**Caenorhabditis elegans**: A Model System for Anti-Cancer Drug Discovery and Therapeutic Target Identification

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**Abstract**

The nematode *Caenorhabditis elegans* (*C. elegans*) offers a unique opportunity for biological and basic medical research due to its genetic tractability and well-defined developmental lineage. It also provides an exceptional model for genetic, molecular, and cellular analysis of human disease-related genes. Recently, *C. elegans* has been used as an ideal model for the identification and functional analysis of drugs (or small-molecules) *in vivo*. In this review, we describe conserved oncogenic signaling pathways (*Wnt*, *Notch*, and *Ras*) and their potential roles in the development of cancer stem cells. During *C. elegans* germline development, these signaling pathways regulate multiple cellular processes such as germline stem cell niche specification, germline stem cell maintenance, and germ cell fate specification. Therefore, the aberrant regulations of these signaling pathways can cause either loss of germline stem cells or overproliferation of a specific cell type, resulting in sterility. This sterility phenotype allows us to identify drugs that can modulate the oncogenic signaling pathways directly or indirectly through a high-throughput screening. *Current in vivo or in vitro* screening methods are largely focused on the specific core signaling components. However, this phenotype-based screening will identify drugs that possibly target upstream or downstream of core signaling pathways as well as exclude toxic effects. Although phenotype-based drug screening is ideal, the identification of drug targets is a major challenge. We here introduce a new technique, called Drug Affinity Responsive Target Stability (DARTS). This innovative method is able to identify the target of the identified drug. Importantly, signaling pathways and their regulators in *C. elegans* are highly conserved in most vertebrates, including humans. Therefore, *C. elegans* will provide a great opportunity to identify therapeutic drugs and their targets, as well as to understand mechanisms underlying the formation of cancer.

**Key Words:** *Caenorhabditis elegans*, Wnt, Notch, Ras, Cancer stem cells, Drug screening

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C. elegans and conserved signaling pathways. (A) An adult wild-type (N2) hermaphrodite stained with DAPI (4’,6-diamidino-2-phenylindole). The hermaphrodite has two gonadal tubes. They produce both sperm and oocytes, and are therefore self-fertile (see embryos). (B) A dissected adult hermaphrodite germline stained with DAPI. In the distal end, somatic gonadal cell, called DTC (see dotted red circle), acts as a germline stem cell niche that is essential for germline stem cell maintenance. The DTC fate is specified at least in part by Wnt/β-catenin signaling in early larval stage (L1). In the distal mitotic region (see dotted yellow lines), GLP-1/Notch signaling maintains germline stem cell self-renewal and promotes mitotic cell cycle of progenitor cells. Once mitotic cells enter mitotic cycle, Ras-ERK MAPK signaling promotes meiotic germ cell progression (see dotted green lines), pachytene exit (see dotted yellow lines), oocyte maturation (see dotted pink lines; circle, oocyte nuclei) and sperm (see dotted blue lines) fate specification. (C) Schematic of an adult C. elegans hermaphrodite gonad. Somatic DTC is located at the distal end. Cells at the distal end of the germline, including germline stem cells, divide mitotically (yellow). As cells move proximally, they enter meiosis (green) and differentiate into either sperm (blue) or oocytes (pink).

Over the past several years, increasing evidence has been found to be in support of the theory of cancer stem cells (sometimes called tumor stem cells or tumor-initiating cells). Cancer cells are heterogeneous, containing abundant proliferative cells (non-cancer stem cells) and rare cancer stem cells. Furthermore, it has been proven that cancer stem cells are similar to normal stem cells in many aspects and exist in multiple cancers such as leukemia, breast cancer, and lung cancer (Visvader and Lindeman, 2012). Therefore, specific therapies targeted at cancer stem cells hold a tremendous promise to increase the efficiency and safety of cancer treatment.

The canonical Wnt/β-catenin is critical for the regulation of embryonic stem cells, adult stem cells, and cancer stem cells (Nussé et al., 2008). In normal stem cells, the self-renewal and differentiation of stem cells are tightly regulated at least in part by Wnt/β-catenin signaling. For example, R-spondin growth factors interact with Leucine-rich repeat-containing G-protein-coupled receptors (Lgr) (Chen et al., 2013; Wang et al., 2013). These R-spondin/Lgr complexes and Wnt ligands...
C. elegans canonical Wnt/β-catenin signaling

Conserved Wnt/β-catenin signaling pathways and core components in C. elegans somatic gonads are summarized in Fig. 2C, and well described in (Eisenmann, 2005). This directly interact with Frizzled receptors on target cells to activate downstream signaling (Birchmeier, 2011). This signaling and downstream activation have been found to be important for the self-renewal and differentiation of stem cells and cancer stem cells (Reya and Clevers, 2005; Holland et al., 2013). Notably, Wnt/β-catenin signaling is likely to control mammary gland stem cell maintenance at different stages of development (Wend et al., 2010; Holland et al., 2013). Mammary gland stem cells can give rise to ductal, basal/myoepithelial and alveolar components (Holland et al., 2013). Therefore, aberrant activation of Wnt/β-catenin signaling contributes to the maintenance of cancer stem cells and results in mammary gland tumorigenesis. In addition, Wnt/β-catenin signaling has been implicated in the regulation of stem cells and cancer stem cells in the nervous system, hematopoietic system, skin, and intestine (Holland et al., 2013). Therefore, the inhibition of Wnt/β-catenin signaling might reduce the capacity of cancer stem cells, which could be of potential therapeutic benefit in treating multiple types of cancer.
review focuses chiefly on canonical Wnt/β-catenin signaling that specifies a germline stem cell niche (known as a DTC in C. elegans) fate specification in the C. elegans gonad (Fig. 2D). The DTC functions as the germline stem cell niche, which is essential for germline stem cell maintenance (Kimble and Crittenden, 2007). During early larval development, the DTC arises initially from an asymmetric cell division of a somatic gonadal progenitor (SGP) cell (Fig. 2C). This division specifies one daughter DTC potential and another non-DTC potential (Kimble and Crittenden, 2007; Byrd et al., 2014). In hermaphrodites, the DTC potential divides asymmetrically once more to differentiate terminally to form the DTC. The DTC fate is specified by divergent Wnt/β-catenin signaling pathways (Fig. 2C). The pathway regulating the asymmetric division of precursor cells, Z1 and Z4, involves Frizzled receptors (LIN-17 and LIN-2C). The pathway regulating the asymmetric division of precur-

