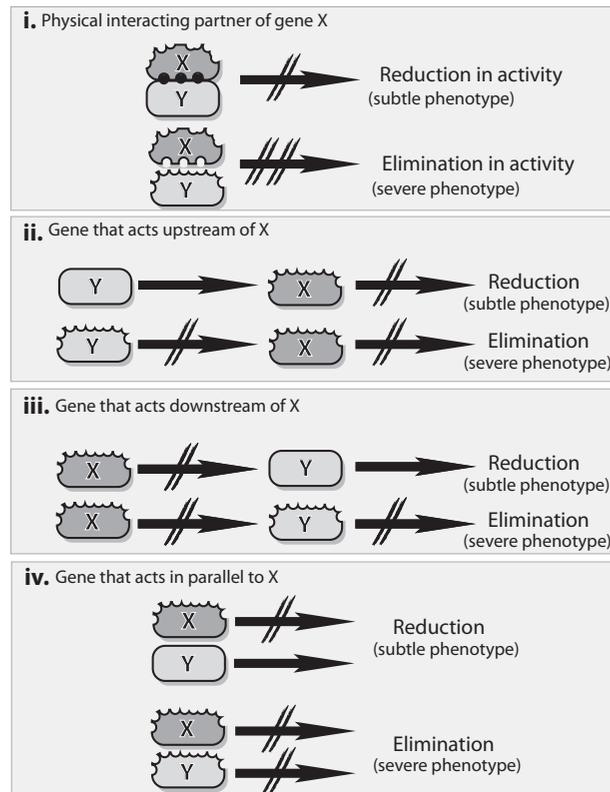


fig0010

**Fig. 2** Possible genetic interactions that can enhance the phenotype of a hypomorphic allele. A hypomorphic mutation (X) causes partial loss of gene function, which only slightly reduces the activity of a corresponding signaling pathway. This reduction can be dramatically enhanced by loss or reduction in the activity of a gene (Y) that encodes a physical interacting partner (i), acts upstream (ii), downstream (iii) of the same pathway, or functions in a parallel pathway that functionally compensates for each other (iv).

1995). EOR-1, a putative transcription factor related to the human oncogene PLZF (Hoepfner *et al.*, 2004), and EOR-2, a novel protein (Hoepfner *et al.*, 2004), act downstream or in parallel to MPK-1 and function redundantly with LIN-1 to regulate transcription of target genes (Howell *et al.*, 2010; Rocheleau *et al.*, 2002). LET-23 signaling is also regulated by several negative regulators, include ARK-1 (Ack) (Hopper *et al.*, 2000), SLI-1 (c-Cbl), which targets activated LET-23 for internalization and degradation (Rubin *et al.*, 2005; Swaminathan and Tsygankov, 2006), UNC-101 and APM-1, which encode medium chains of the AP-1 adaptin and promote LET-23 endocytotic recycling (Lee *et al.*, 1994; Shim *et al.*, 2000), and GAP-1, which stimulates LET-60 GTP hydrolysis (Hajnal *et al.*, 1997). More details about this pathway can be found elsewhere (Sundaram, 2006). The mammalian homologs of these genes are indicated in parentheses.

RTK-Ras-MAPK component encoding a Raf protein, Rocheleau *et al.* (2002) identified novel alleles of known components of this pathway that function either upstream or downstream of *lin-45*, including *sem-5*, *sos-1*, *lin-1*, and *ksr-1*, and alleles in two new components, *eor-1* and *eor-2*, likely acting downstream of or in parallel to *mpk-1* (Fig. 1). The spectrum of genes identified highlights the efficiency of using hypomorphic alleles in enhancer screens.

par0085

Another example showing the usage of hypomorphic alleles in identifying genes with redundant functions comes from a screen carried out by Schwabiuk *et al.* (2009). They discovered a novel function for a gene, *sdn-1*, in regulating migration of the distal tip cells (DTC) that lead to gonad extension. *sdn-1*, which encodes an ortholog of a type I transmembrane proteoglycan syndecan-2, was previously implicated in axon guidance (Rhiner *et al.*, 2005) and in epidermal enclosure (Hudson *et al.*, 2006). Its new functional role in DTC migration was found in a screen for mutations that enhanced the DTC migration defects caused by a hypomorphic allele of *unc-5*, which encodes a receptor for the axon guidance cue, netrin. *sdn-1* would not otherwise have been identified in simple screens using wild-type worms or enhancer screens using a null allele of *unc-5*, because the ensuing functional characterization revealed that all *sdn-1* alleles (null and hypomorphic) were phenotypically silent in DTC migration on their own and importantly no enhancement was observed in the double mutants between an *unc-5* null allele and any of *sdn-1* alleles, indicating that *sdn-1* function is linked to *unc-5* activity.

Q1

par0090

For some enhancer screens, temperature-sensitive alleles (“hypomorphic” equivalents) can also be used. These alleles often display incomplete phenotypic penetrance at intermediate temperature between restrictive and permissive temperatures. Additional mutations that increase original penetrance of phenotypes at intermediate temperature are isolated and cloned. This temperature strategy has been used in a number of enhancer screens, such as a screen to identify genes involved in the neddylation process (Dorfman *et al.*, 2009), as well as LIN-12/Notch signaling (Qiao *et al.*, 1995).

#### sec0050 Synthetic Lethality Screen Using Extrachromosomal Arrays

par0095

In some enhancer screens, the combination of two mutations can cause synthetic lethality: the disruption of a single gene displays no discernable or a very subtle phenotype, whereas simultaneous disruption of two or more genes causes lethality. Synthetic lethality poses a challenge for mutation recovery as it is usually unknown whether a starting mutation has synthetic lethal partners. One way to recover synthetic lethal mutations is to devise an enhancer screen using a starting mutant carrying an extrachromosomal array that rescues potential lethality (Fig. 3). These arrays are transgenes introduced into worms by gonad microinjection (Mello and Fire, 1995). Due to their extrachromosomal nature, these transgenes are stochastically lost during meiosis and mitosis, and are only expressed in the progeny or daughter cells containing them. For enhancer screens, the transgenes carried by the starting mutant are often designed to express two proteins simultaneously: (1) a wild-type copy of a starting-mutation-harboring gene for avoiding synthetic

lethality; (2) a dominant visible marker (e.g., GFP) driven by a ubiquitous promoter to indicate the presence of the transgenes. The progeny of this transgenic strain will be comprised of two populations: the marker-positive worms (*transgene*<sup>+</sup>) and the marker-negative worms (*transgene*<sup>-</sup>), the frequency of each being dependent on the frequency of stochastic loss of the extrachromosomal array. If a synthetic lethal mutation were to be introduced into one of these transgene-containing worms, only progeny containing the transgene would be viable, as the marker-negative population would be all dead due to the absence of an extrachromosomal array expressing the wild-type protein. Conversely, both *transgene*<sup>+</sup> and *transgene*<sup>-</sup> progeny would be present and viable with nonlethal enhancers.

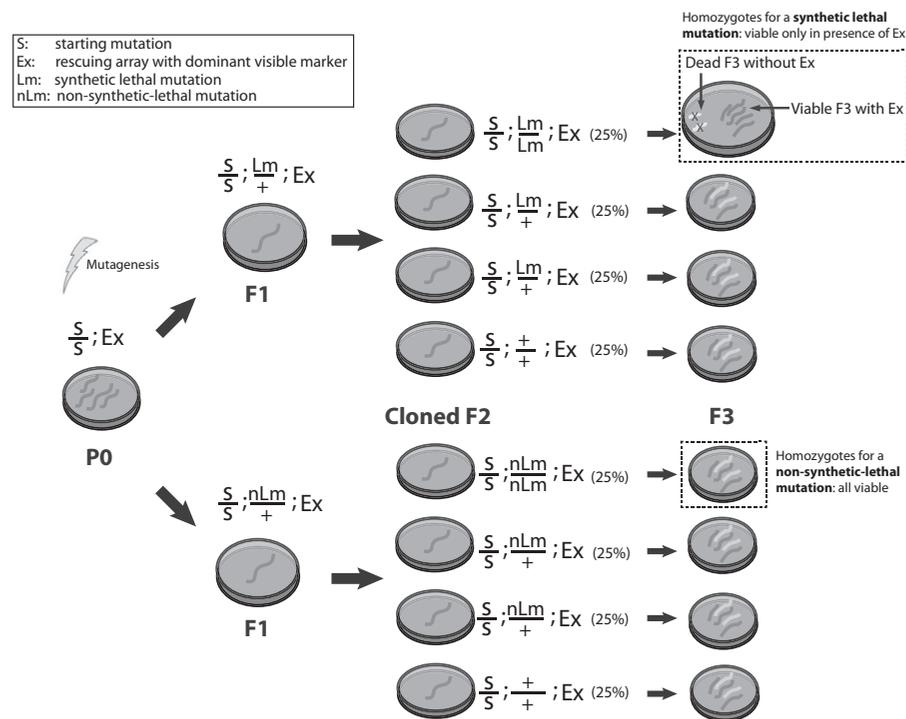


fig0015

**Fig. 3** Synthetic lethality screen using extrachromosomal arrays. The starting strain (P0) with an initial mutation (S) and a rescuing array containing a dominant visible marker (Ex) is mutagenized. The progeny (F1) carrying the visible marker (green) are cloned onto individual plates. As an F1 worm is heterozygous for a recessive mutation, only 25% of the progeny (F2) will be homozygous. Thus, several F2 progeny carrying the marker from each F1 plate are then cloned onto individual plates (here, we show four F2 worms per F1). The progeny (F3) of each cloned F2 are inspected for the presence of the marker. For an F2 that is homozygous for a synthetic lethal mutation (Lm), all viable F3 progeny will be marker-positive (green) because the progeny lacking the marker (white) will be dead (dotted box). In the case of F2 worms derived from an F1 worm carrying a nonlethal mutation (nLm), the progeny F3 of each F2 worm will be both marker-positive (green) and marker-negative (white). This screening process is also known as an F2 clonal screen.

par0100

Using this screening strategy, Fay *et al.* (2002) discovered that the gene *fzr-1* functions redundantly with *lin-35*, the *C. elegans* ortholog of the Rb (Retinoblastoma protein) tumor suppressor gene, in controlling cell proliferation. Unlike Rb knockout mutants in flies and mice (Clarke *et al.*, 1992; Du and Dyson, 1999; Jacks *et al.*, 1992), worms with strong loss of function of *lin-35/Rb* are viable, and display relatively subtle defects in development (Fay *et al.*, 2002; Lu and Horvitz, 1998), suggesting the existence of genes acting redundantly with *lin-35*. To identify these genes, they performed a synthetic lethality screen on *lin-35* mutants carrying an extrachromosomal array expressing the wild-type LIN-35 protein and a ubiquitously expressed GFP marker for tracking ~~the array and worms~~. After mutagenesis, F3 worms derived from each of 10,000 single cloned F2 (four F2 per F1) were examined for the presence or absence of the GFP marker (see Fig. 3 for details). They ~~then~~ recovered seven mutations defining seven distinct loci. The gene characterized in this study was *fzr-1*, encoding a protein orthologous to a regulatory subunit of the anaphase-promoting complex required for anaphase initiation and exit from mitosis (Dawson *et al.*, 1995; Schwab *et al.*, 1997; Sigrist *et al.*, 1995). In addition to synthetic lethality, the double mutants of *fzr-1* and *lin-35* also showed extensive tissue hyperproliferation affecting a wide range of cell types, indicating new functional roles of *lin-35*. As hyperproliferation caused by mutations in mammalian Rb genes is an important genetic event during multi-step carcinogenesis, this study provided support that uncontrolled proliferation in *C. elegans* follows the same genetic pattern of oncogenesis in mammals and revealed a possible connection of this process with the anaphase-promoting complex.

sec0055

## 5. Suppressor Screens

par0105

Suppressor screens are a powerful way to identify interacting genes that regulate biological processes. The “suppression” design enables this type of screen to bypass synthetic lethality, which can be a limitation of an enhancer screen. A suppressor refers to a gene whose dysfunction (loss, reduction, or gain of function) can suppress the well-defined phenotypic defect of a mutation in another gene (starting allele). Prior to screening, it is of great importance to decide the appropriate starting alleles for suppression. Alleles identified in direct simple screens often exhibit highly penetrant phenotypic defects, which may limit their utility in enhancer screens but makes them potentially ideal starting alleles for suppressor screens. Second, the genetic nature (null, hypomorphic, or hypermorphic, i.e., gain of function) of a starting allele should be taken into careful consideration as it determines the types of mutants that can be recovered from the screen.

sec0060

## Suppression of a Null Allele

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Suppression of a null allele in a gene of interest allows isolation of mutations in genes that bypass the need for the original gene. In addition, the screen can also identify gain-of-function (*gf*) mutations in downstream genes that are positively

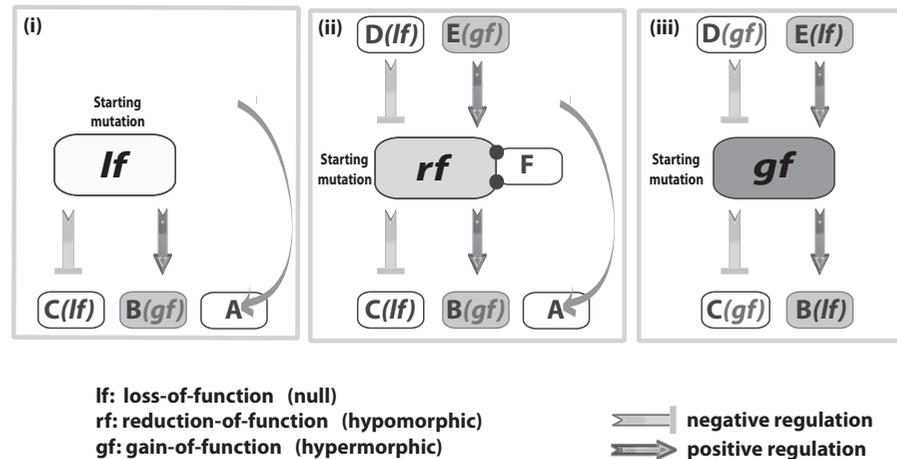


fig0020

**Fig. 4** The choice of starting mutations affects the types of mutations recovered from suppressor screens. (i) A suppressor screen using a loss-of-function (*lf*) mutation as a starting mutation allows isolation of mutations (A) in genes that bypass the gene of interest, gain-of-function (*gf*) mutations (B) in downstream genes that are positively regulated by the gene of interest, and loss-of-function (*lf*) mutations (C) in downstream genes that are negatively regulated by the pathway. (ii) In addition to the similar spectrum of mutations recovered from suppression of a null mutation, suppression of a reduction-of-function (*rf*) mutation can identify *lf* mutations (D) in upstream negative regulators and *gf* mutations (E) in upstream positive regulators, and mutations (F) in direct physical interactors. (iii) Suppression of a *gf* mutation can obtain a wide range of mutations with attributes opposite to those identified in suppression of a *lf* mutation.

regulated by the gene of interest as well as loss-of-function (*lf*) mutations in downstream genes that are negatively regulating the pathway (Fig. 4i).

par0115

Suppressor screens using ~~starting~~ null mutations are in general not effective in recovering mutations in genes acting upstream of a pathway or directly interacting with the starting genes, because null alleles produce no protein products for upstream genes to regulate and for direct interactors to modulate. A way to identify genes that act upstream and direct interacting genes is to use hypomorphic alleles as the basis of a suppressor screen.

sec0065

#### Suppression of a Hypomorphic Allele

par0120

Suppression of hypomorphic alleles, that is, reduction-of-function (*rf*) mutations, not only yields the range of mutations similar to suppression of null mutations, but also allows recovery of mutations in upstream genes, such as *lf* mutations in upstream negative regulators and *gf* mutations in upstream positive regulators, and mutations in genes directly interacting with the gene of interest (Fig. 4ii). For example, UNC-4, a homeodomain protein, regulates synaptic connectivity of VA motor neurons, which mediates backward movement in *C. elegans*. *unc-4* mutants

are unable to crawl backwards. To identify gene products that directly interact with UNC-4, Miller *et al.* (1993) screened for mutants that suppressed a hypomorphic temperature sensitive allele of *unc-4* and successfully identified a *gf* mutation in a gene, *unc-37*, which was found to encode a Groucho-like protein forming a complex with UNC-4 to regulate transcription (Winnier *et al.*, 1999). Importantly, this mutation in *unc-37* was not able to suppress *unc-4* null alleles, suggesting that this suppression was dependent on UNC-4 activity.

sec0070      Suppression of a Hypermorphic Allele

par0125

Suppression of hypermorphic alleles, that is, *gf* mutations, yields a spectrum of alleles in genes with attributes opposite to those identified in suppression of *lf* mutations (Fig. 4iii). This type of suppression is a powerful way to identify components of a biological pathway. A good example comes from the highly conserved RTK/Ras/MAPK pathway, which is involved in vulval induction in *C. elegans* (see Fig. 1). The worm Ras gene, *let-60*, is a crucial component of this pathway. Gain-of-function mutations in *let-60* cause a multiple-vulva (Muv) phenotype. A group of key components acting downstream of *let-60*, including *lin-45* (the worm Raf gene) (Hsu *et al.*, 2002), *mpk-1* (the worm ERK gene) (Wu and Han, 1994), *mek-2* (the worm MEK gene) (Wu *et al.*, 1995), *ksr-1* (a *C. elegans* kinase suppressor of Ras) (Sundaram and Han, 1995), and *sur-2* (Singh and Han, 1995), were all identified in screens for suppressors of the Muv phenotype caused by a *gf* allele of *let-60*.

sec0075      Suppression of Engineered Gain-of-Function (*gf*) Alleles

par0130

*gf* mutations used for suppressor screens are not limited to genetically defined alleles. By engineering into worms transgenes (“artificial *gf* alleles”) expressing mutant proteins, one can produce a phenotype that facilitates selection and identification of suppressors. For instance, Zheng *et al.* (2004) devised a suppressor screen hunting for genes interacting with an important class of neurotransmitter receptor, ionotropic glutamate receptors (iGluRs), that mediate most excitatory synaptic signaling between neurons (Zheng *et al.*, 2004). The screen was performed in a transgenic strain engineered to express a non-N-methyl-d-aspartate (NMDA) type ionotropic glutamate receptor (GLR-1) subunit containing a dominant mutation. Expression of this transgene under the control of the *glr-1* promoter resulted in a hyper-reversal phenotype, that is, transgenic animals show a higher frequency of reversing direction during movement than wild-type worms. By searching for mutations suppressing this phenotype, Zheng and colleagues identified a gene encoding a type I transmembrane protein, SOL-1, that can bind to GLR-1 and participate in the gating of non-NMDA iGluRs.

sec0080      Suppressors of Engineered Pathological Processes

par0135

Greater than 40% of human-disease-related genes have clear *C. elegans* orthologs (Culetto and Sattelle, 2000). *C. elegans* has been used to model human diseases,

including human ADPKD (Barr *et al.*, 2001; Barr and Sternberg, 1999), muscular dystrophy (Kim *et al.*, 2004), cancer (Bergamaschi *et al.*, 2003; Polanowska *et al.*, 2004), diabetes and obesity (Pierce *et al.*, 2001), and neurodegenerative diseases (Lakso *et al.*, 2003). The applications of *C. elegans* for various disease models have been extensively reviewed (Dimitriadi and Hart, 2010; Kaletta and Hengartner, 2006; Kirienko *et al.*, 2010). With the unique genetic advantages of *C. elegans*, large-scale screens that are impractical in vertebrates can be readily performed in *C. elegans* to identify highly conserved genes that may modulate human diseases. To identify those genes, suppressor screens are often performed on a transgenic strain generated to resemble a pathological process of interest. For example, one hallmark of some notable neurodegenerative diseases is the abnormal aggregation of proteins, such as wild-type or mutated tau protein that normally functions to stabilize microtubules and promote their polymerization. The aggregation of tau is seen in a group of neurodegenerative diseases, including Alzheimer's disease and frontotemporal dementia with parkinsonism chromosome 17 type (FTDP-17T) (Lee *et al.*, 2001). To identify genes participating in tau neurotoxicity, Guthrie *et al.* (2009) carried out a forward genetic screen for suppressors of the Unc (uncoordinated movement) phenotype caused by accumulation of exogenous mutated human tau in a transgenic strain that was engineered to express this tau protein in all neurons. Using this transgenic worm as a model of human tauopathy disorders, they revealed that loss of function in a gene, *sut-2*, which encodes a highly conserved subtype of CCCH zinc finger protein, was able to suppress tau neurotoxicity. The identification of this gene suggested a novel neuroprotective strategy to interrupt tau pathogenesis (Guthrie *et al.*, 2009).

sec0085      Some Considerations Regarding Nature of Suppressors

par0140

There are two specialized types of suppression that can arise when performing a suppressor screen, which may not provide insight into the biological processes of interest. These are important to be aware of when interpreting results of suppressor screens. One is informational suppression caused by mutations in genes involved in the general machinery of transcription, RNA processing, and protein translation. This suppression is allele-specific, gene-nonspecific. A large number of EMS-induced null alleles are nonsense point mutations causing early stop codons. Suppressor screens using these nonsense alleles can produce tRNA mutations that recognize stop codons as sense codons so that the starting allele can be translated to the protein with biological activity. As an informational suppressor is allele-specific, it may not suppress the phenotype of other alleles of the gene of interest, which provides a good way to test if a suppressor mutant is an informational mutation. Similarly, mutations of components in the nonsense decay system that is responsible for degrading premature mRNA can also suppress the phenotype of the starting mutation. Informational mutations are valuable for studies on regulation of transcription, RNA processing, and translation; however, they do not provide insights into the genetic networks controlling the biological processes likely to be of interest in the screens.

par0145

Another type of suppression is referred to as intragenic suppression, where a suppressor mutation in the same gene that harbors the starting mutation reverses its defects. For example, the suppressor may introduce a functional favorable mutation into the protein, which may compensate for the reduction of activity caused by the original deleterious mutation. Sometimes, intragenic suppressor mutations can affect splicing, which causes a skip of the original mutation and produces a protein with biological activity. More details on this topic can be found in a review by Hodgkin (2005).

sec0090

## 6. Selection Screens

par0150

Depending on the design of the genetic screen, the isolation of mutants can be laborious. Many screens require careful examination of nearly every single worm for the presence or absence of a desired phenotype. For example, in the previously mentioned screen for suppressors of an *unc-4* allele, 500,000 progeny of mutagenized worms were individually tapped on the head with a platinum pick to test if the worms were able to move backward (Miller *et al.*, 1993). Selection screens are designed to rapidly facilitate identification of mutants with a specific phenotype by eliminating animals that do not carry a desired mutation. A drug screen is one type of selection screen in which mutants resistant to a particular drug will be readily selected because all other worms are either killed or display a specific phenotype when lacking resistance. For example, acetylcholine, a neurotransmitter, is released from synaptic vesicles at neuromuscular junctions, where it induces muscle contraction. Acetylcholine is normally degraded by the enzyme acetylcholinesterase. Pesticides, such as Aldicarb, block the activity of this enzyme and cause accumulation of acetylcholine, which ultimately kills animals, including worms, because of excessive excitation of muscles. A screen searching for mutants that are resistant to Aldicarb (e.g., viable in its presence) identified *unc-17*, which was later cloned and found to encode a broadly conserved acetylcholine transporter involved in uptake of acetylcholine into synaptic vesicles (Alfonso *et al.*, 1993; Brenner, 1974).

par0155

Inducible transgenes can also be used for selection screens, where a transgene engineered into a strain induces a particular phenotype that when suppressed enables easy selection of corresponding mutations. For example, GOA-1, a *C. elegans*  $\alpha$ -subunit of the major heterotrimeric G protein in the nervous system, regulates many behaviors, including locomotion and egg laying (Hajdu-Cronin *et al.*, 1999). To identify genes that interact with G<sub>o</sub> signaling, Hajdu-Cronin *et al.* (1999) generated a transgenic strain overexpressing a constitutively active GOA-1 mutant protein under the control of a heat-shock promoter. Upon heat shock, this strain displays a severe phenotype, paralysis. By screening for mutants that restored locomotion, they identified two regulators of G<sub>o</sub> signaling, *dgk-1* (first identified and named as *sag-1* in the study), which encodes a diacylglycerol kinase (Miller *et al.*, 1999; Nurrish *et al.*, 1999), and *eat-16*, which encodes a protein orthologous to the mammalian regulators of G protein signaling (RGS) 7 and RGS9 (Hajdu-Cronin *et al.*, 1999).

par0160

In addition, selection screens can be conducted using temperature sensitive (*ts*) lethal mutants. Growing such mutants at restrictive temperature usually induces embryonic or larval lethality. In screens for suppressors of lethality of *ts* alleles, mutants that restore the viability of embryos or larvae can be easily identified. For instance, PAR (partitioning defective) proteins, first identified in forward genetic screens in *C. elegans*, are highly conserved regulators of cell polarity and asymmetric cell division (Kemphues *et al.*, 1988). To gain insight into the precise mechanisms by which PAR-1, a Ser/Thr kinase, regulates embryonic asymmetric cell division, Spilker *et al.* (2009) performed a genome-wide RNAi screen on a temperature sensitive *par-1* allele and identified several genes that when their activity is reduced specifically suppress the embryonic lethality of *par-1*. One of the identified suppressors was *mpk-1*, which encodes a mitogen-activated protein (MAP) kinase. Reduced activity of *mpk-1* restored the asymmetric distribution of cell-fate specification markers in *par-1* mutants. In addition, disrupting the function of other components of the MAPK signaling pathway also suppressed *par-1* embryonic lethality. These results revealed that MAP kinase signaling is involved in antagonizing PAR-1 activity during early *C. elegans* embryonic polarization.

sec0095

## 7. Sequential Screens

par0165

The specific phenotypes of a biological process of interest may not always be suitable for large-scale forward genetic screens. One common reason is the difficulty in observing the phenotype. To make genetic screens applicable for such a biological process, it is often possible to score a more readily detected phenotype, such as lethality or uncoordinated movement (primary screen), that allows isolation of a broader scope of mutations including those specific for the process of interest. The specific mutations are then identified through a secondary screen. A good example using a sequential strategy was a screen aimed at identifying genes involved in regulation of presynaptic terminal formation. This was accomplished by seeking suppressors of a mutation in the RING finger/E3 ubiquitin ligase gene *rpm-1*, a key regulator of synapse formation (Nakata *et al.*, 2005). Mutations in *rpm-1* result in a disorganized presynaptic structure but they cause no defects in locomotion. Although this phenotype can be observed with fluorescent synaptic markers, its subcellular microscopic-level nature limits the screening scale. To facilitate the identification of genes interacting with *rpm-1*, they devised a sequential screen for suppressors of an *rpm-1* mutation. In the primary screen, instead of screening solely in the *rpm-1* mutant background, they screened for suppressors of a set of easily scored phenotypes, severe defects in locomotion, and reduction in body size, caused by introducing (with *rpm-1*) a mutation in a synaptogenesis gene, *syd-1*. The SYD-1 protein regulates the distribution of presynaptic components and when mutated leads to mild defects in locomotion and egg-laying (Egl) behavior. Specific suppressors of *rpm-1* could be identified by restoration of locomotion and body size but not the Egl phenotype of the *syd-1* mutation. The alternative easily scored phenotypes used for suppression in the primary screen allowed a large

screening scale. As suppressors isolated in the primary screen may not all be specific to loss of *rpm-1* activity, a secondary screen at the microscope level was then conducted to identify *rpm-1*-specific suppressors that restored synaptic morphology using a synapse-specific fluorescent marker. The highlight from this screen was the identification of three MAP kinases, *dlk-1* (MAPKKK), *mkk-4* (MAPKK), and *pmk-3* (p38-like MAPK), which were found to form a previously uncharacterized p38 MAP kinase cascade that is negatively regulated by RPM-1 during synapse formation. The success of this screen speaks to the specificity and utility of a sequential screening strategy when designed appropriately.

## sec0100 8. Evaluation and Limitations of Forward Genetic Screens

par0170

Regardless of various screening designs, a high-quality screen should be able to identify many or most of the nonredundant components of a biological pathway of interest. A key question that then arises is how to determine the degree of saturation (all genes that can be mutated to display a specific phenotype) that a screen reaches. Empirically, if a screen is saturated, (1) mutations in the same gene, particularly in small-size genes that usually are less frequently hit, will be repeatedly isolated. This will be reflected by the fact that multiple alleles fail to complement each other in a complementation test, which is a genetic experiment to determine if two alleles reside in the same gene by comparing the phenotype of transheterozygotes of these two alleles with that of homozygotes for each allele. If the phenotypes of transheterozygotes and homozygotes are the same, it indicates that the two alleles fail to complement each other, suggesting that the two alleles likely correspond to the same gene. Conversely, if a screen is not saturated, it is common to see that each mutation defines a distinct locus. For example, in the *lin-35/Rb* synthetic lethality screen described in Section II.A.4.3, Fay and his colleagues recovered seven mutations defining seven distinct loci, indicating the screen was not saturated. (2) Unusual hypomorphic alleles of lethal genes will be identified that normally are less likely to be recovered than null alleles because these alleles require changes in specific amino acids. Besides qualitative judgment, the degree of saturation can also be analyzed in a quantitative manner. A statistic method using Bayesian and maximum-likelihood calculation may be used to estimate the number of alleles that remain to be found (Pollock and Larkin, 2004).

par0175

Many components of genetic pathways have been identified through mutagenesis-based forward genetic screens. However, as an experimental approach, forward genetic screens have intrinsic weaknesses in identifying some pathway components. Even though a forward genetic screen can be performed at a large scale, many genes may still be missed or rarely hit for several reasons: (1) small-size genes may be missed because they are too small to be effective targets for mutagenesis; (2) genes with pleiotropic functions might not be identified because they preferentially give a phenotype that masks their other functions (Jorgensen and Mango, 2002); (3) genes that when mutated confer early lethality often prevent identification of their later functions; (4) functional redundant genes that ensure robustness and plasticity to

biological processes often have no observable phenotypes when individually mutated (Wagner, 2000). Although an enhancer screen can overcome some redundancy, this approach does not always exhaust its multiple layers. RNAi-mediated forward genetic screens can also bypass substantial redundancy and lethality by causing partial loss of gene function; however, RNAi has its own limitations. RNAi phenotypes are often variable in penetrance and RNAi is ineffective in neurons (Tavernarakis *et al.*, 2000; Tewari *et al.*, 2004). To find genes that are not easily identified through forward genetic screens, functional genomic and systems-level approaches that complement conventional genetic screens can be used.

sec0105  
par0180

## B. Functional Genomic Approaches to Identify Components of Pathways

The completion of sequencing *C. elegans*, *D. Melanogaster*, and *H. sapiens* genomes along with rapidly evolving high-throughput techniques have changed the methodological ways biologists study gene function and dissect genetic pathways. The genome-sequencing project in *C. elegans* revealed a significant number of novel genes with unknown function. Undoubtedly, these genes are involved in a wide variety of biological functions. To decipher their function, traditional forward genetic screening still remains useful but now functional genomic studies can also be utilized. Functional genomics uses high-throughput approaches, such as genome-wide RNAi, DNA microarray, Serial Analysis of Gene Expression (SAGE), *cis*-regulatory analysis, yeast-two-hybrid/yeast-one-hybrid techniques, and mass spectrometry, to acquire information about genome-wide patterns of gene expression, protein–DNA interactions, and protein–protein interactions. By analyzing this information, biologists can begin to elucidate the organization and regulation of genetic pathways at a global level. Nevertheless, during analysis, experimental validation using conventional single-gene genetic approaches, including genetic perturbation analysis and reverse genetic approaches, is indispensable for confirming results obtained from functional genomic studies. The combination of functional genomic approaches with conventional methods has emerged as an effective way to gain a more complete understanding of the gene networks that guide biological processes (see also reviews by Grant and Wilkinson, 2003; Kim, 2001; Piano *et al.*, 2006).

sec0110  
par0185

### 1. Genome-Wide RNAi Screens

RNAi is an endogenous cellular process during which double-stranded RNA (dsRNA) complementary to sequences of target messenger RNAs (mRNA) mediates degradation of these mRNAs, resulting in reduction of expression of corresponding genes (Boutros and Ahringer, 2008). Since RNAi was discovered (Fire *et al.*, 1998), it has rapidly been adopted as an experimental means to silence expression of genes in a range of organisms (Boutros and Ahringer, 2008; Gildsorf *et al.*, 2010). In *C. elegans*, RNAi assays can be conveniently carried out by feeding worms with bacteria containing dsRNA constructs (Ahringer, 2006), soaking them in nematode growth media containing these bacteria (Lehner *et al.*, 2006), or injecting dsRNA

into gonads of young adult hermaphrodites to obtain progeny with mutant phenotypes. Essentially, RNAi can be used, instead of a mutagen, for variously designed forward genetic screens already discussed. RNAi-mediated forward genetic screens have some unique advantages. For instance, the identity of genes whose inactivation causes phenotypes is immediately known, in contrast to the time-consuming cloning of mutation-harboring genes from forward mutagenesis screens. RNAi also has temporal flexibility. It can be applied to animals at different developmental stages to avoid embryonic or larval lethality caused by inactivation of corresponding genes at the early developmental stages. Moreover, RNAi usually results in reduction of function of gene activity rather than complete loss, which allows effective investigation of the roles of essential genes (Kemphues, 2005).

par0190

There are currently two RNAi feeding libraries available for *C. elegans* research. One library constructed by the Vidal lab has 11,511 clones containing full-length gene cDNAs that were cloned into a double T7 vector by the Gateway cloning method (Rual *et al.*, 2004). This library is commercially named the *C. elegans* ORF-RNAi Collection V1.1, available through Open Biosystems. The other library was constructed by the Ahringer lab and has 16,757 clones containing the genomic sequences of genes (Fraser *et al.*, 2000; Kamath *et al.*, 2003). This library is commercially available through Geneservice. With the availability of two RNAi libraries, which together target 94% of *C. elegans* genes (Ahringer, 2006), RNAi screens are often carried out at a genome-wide scale and more frequently in an automated high-throughput fashion. For example, to identify genetic interactors with the RTK/Ras/MAPK pathway, Lehner *et al.* (2006) performed an RNAi screen in the background of loss-of-function mutations in the 12 known components of the RTK/Ras/MAPK pathway (Kamath *et al.*, 2003). To perform this screen in a high-throughput manner, the RNAi was delivered in 96-well plates in which mutants were soaked in a liquid containing RNAi feeding bacteria. They screened for RNAi clones that produced synthetic lethality with any of these 12 known components. Notably, 16 genes that had no previously reported roles in RTK/Ras/MAPK signaling were found to genetically interact with two or more components of the pathway. Nine out of these 16 genes were shown to regulate RTK/Ras/MAPK signaling during vulval induction (see Fig. 1), perhaps the best-characterized function of this pathway. This study highlights how high-throughput functional genomic approaches can rapidly identify new components of a specific pathway.

par0195

The growing number of large-scale RNAi studies carried out in *C. elegans* have produced a wealth of RNAi-induced phenotypic information. This information is being deposited into online databases, such as Wormbase (Harris *et al.*, 2010; Rogers *et al.*, 2008), RNAiDB (Gunsalus *et al.*, 2004), and PhenoBank (Sonnichsen *et al.*, 2005), to facilitate gene function studies. For example, using these databases, our group narrowed our search for genetic regulators of anchor cell invasion in a sequential RNAi screen (Matus *et al.*, 2010). Anchor cell invasion through basement membranes, which mediates formation of uterine–vulval attachment in *C. elegans*, has been used as a simple *in vivo* model for investigating cell invasion (Sherwood, 2006; Sherwood *et al.*, 2005; Ziel *et al.*, 2009). As a failure of anchor cell invasion

causes a Protruding-vulva (Pvl) phenotype, we first compiled a list of 539 genes whose reduction in activity was reported to result in the Pvl phenotype from a number of whole-genome RNAi screens (Kamath *et al.*, 2003; Rual *et al.*, 2004; Simmer *et al.*, 2003). We then performed a focused RNAi screen on these genes by examining anchor cell invasion using differential interference contrast (DIC) optics. Through these efforts, we identified 99 genes that are required for anchor cell invasion. Most of these genes have not been previously implicated in cell invasion, potentially expanding new targets for cancer therapeutics.

sec0115      2. Gene Expression Profiling Approach

par0200

Examination of gene expression at a genome-wide scale within the whole organism, specific tissues, and even single cells has proven to be a valuable approach in identifying pathway components and characterizing gene function (Grant and Wilkinson, 2003). Genes responsible for the same biological process tend to be regulated in a similar manner. By profiling gene expression changes associated with a biological process, it is possible to identify a common set of genes whose expression dynamics and spatiotemporal localization share the same pattern under different conditions and in various mutant backgrounds.

par0205

A way to measure expression levels of genes is to quantify transcripts of corresponding genes, which can now be readily achieved through a variety of high-throughput technologies at a genome-wide level, including hybridization-based approaches and sequence-based approaches (Wang *et al.*, 2009). A typical example of hybridization-based approaches is a DNA microarray assay that profiles expression of individual genes at a genomic scale through hybridization of oligonucleotide DNA probes with fluorescently labeled cDNAs of nearly every gene. Unlike hybridization techniques, sequence-based approaches obtain quantitative gene expression data by sequencing gene transcripts. For example, SAGE, a sequencing-based method, quantifies gene expression by counting the number of times a particular transcript is found in a pool of short diagnostic sequence tags isolated from an mRNA sample (Velculescu *et al.*, 1995). Recently, with advances in deep sequencing technologies, RNA-Seq, a new high-throughput and more precise sequencing-based method, allows quantification of all transcripts by directly sequencing fragmented cDNA (30–400 bp) converted from a population of RNA (Wang *et al.*, 2009). In addition to acquiring information about levels of gene expression, a project aimed to profile spatiotemporal patterns of gene expression at a large scale (localizome) has been initiated (Dupuy *et al.*, 2007). In this project, worms have been engineered to express transgenes in which the open reading frame of GFP was placed downstream of the proximal promoters of 1610 predicted genes. The expression of GFP from these promoters has been characterized using a worm sorter that profiles tissue expression at various developmental stages in a high-throughput fashion. The relevant expression data can be found at the web site <http://localizome.dfci.harvard.edu/>. The ultimate goal of this project is the characterization of all of the ~20,000 genes in the *C. elegans* genome.

par0210

Excellent examples of how expression analyses have facilitated identification of the genetic networks controlling diverse biological processes include aging (Budovskaya *et al.*, 2008; Murphy *et al.*, 2003), development (Baugh *et al.*, 2009), and innate immunity (Styer *et al.*, 2008). Illustrating its utility in the aging field, Budovskaya *et al.* (2008) recently discovered a development-related transcriptional circuit that guides the aging process. By comparing DNA microarray profiles, they identified a common set of 1254 genes that showed age-dependent expression changes (both upregulated and downregulated) during normal aging. This pattern of gene expression was found to be shared with ~~that associated with~~ dauer larvae (developmentally arrested worms whose life spans are ten times longer than normal worms), and longevity mutants displaying either extended or shortened life spans. For example, genes that show increased expression with age tend to have increased expression in dauer larvae and long-lifespan mutants, but show decreased expression in short-lifespan mutants. To search for transcription factors that regulate these age-dependent expression changes, they analyzed the upstream regulatory regions of these 1254 genes and identified a common consensus motif recognized by a GATA transcription factor, *elt-3*, in 602 of them. The importance of *elt-3* was validated by showing that RNAi depletion of *elt-3* activity resulted in decreased expression of 12 representative GATA-site-containing age-regulated genes. The expression of *elt-3* itself over the normal life span was negatively regulated by two other GATA transcription factors, *elt-5* and *elt-6* (Fig. 5), which were previously known to function with *elt-3* to regulate hypodermis differentiation in embryos (Gilleard *et al.*, 1999; Gilleard and McGhee, 2001). Consistent with a role for these transcription factors in regulating longevity, ~~*elt-3* was found to be required for longevity, as loss of function of *elt-3*~~ suppressed the long-lifespan phenotype of a longevity mutant of *daf-2* (encoding a *C. elegans* insulin/IGF receptor), whereas *elt-5* or *elt-6* promoted aging as reduction in activity of either *elt-5* or *elt-6* caused lifespan extension. Thus, using a combination of transcriptional profiling, *cis*-regulatory analysis, and reverse genetic approaches for validation, this study identified a development-related transcriptional circuit consisting of three GATA transcription factors and revealed its novel role in regulating aging late in life.

sec0120

### 3. Protein Interaction Screens

par0215

Many genetic interactions are realized in the form of direct protein–protein interactions. Analyzing interactions among proteins not only provides insight into the function of their corresponding genes, but also helps unravel the genetic topology of pathways and networks regulating biological processes. To acquire interaction information between proteins in *C. elegans*, two strategies are often employed: yeast two-hybrid (Y2H) screens (Fields and Song, 1989) and affinity-based protein isolation coupled with mass spectrometry (MS) analysis. In a typical Y2H assay, one protein is used as a bait and fused with the DNA-binding domain of a transcription factor, whereas the other protein functions as a prey and is fused with the activating domain of the transcription factor. These two fusion proteins are introduced into the

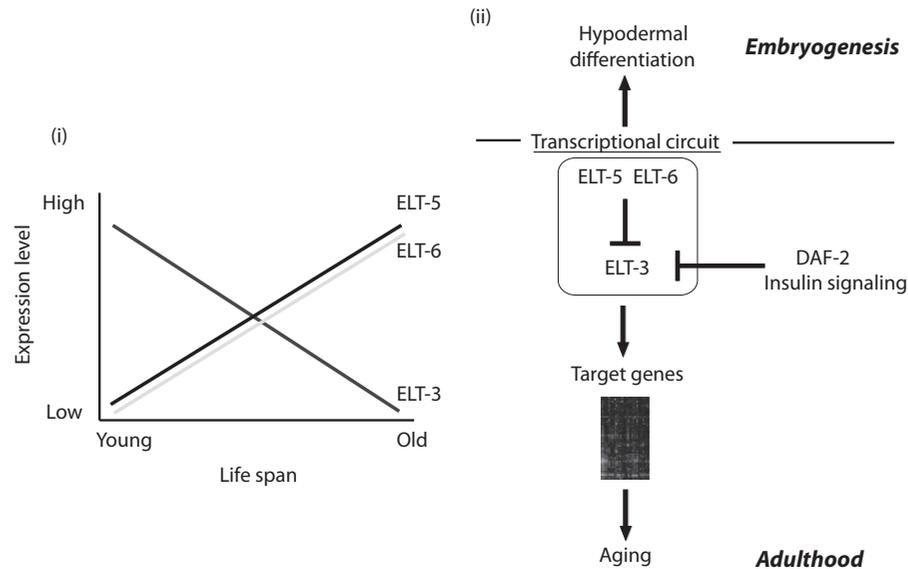


fig0025

**Fig. 5** Expression changes of *elt-3/elt-5/elt-6* during normal aging and the model for this transcriptional circuit. (i) During the normal aging process, the expression of the GATA transcription factors *elt-5* and *elt-6* increases. This represses the expression of the GATA transcription factor *elt-3*. (ii) The transcriptional circuit consisting of *elt-3*, *elt-5*, and *elt-6* regulates hypodermal differentiation during embryogenesis and aging during adulthood. *elt-5* and *elt-6* promote normal aging by negatively regulating *elt-3* expression. ELT-3 regulates a group of age-dependent genes identified from DNA microarray analysis. Consistent with a functional role in aging, loss of function of *elt-3* suppresses the long-lifespan phenotype of *daf-2* mutants. The expression of *elt-3* is repressed by the DAF-2 mediated insulin signaling, indicating that this transcriptional circuit may also modulate the effects of the insulin signaling.

yeast system. If the two proteins physically interact, the two domains are brought into close proximity, which triggers transcription of a reporter gene indicating that an interaction has taken place. As an effort toward understanding protein–protein interactions, a large-scale protein interaction (interactome) mapping project based on Y2H screens is in progress ([http://interactome.dfci.harvard.edu/C\\_elegans/](http://interactome.dfci.harvard.edu/C_elegans/)) (Simonis *et al.*, 2009). This interactome map (Worm Interactome version 8) currently contains 3864 known binary protein–protein interactions. Through interactome mapping, many interactive connections involving novel proteins have been found between disparate biological processes in *C. elegans* (Boulton *et al.*, 2002; Li *et al.*, 2004; Reboul *et al.*, 2003). For example, using the Y2H screening strategy, Tewari *et al.* (2004) uncovered eight *daf-7/TGF- $\beta$*  pathway modifiers that were not identified by conventional means. They first employed six known components of the *daf-7/TGF- $\beta$*  pathway as bait for the first round Y2H screen using a *C. elegans* cDNA library. In order to identify more novel interaction links, they conducted the second round of Y2H screening using genes identified in the initial screen as bait. Such a sequential multi-round Y2H screening approach is termed

“interactome walking”, which can identify novel interactors linking distinct functions (Cusick *et al.*, 2005). In this study, they identified 71 interactions among 59 proteins, comprising a complex interactome map. Because Y2H screens might not reflect the *in vivo* functional relationships between two genes, other functional genomic techniques and conventional genetic approaches are often used for independent experimental validation. In this study, the identified interactions were confirmed by co-affinity purification assays and functionally validated by double genetic perturbation analysis in which RNAi was used to inactivate genes in loss-of-function mutant backgrounds of known *daf-7*/TGF- $\beta$  pathway genes. Through these approaches, nine genes were confirmed to interact with the *daf-7*/TGF- $\beta$  pathway, eight of which had not previously been reported to have roles in the *daf-7*/TGF- $\beta$  pathway. Given the high false-negative rate (approximately 60–70%) of Y2H screens (Walhout *et al.*, 2000) and intrinsic limitations of RNAi (discussed above), they speculated that many more interactors were likely missed in this study. Nevertheless, this study highlights the power of coupling large-scale protein interaction mapping with conventional genetic perturbation to identify components of signaling pathways in *C. elegans*.

par0220

Another strategy for identifying interacting proteins is affinity-based protein isolation coupled with MS analysis (Aebersold and Mann, 2003). This strategy is used to identify proteins that are associated with a protein of interest. These associated proteins can be isolated through two affinity purification techniques: (1) immunoprecipitation, an antibody-based purification, in which an antibody against a protein of interest is used to isolate it and its associated proteins; (2) tandem affinity purification (TAP), in which two tags separated by an enzyme-cleavable site are fused to a protein of interest and the associated proteins are isolated in two steps sequentially using antibodies or binding proteins against the two tags (Fig. 6). The tags can be fluorescent proteins, such as GFP, which allow both dynamic imaging studies and proteomic analysis. This type of tag is also referred to as the “localization and affinity purification” (LAP) tag (Cheeseman and Desai, 2005; Rigaut *et al.*, 1999). Compared with the Y2H technique in which the proteins cannot undergo some of the post-translation modifications required for particular interactions in metazoans, the tag-based strategy has several advantages: (1) the fully processed and modified protein can be used as a bait; (2) bound proteins are isolated from the native cellular environment where interactions take place; (3) multiple associated components can be isolated and analyzed at a single time (Ashman *et al.*, 2001). An example of the application of TAP/LAP coupled with MS for identifying genes was a proteomic study on *C. elegans* kinetochores (Cheeseman *et al.*, 2004), a specialized organelle that regulates chromosome segregation in mitosis and meiosis (Maiato *et al.*, 2004). To isolate the proteins involved in the assembly and function of kinetochores, they first generated a transgenic strain expressing two newly identified kinetochore proteins that were used as bait and fused with two tags, GFP and the S peptide domain. These two tags were separated by a sequence recognized by the tobacco etch virus (TEV) protease. Two sequential rounds of affinity isolation were performed. The bound proteins were first isolated using an antibody against

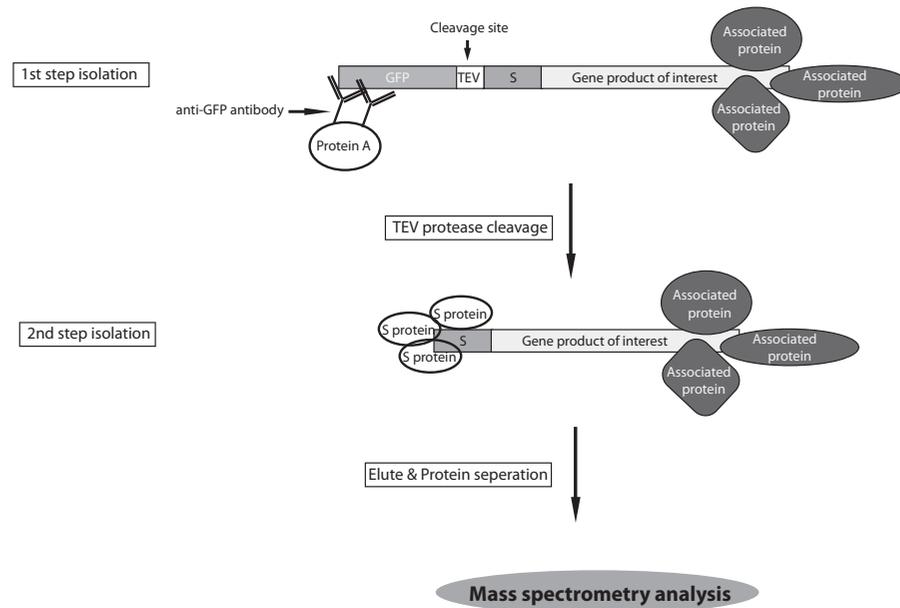


fig0030

**Fig. 6** Tandem affinity purification (TAP) coupled with mass spectrometry (MS) analysis. The two tags, GFP and S peptide domain, are fused to a gene product of interest. These two tags are separated by a tobacco etch virus (TEV) protease cleavage site. The proteins associated with the gene product of interest are isolated using the antibody against the first tag, GFP, which is then released by TEV protease cleavage. S protein that binds to the S peptide domain is used for the secondary affinity purification. The associated proteins are then separated from the gene product of interest and subject to MS analysis.

GFP that was removed by TEV digestion. A second round of affinity purification using the second tag enriched the kinetochore complex components, which were then subject to the MS analysis. MS analysis indicated that this two-step purification process removed most of the nonspecific proteins that were present in the single-step antibody-based immunoprecipitation that was also performed in parallel. The study identified 10 kinetochore proteins, of which seven were previously uncharacterized.

sec0125

## 4. Using Bioinformatics Tools

par0225

Bioinformatics is the analysis of biological systems, especially systems involving genetic materials, using computer science, statistics, engineering, and information theory. Bioinformatic tools have been widely applied in biological research, ranging from sequence-based analysis, transcriptome analysis to computational proteomics (Rhee *et al.*, 2006). In *C. elegans*, bioinformatic tools in conjunction with functional perturbation are effective in dissecting biological processes in some circumstances, such as microRNA prediction (Grad *et al.*, 2003), gene identification by homology search (Berset *et al.*, 2001; Chen and Greenwald, 2004), and *cis*-regulatory sequence

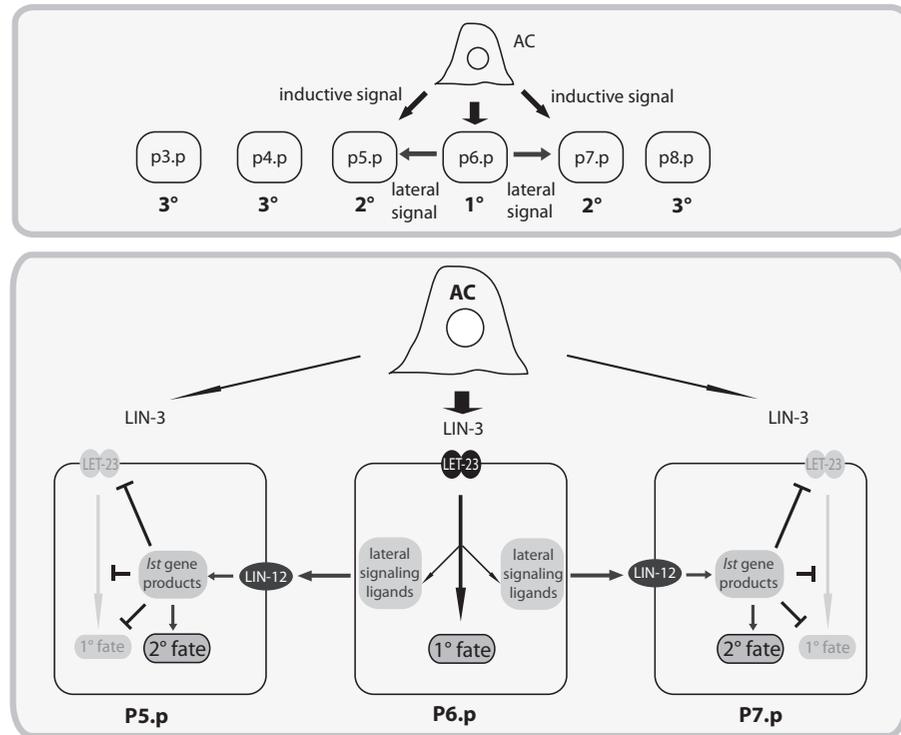


fig0035

**Fig. 7** Model for the crosstalk between LIN-3/LET-23 (ligand/receptor tyrosine kinase) signaling and LIN-12/Notch signaling in specification of vulval cell fate. Through LET-23/EGFR receptor, the graded inductive signal, LIN-3/EGF, secreted from the anchor cell (AC) promotes the 1° fate and activates expression of genes encoding ligands for the LIN-12 receptor in P6.p. Lateral signaling mediated by these ligands via LIN-12 promotes gene expression specific for the 2° fate in P5.p and P7.p, including *lst* (lateral signal target) genes. The *lst* genes that encode inhibitors of the LIN-3 signaling pathway antagonize the 1°-fate inductive effects of LIN-3 on P5.p and P7.p.

prediction (Budovskaya *et al.*, 2008; Hwang *et al.*, 2007; Yoo *et al.*, 2004). An excellent example showing the ~~potential~~ power of this approach was a study on LIN-12/Notch signaling in vulval development in *C. elegans* (Yoo *et al.*, 2004) (Fig. 7). Vulval patterning is precisely regulated through crosstalk between two pathways: the RTK/Ras/MAPK pathway mediated by LIN-3/LET-23 (ligand/receptor tyrosine kinase) and the Notch signaling pathway mediated by LIN-12 (a Notch-like receptor). *C. elegans* has six vulval precursor cells (VPCs) named consecutively P3.p to P8.p that adopt one of the three cell fates: primary fate (1°), secondary fate (2°), or tertiary fate (3°). Only the descendants of the 1° and 2° cells form the vulva. In wild-type animals, P6.p adopts the 1° fate, whereas P5.p and P7.p adopt the 2° fate. The LIN-12/Notch signaling pathway signals through a ~~complex of a~~ proteolytically freed intracellular fragment of LIN-12 receptor complexed with the

transcription factor LAG-1. To identify transcriptional target genes of LIN-12/Notch that antagonizes LIN-3/LET-23 signaling, Yoo *et al.* (2004) utilized computational programs to determine genes whose promoter regions contain clusters of the binding sites for LAG-1. One hundred sixty three genes were identified, two of which were previously reported to, respectively, antagonize or interact with the LIN-3/LET-23 signaling pathway during vulval development. By comparing the 5' regulatory regions of these two genes, they deduced two additional motifs that they postulated conferred tissue specificity of the 2° fated vulval cells. Through searching for genomic regions containing these motifs in the vicinity of the LAG-1 binding site clusters, they identified 10 candidate LIN-12 target genes that might act to antagonize LIN-3/LET-23 signaling in the 2° vulval cells. Of these 10 genes, five were experimentally verified as novel negative regulators of LIN-3/LET-23 signaling as the depletion of their activity using RNAi resulted in the increased activity of the LIN-3/LET-23 pathway in the 2° vulval cells. Importantly, in no case did elimination of activity of any of these genes ~~on their own~~ disrupt 2° fate specification, suggesting that they function redundantly to inhibit LIN-3/LET-23 activity in the vulval cells. This work powerfully underscores the usefulness of functional genomic approaches, such as bioinformatic analysis, in identifying gene regulatory networks and in circumventing genetic functional redundancy.

sec0130

### C. Using Systems Biology Approaches to Identify Components of Pathways

par0230

Systems biology approaches biological questions from the holistic and system-wide perspective rather than by a reductionist's gene-by-gene method. Though sometimes confusing in its definition, systems biology is emerging as an important approach for identifying and understanding gene networks in biology. All of the previously discussed genomic approaches are tools utilized in systems biology. These functional genomic approaches produce a large number of heterogeneous datasets on a genome-wide scale, such as gene expression data (transcriptome), protein-protein interaction data (interactome), and RNAi phenotypic data (phenome). Computational integration and systematic analysis of these datasets can reveal meaningful correlations that point to functionally associated components and lead to the generation of testable models that may assemble a more comprehensive picture of how gene regulatory networks control a particular biological process.

par0235

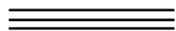
Evidence of correlations between any two types of datasets – transcriptome, interactome, and phenome – obtained from studies in yeast and worm has suggested that interacting genes appear to share similar expression, protein-protein interaction, and phenotypic profiles (Boulton *et al.*, 2002; Ge *et al.*, 2003; Jansen *et al.*, 2002; Jeong *et al.*, 2001; Kamath *et al.*, 2003; Li *et al.*, 2004; Oltvai and Barabasi, 2002; Piano *et al.*, 2002; Walhout *et al.*, 2002). Correlations across all these three types of data have been utilized to study and model the mechanisms underlying early embryogenesis in *C. elegans* (Gunsalus *et al.*, 2005). By integrating coexpression, protein-protein interaction, and phenotypic similarity datasets, Gunsalus *et al.* (2005) generated a network of 661 genes involved in early embryogenesis, including

the ribosome, proteasome, anaphase-promoting complex, and COPI coatomer, as well as complexes involved in translation initiation, nucleocytoplasmic transport, and cell polarity. They validated their predictions by testing localization of ten function-unknown genes that were predicted to associate with these “molecular machines”. Their results suggest that early embryogenesis in *C. elegans* is regulated by the coordination of a limited set of molecular machines, and provided hundreds of new putative molecular components of these machines.

par0240

Integration of functional genomic datasets can also be used across species as well as within. Because of functional conservation of orthologous genes and their genetic interactions, integration of disparate datasets from multiple organisms can provide stronger predictability for genetic interactions. An example illustrating the strength of this strategy comes from a study aimed at acquiring a global view of genetic interactions in *C. elegans* (Zhong and Sternberg, 2006). Zhong and Sternberg used a probability-based scoring system to integrate different datasets (interactome data, gene expression data, phenotype data, and functional annotation data curated from the literature) across three organisms (*S. cerevisiae*, *C. elegans*, *D. melanogaster*). They then generated a genetic interaction network consisting of 2254 genes and 18,183 predicted interactions with probability values for interactions. As a part of their experimental validation, they chose to verify the predicted interactions for two genes that have been the subjects of a number of genetic screens, *let-60*, a worm Ras gene, which plays a critical role in vulval induction, and *itr-1*, a worm 1,4,5-trisphosphate (IP3) receptor gene, which regulates pharyngeal pumping. As individual disruption of most of putative interacting genes caused no phenotype, double genetic perturbation was used for interaction validation. These genes were depleted by RNAi one at a time in the *let-60* or *itr-1* mutant backgrounds. Resulting enhancement or suppression of the phenotypic defects caused by the *let-60* or *itr-1* mutation indicated that interactions took place. Twelve of 49 predicted genes were confirmed to interact with *let-60*, and two of six genes for *itr-1*. Importantly, these 14 verified genes were novel modifiers that appeared to be missed in conventional screens, once again highlighting the ability of functional genomic/systems approaches in effectively identifying new genes and in particular functionally redundant genes or weak modifiers.

sec0135



### III. Ordering Genes into Pathways

Historically, the ordering of genes into pathways in *C. elegans* was accomplished with genetic analysis [see review by Huang and Sternberg (1995)] with an emphasis on arranging linear genetic pathways controlling developmental processes. One realization over the past decade is that most pathways controlling biological processes are not simply linear, but rather are highly regulated, buffered with redundancy, and often branched with multiple feedback or feedforward mechanisms. Modern pathway analysis involves the combination of genetic, biochemical, cell biological, and functional genomic approaches. We outline here how these diverse strategies are used to order genes into pathways that control biological processes.

sec0140  
par0250

### A. Determining Whether Two Genes Function in the Same Pathway

Following the recovery of mutations based upon the strategies discussed above, great effort should be put to carefully characterize the phenotypes and genetic nature of mutations (i.e., null, hypomorphic, or hypermorphic). This information is essential for genetic interaction analysis in which the defects of double mutants are compared to those of single mutants, allowing one to determine whether mutated genes act in the same or distinct pathways that regulate a particular biological process. It is particularly important for this analysis to use null alleles or a null allele and a strong-loss-of-function allele with no activity in the assayed biological process for double mutant construction. Double mutants with a phenotypic severity similar to that of the more severe single mutant suggest that the two genes work together or in series within the same pathway (Fig. 8i). Lacking a genetic interaction, the phenotype of the double mutant would be expected to be equal to the additive defects of the single mutants (Fig. 8ii). Double mutants displaying a more severe phenotype than the expected combined loss of each indicate a synergistic (or synthetic) interaction (Fig. 8iii) (Boone *et al.*, 2007; Guarente, 1993; Mani *et al.*, 2008). The assumption in this case is that the two genes likely act in parallel pathways that converge on a common function or activity. These interpretations do not apply for genetic interactions between two hypomorphic alleles. Because of their residual activity, the linearity of the pathway where they reside is not completely interrupted, any scenario above can be caused by two hypomorphic alleles that either function in the same pathway or the distinct pathways. Other functional information is often needed to

Types of genetic interactions		Implications of interactions
(i) Similar to whichever is more severe	$AB = A \text{ (or B)}$	 The same pathway
(ii) Additivity	$AB = A+B$	No genetic interaction
(iii) Synergism	$AB > A+B$	 Converge on a common function

fig0040

**Fig. 8** Implications of genetic interaction between two null alleles or a null allele and a strong-loss-of-function allele with no activity in the assayed biological process. To determine whether two alleles (A and B) function in the same pathway, the double mutant (AB) homozygous for both alleles is constructed. Comparing the severity of this double mutant with that of single mutants can provide insight into their functional relationship. (i) If the phenotypic severity of the double mutant is similar to that of the single mutant with the more severe phenotype, it implicates that the two genes act in the same pathway; (ii) if the phenotypic severity of the double mutant is the sum of that of the single mutants, it implicates that these two genes have no genetic interaction; (iii) if the phenotypic severity of the double mutant is more than the sum of that of the single mutants, it indicates that the two genes act in parallel pathways that converge on a common function.

reveal the genetic relationship of two hypomorphic alleles. For genes that act in the same pathway, determining their genetic hierarchy often requires comprehensive analysis of the genetic, cell biological, and biochemical information about how genes and their products interact. There are no universal rules to integrate all of this information for ordering genes into biological pathways. Indeed, many strategies that successfully revealed the order of genes are highly context-specific. Below, we discuss several examples as case studies for developing approaches to order genes that regulate a biological process.

sec0145 **B. Genetic Ordering of Pathways**

sec0150 1. Epistatic Analysis for Gene Ordering

par0255

As some components in a genetic pathway may play positive regulatory roles and others play negative roles, it is common that genes in the same pathway exhibit the opposite phenotypes when mutated. Epistatic analysis is a powerful way to order these components into a signaling hierarchy. The term “epistatic” was first coined in 1909 by Bateson to describe a masking effect in which an allele at one locus prevents the allele at another locus from exhibiting its phenotypes (Bateson, 1909; Cordell, 2002). Similarly, epistasis defined by molecular geneticists refers to a genetic situation in which the phenotype of a mutation in one gene is masked by the phenotype of the mutation in the other (Avery and Wasserman, 1992). This definition views a phenotype as a qualitative trait; so it is also termed “compositional epistasis” to set it apart from “statistical epistasis” used by population geneticists for quantitative differences of allele-specific effects in a population (Phillips, 2008).

par0260

Compositional epistatic analysis is particularly suitable for ordering genes whose mutations cause opposite phenotypes. It has been used in *C. elegans* to successfully construct pathways in various developmental processes such as the development of the vulva (Sternberg and Horvitz, 1989), sex determination (Goodwin and Ellis, 2002), and dauer formation (Thomas *et al.*, 1993). To perform epistatic analysis, double mutants carrying two mutations giving opposite phenotypes are constructed. The mutant phenotype that the double mutant adopts indicates the gene that is epistatic (downstream of) to the other. Two assumptions should be met prior to epistatic analysis. First, the two genes analyzed should be involved in the same pathway. Second, the opposite defective phenotypes should be direct opposite states of a genetic event assayed. For example, in vulval development, there are six VPCs. Normally, only three of these six VPCs give rise to progeny that form the vulva. A mutation in *lin-1*, encoding a transcription factor, causes more than three VPCs to adopt vulval fates, which produces the Muv phenotype. Conversely, mutations in *lin-3*, encoding an inductive cue for vulval formation, cause a reduction in vulval induction, which can lead to the Vulvaless (Vul) phenotype. These opposite phenotypes, Muv and Vul, are two opposite states in the same vulval induction pathway. Thus, epistatic analysis is applicable for ordering these two genes. As the *lin-1;lin-3* double mutant displays a Muv phenotype, *lin-1* is epistatic (downstream) of *lin-3*.

Moreover, since the LIN-3 protein is normally required for VPCs to adopt vulval fates and the LIN-1 protein inhibits the adoption of vulval fates of VPCs, the interactive relationship between them can be inferred as LIN-3 negatively regulates LIN-1 (see Fig. 1).

par0265

In cases where mutations involved in a common pathway display the same phenotype, epistatic analysis is not possible. However, some genes may have both *gf* and *lf* alleles that display the opposite phenotypes. Such gain-of-function alleles have proven to be very useful in deducing genetic hierarchies. The *gf* alleles can be either mutagen-induced or artificially engineered. One example using an artificially engineered transgene was a study examining the role of netrin signaling in axon outgrowth. A *lf* mutation in *unc-40*, encoding a netrin receptor, causes defects in axon guidance. Several *lf* mutations in genes involved in actin cytoskeleton regulation also display similar defective phenotypes. To order these genes into the *unc-40* pathway, Gitai *et al.* (2003) generated an artificial *gf* allele of *unc-40* by overexpressing and targeting the UNC-40 intracellular domain to the plasma membrane of the neuron. The engineered transgenic strain displays excessive axon outgrowth. Through epistatic analysis of the double mutants of this artificial *unc-40 gf* allele and the other actin-regulating mutants, they found that these actin-regulating genes are epistatic to *unc-40* because the mutations in these actin-regulating genes suppress the excessive axon outgrowth of the *unc-40 gf* allele. Further, these genes were found to form two bifurcated pathways downstream of *unc-40* as pairwise combinations of these mutations showed synergistic suppression of the *unc-40 gf* phenotype.

sec0155  
Analysis

## 2. Consideration Regarding Mutants Used for Epistatic Analysis and Limitations of Epistatic

par0270

It is important to use null mutants or hypomorphic alleles with no activity in the biological process being analyzed for epistatic analysis. An appropriate choice of mutants (null or hypomorphic) based on their dosage effects is critical for successful epistatic analysis. Using any mutants with residual activity may lead to a misinterpretation of the results of epistatic analysis, particularly in cases where one tests a gene with a hypomorphic allele that acts downstream of a gene with a null allele. Because the phenotype of the double mutants of these two alleles will be similar to that of the null allele, it will lead to an inaccurate conclusion that the gene with the null allele is epistatic to (downstream of) the gene with the hypomorphic allele (Fig. 9).

par0275

~~Epistatic analysis is a powerful tool for ordering genes.~~ An assumption of epistatic analysis is that the genes being ordered function in a linear genetic pathway. However, many pathways regulating biological processes have more complicated topological structures. They are nonlinear and often contain feedback/feedforward loops or autoregulatory elements. Moreover, the genetic hierarchy between the same components is sometimes context-dependent and can change spatially or temporally. Such a complexity limits the applicable scope of epistatic analysis. Fortunately, other tools are also available to *C. elegans* researchers to complement epistatic analysis in constructing gene regulatory pathways.

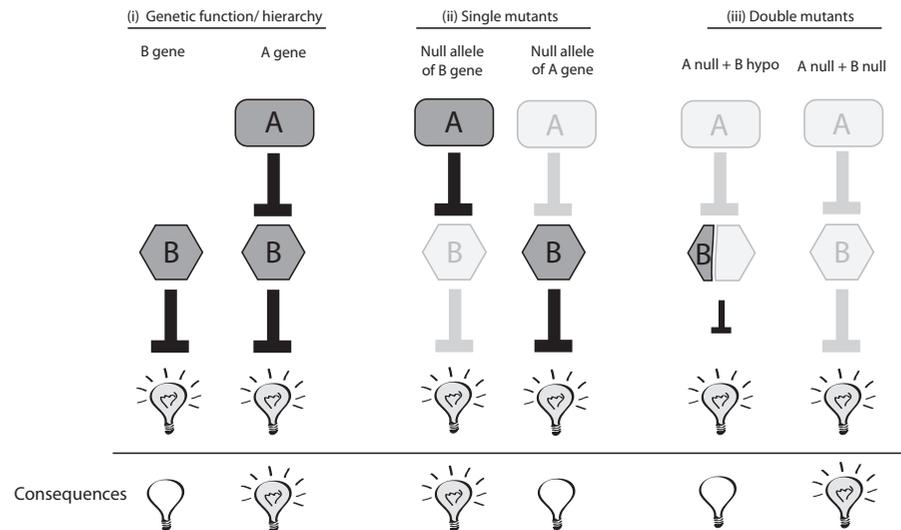


fig0045

**Fig. 9** Consideration regarding alleles used for epistatic analysis (i) Normally, gene B negatively regulates a functional state (illustrated as a bulb with light), causing the inactivation of this state (illustrated as a bulb without light). Gene A, however, acts upstream of gene B in this pathway and negatively regulates it, thereby maintaining the functional state. (ii) Complete loss of gene B activity abolishes its negative regulatory effects on the functional state (“light on”). In contrast, complete loss of gene A activity removes its negative regulation on gene B, releasing gene B inhibitory effects on the functional state, therefore inactivating the functional state (“no light”). The single mutants of gene B and A display the opposite phenotypes, which makes epistasis analysis applicable for organizing genes B and A. (iii) When one performs epistasis analysis using the null allele of gene A and the hypomorphic allele for gene B, the constructed double mutant might inactivate the functional state as the hypomorphic allele of gene B preserves some residual inhibitory activity. Thus, the possible phenotype of this double mutant (“no light”) would be similar to that of the single null of gene A (“no light”), leading to a misinterpretation that gene A is epistatic to (downstream of) gene B. Using null alleles of both genes A and B, however, reveals the correct epistatic relationship of gene B downstream of gene A.

sec0160

### C. Properties of Gene Products for Gene Ordering

par0280

The properties of proteins, including conserved function of their homologs/orthologs, site of action, and cellular/subcellular localization, are also useful for ordering genes into pathways. Comparative genomic analysis based on sequence homology and conservation across species can be used to deduce the potential functions of genes identified from screens. This information can provide clues for gene ordering. For example, in the case of two genes with the same phenotype when mutated, if a gene A is predicted to encode a potential transmembrane receptor and gene B encodes a potential cytoplasmic signaling transducer, this suggests that gene A likely acts upstream of gene B in a pathway, a hypothesis that can be further tested with cell biological analysis. If this hierarchical relationship between genes A and B is also conserved in other species, it would make this conclusion more solid. A good example

of this approach is previous work with the TGF- $\beta$  signaling pathway in *C. elegans*. TGF- $\beta$  signaling is involved in diverse developmental processes. A mutation in *daf-4*, encoding a receptor for TGF- $\beta$  superfamily ligands, results in small body size and morphological defects in *C. elegans* male tails. These phenotypes are identical to those of *sma-2*, *sma-3*, and *sma-4* mutants, which initially suggested that these genes were likely involved in the same biological process. To determine the hierarchical relationship between *daf-4* and *sma-2*, *sma-3* and *sma-4*, Savage *et al.* (1996) cloned and sequenced *sma-2*, *sma-3*, and *sma-4*, and found that they encode related proteins homologous to a *Drosophila* TGF- $\beta$  signaling component, *Mad*, suggesting that the three genes might be also required for TGF- $\beta$  signaling in *C. elegans*. This notion was supported by the ensuing genetic analysis revealing that the sites of action of *daf-4* and *sma-2* were in the same cell. The protein sequences of *sma-2*, *sma-3*, and *sma-4* further suggested a cytoplasmic or nuclear localization as no motifs were found to specify extracellular or transmembrane localization. Two possibilities for function arose: (1) *sma-2*, *sma-3*, and *sma-4* might function as cytoplasmic targets downstream of *daf-4*/TGF- $\beta$  signaling, or (2) *sma-2*, *sma-3*, and *sma-4* might act upstream of *daf-4* by regulating *daf-4* expression. The latter possibility was ruled out by showing that functional *daf-4* driven by a heat-shock promoter failed to rescue the defects of *sma-2*, *sma-3*, and *sma-4* mutants. They then concluded that SMA-2, SMA-3, and SMA-4 acted downstream of *daf-4*, which was confirmed by later studies showing that SMA-2, SMA-3, and SMA-4 form heterotrimers for propagation of TGF- $\beta$  signaling (Savage-Dunn *et al.*, 2000; Wu *et al.*, 2001).

sec0165  
par0285

#### D. Localization Dependency of Gene Products for Gene Ordering

The function of a protein often relies on its precise cellular and subcellular localization. In cases where this localization is tightly regulated, mutations in the corresponding genes that genetically interact with these components may alter protein localization. Therefore, examining localization patterns of a gene product in different mutant backgrounds may provide useful information regarding the relative positions of two genes in a pathway or interacting genetic network. This strategy is termed as “molecular epistasis” in some studies. However, as it is so distinct from the definition of “epistasis” discussed above, it would be more precise to term this strategy “localization dependency” to reflect its nature. Our lab (Hagedorn *et al.*, 2009) utilized this strategy to determine the hierarchical relationship for two genes, *ina-1* (encoding an  $\alpha$ -integrin) and *unc-40* (encoding a receptor for the netrin ligand), during anchor cell invasion into the vulval epithelium. Both *ina-1* and *unc-40* mutants are defective in AC invasion and both disrupt F-actin localization at the invasive cell membrane. By tracking GFP fusion proteins, we found that the normal localization pattern of UNC-40 in the AC was disturbed in worms whose *ina-1* activity is depleted by RNAi. In contrast, the localization pattern of INA-1 remained normal in *unc-40* mutants. Based on this result, we concluded that INA-1 acts upstream of UNC-40 functionally in regulating F-actin formation in the AC during AC invasion.

par0290

Another example of how this approach can be used to determine the relationships of interacting gene networks involves work on the PAR proteins, which play crucial roles in establishing the anterior–posterior axis of embryos after fertilization (Munro *et al.*, 2004). The hierarchical relationships between PAR proteins are highly spatially dependent. The PAR-3 protein is normally polarized in the anterior of embryos, whereas the PAR-2 protein is enriched in the posterior. In *par-2* mutants, PAR-3 is mislocalized to the posterior, which suggests that PAR-2 excludes PAR-3 from the posterior. This indicates that *par-2* may function upstream of *par-3* in the posterior. Conversely, in the anterior of the embryo, *par-3* is upstream of *par-2* because in *par-3* mutants PAR-2 is aberrantly accumulated in the anterior. In cases like cell polarity, careful localization dependency analysis is crucial in resolving the dynamic relationship between gene products in controlling a biological process.

sec0170

### E. Gene Expression Dependency for Gene Ordering

par0295

Many pathways and networks that control developmental events, such as cell-fate specification (Chang *et al.*, 2004), aging (Lin *et al.*, 2001), and developmental timing regulation (Lee *et al.*, 1993; Reinhart *et al.*, 2000), often involve transcription factors and/or components subject to post-transcriptional regulation. In these pathways and networks, interactions between two components are sometimes manifested by one component regulating expression of another. The hierarchical relationship among these components can be determined by their gene expression dependency. To determine this dependency expression of a full-length GFP reporter transgene carrying one gene (gene A) under control of its endogenous promoter is often examined in the mutant background of the other (gene B). In the case where expression of gene A is reduced in the mutant background of gene B, it suggests that gene B likely acts upstream of gene A as a positive regulator. For example, using this strategy, Johnston and Hobert (2003) placed a microRNA gene, *lisy-6*, into a transcriptional cascade of three homeobox transcription factors (*ceh-36*, *lim-6*, and *cog-1*) that regulate left–right functional asymmetrical expression of a guanyl cyclase (*gcy*) chemoreceptor gene, *gcy-5*, in ASE left (ASEL) neuron, but not in ASE right (ASER) neuron (Chang *et al.*, 2003) (Fig. 10). To order *lisy-6* into this regulatory hierarchy, they analyzed the expression of the transcription factors *ceh-36*, *lim-6*, and *cog-1* in a *lisy-6* mutant background. Expression of *ceh-36* gene was unaffected, but expression of the normally left-expressed *lim-6* gene was lost, suggesting that *lisy-6* acted upstream of *lim-6* in ASEL. Conversely, expression of *cog-1*, a negative regulator of *lim-6*, was upregulated in ASEL, indicating that *lisy-6* acted upstream of *cog-1* as a negative regulator. Consistent with this notion, the removal of *cog-1* activity in a *lisy-6* mutant background resulted in upregulation of *lim-6* expression, suggesting that *lisy-6* acts through *cog-1* to regulate *lim-6* expression.

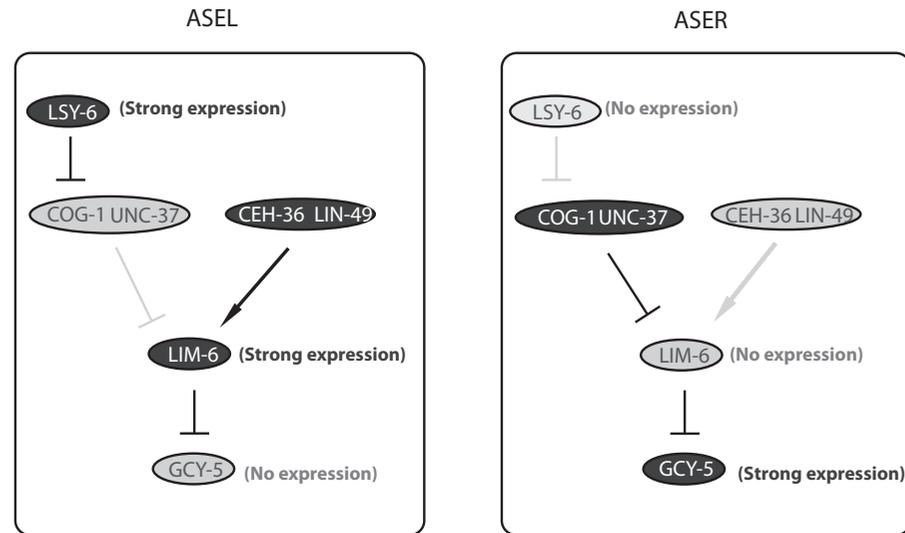


fig0050

**Fig. 10** Schematic diagram of the genetic pathway leading to *gcy-5* expression in ASER neuron. Despite bilaterally morphological symmetry, the ASEL and ASER neurons display distinct chemosensory capacities that correlate with the left–right asymmetric expression of three putative sensory receptor genes, *gcy-5*, expressed only in ASER, and *gcy-6* and *gcy-7* (not shown here) expressed only in ASEL (Chang *et al.*, 2003). A cascade consisting of a microRNA and several transcription factors act sequentially to restrict *gcy-5* gene expression to the ASER: a microRNA encoded by *lsy-6* gene, the homeobox transcription factors *cog-1*, *ceh-36*, and *lim-6*, and the transcriptional cofactors *unc-37*/Groucho and *lin-49*. In ASEL and ASER, a tightly balanced antagonistic effect between a repressor (COG-1/UNC-37) and a putative activator (CEH-36/LIN-49) complex regulates expression of *lim-6* (Chang *et al.*, 2003). In ASEL, *lsy-6* acts by targeting a complementary site in the *cog-1* 3' UTR to repress *cog-1* expression. CEH-36/LIN-49 then induces expression of *lim-6*, which subsequently represses expression of *gcy-5*. Thus, ASEL normally does not express *gcy-5*. In ASER, *lsy-6* is not expressed. The raised activity of COG-1/UNC-37 represses expression of *lim-6* by overcoming CEH-36/LIN-49-mediated induction of *lim-6* expression. Consequently, LIM-6-mediated repression of *gcy-5* expression is released, which leads to the differential expression of *gcy-5* in ASEL and ASER. More details about this pathway can be found elsewhere (Hobert, 2006).

sec0175  
par0300

## F. Functional Genomic Approaches for Gene Ordering

The functional genomic approaches discussed above are not only powerful in identifying genes that regulate a particular biological process, but also useful in ordering these genes, because some functional genomic approaches by their nature reveal the hierarchical relationships between components of pathways. For example, in Section II.B.2., Budovskaya *et al.* (2008) bioinformatically searched for transcription factors that act upstream to control expression of several hundred genes that were found to share similar age-regulated expression changes identified by their DNA microarray analysis. In this study, the direction of the searching strategy was from the age-regulated target genes (downstream) to transcription factors (upstream). Once

the transcription factor ELT-3 was found and its transcriptional regulation on the target genes was experimentally verified, the hierarchical relationship between them was evident. Conversely, in Section II.B.4., Yoo *et al.* (2004) searched for downstream target genes of the known transcription factor LAG-1, and identified and placed five novel targets genes into the LIN-12/Notch signaling pathway.

sec0180

## IV. Future Outlook

*C. elegans* is a powerful model organism to decode the cellular and molecular mechanisms underlying a variety of biological processes. Looking into the future, we anticipate forward genetic screens to continue to be an important approach for gene identification. More sophisticated genetic screens, such as enhancer screens on genetic backgrounds with tissue-specific gene perturbations, are expected to be carried out to cope with genetic lethality and substantial redundancy. Emerging cytometry-based automated screening techniques and whole genome sequencing for pinpointing mutation lesions will simplify mutant isolation and identification, allowing a more exhaustive and effective interrogation of genetic pathways involved in many biological processes. Technological advances in functional genomics and systems biology are revolutionizing *C. elegans* studies by providing diverse approaches to complement classic genetic screens to dissect genetic pathways/networks at unprecedented scales. Given the wealth of this functional data, we anticipate that strategies involving traditional genetics, cell biology, biochemistry, and functional genomics, will be further refined to effectively construct genetic pathways. The development of new technologies coupled with the established powerful traits of the *C. elegans* model system ensures that this organism will continue to serve an important role at the forefront of biological discovery.

## Acknowledgments

par0310

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