| Signaling pathway | Mutants or transgenic lines | Phenotypes | Reference |
|-------------------|-----------------------------|------------|-----------|
| Wnt/β-catenin signaling | pop-1(q645) | DTC loss (100%) and sterile | (Siegfried and Kimble, 2002) |
|                    | sys-1(q544) | DTC loss (100%) and sterile | (Kidd et al., 2005) |
|                    | lin-17(n671) | DTC loss (<10%) and partially sterile | (Phillips et al., 2007) |
|                    | ceh-22(q632) | DTC loss (~40%) and partially sterile | (Lam et al., 2006) |
|                    | hs::sys-1 | Extra DTCs and fertile | (Kidd et al., 2005) |
|                    | hs::ceh-22 | Extra DTCs and fertile | (Lam et al., 2006) |
| GLP-1/Notch signaling | glp-1(q46) | No GSCs ("Glp" phenotype) | (Austin and Kimble, 1987) |
|                    | glp-1(bn16)ts | Fertile at 20°C | (Nadarajan et al., 2009) |
|                    | glp-1(q224)ts | "Glp" phenotype at 25°C | (Maine and Kimble, 1989) |
|                    | glp-1(ar202)ts | Fertile at 20°C | (Pepper et al., 2003a) |
| Ras-ERK MAPK signaling | mpk-1(ga117) | Pachytene arrest and sterile | (Lackner and Kim, 1998) |
|                    | mpk-1(qa111)ts | Pachytene arrest and sterile at 25°C | (Lackner and Kim, 1998) |
|                    | let-60(n1046) | Multi-layered small oocytes and multivulva (Muv) phenotype | (Hajnal and Berset, 2002) |
|                    | puf-8(q725); lip-1(zh15) | Mog sterile at 20°C | (Beitel et al., 1990) |
|                    | hs::ceh-22 | Germline tumors at 25°C | (Morgan et al., 2010) |
|                    | hs::sys-1 | Germline tumors at 25°C | (Cha et al., 2012) |

Table 1. Summary of oncogenic signaling pathways and mutant phenotypes

In C. elegans, the DTC functions as a germline stem cell niche, and it employs GLP-1/Notch signaling to promote con-
thin mitotic divisions. The DTC fate is regulated by Wnt/β-catenin signaling and its direct target, CEH-22/Nkk2.5 (Lam et al., 2006; Kimble and Crittenden, 2007). Therefore, weak or no Wnt signaling results in DTC loss and consequently no germline stem cells, which cause either partially or completely sterile (Fig. 2E). To visualize DTCs, a molecular marker lag-

2::GFP reporter gene can be used. This GFP marker is expressed brightly in DTCs (Fig. 2D, see green) (Bielloch and Kimble, 1999). The lag-2 is one of C. elegans Notch ligands and expressed in the DTC (Henderson et al., 1994; Bielloch et al., 1999). Normally, wild-type hermaphrodites have two DTCs (Byrd and Kimble, 2009) (Fig. 2D). However, loss of POP-1, SYS-1 or CEH-22 eliminates DTC fate (Fig. 2E), and ecto-
ic expression of SYS-1 or CEH-22 produces extra DTCs and generates new germline stem cell populations (Fig. 2F, Table 1) (Kimble and Crittenden, 2007; Byrd et al., 2014). Indeed, the ceh-22 gene is a direct target of transcriptional activation by POP-1/ TCF and SYS-1/β-catenin (Fig. 2C) (Lam et al., 2006) and is expressed in the cells with DTC potential (Lam et al., 2006). Notably, about 40% of ceh-22 mutants are missing both DTCs and are completely sterile. 40% of them are missing one of two DTCs and are partially sterile, and 20% of them produce two DTCs and are fertile (Lam et al., 2006). Amazingly, ecto-
ic expression of ceh-22 gene produces extra DTCs (Fig. 2F, Table 1) (Lam et al., 2006). Therefore, ceh-22 (q632) loss-of-function mutant is an attractive allele to identify drugs that can inhibit or activate the Wnt/β-catenin signaling pathway. If a drug inhibits Wnt/β-catenin directly or indirectly, it will enhance DTC loss and produce the sterile phenotype of ceh-22 mutants (Fig. 2G left). By contrast, if the drug activates Wnt/β-catenin signaling, it will retain the fertility of ceh-22 mutants and will result in two or more DTCs (Fig. 2G right). Furthermore, if the drug suppresses the inhibitor of Wnt/β-catenin signaling directly or indirectly, it can activate Wnt/β-catenin signaling and may retain the fertility of ceh-22 mutants. The DTC fate is also regulated at least in part by cell cycle regulators. Tilmann and Kimble have previously reported that Cyclin D is required for DTC fate specification (Tilmann and Kimble, 2005). More-
over, we also found that cell cycle regulators work together with Wnt/β-catenin signaling to specify DTC fate (Lee et al., 2014). Therefore, it suggests a possibility that the identified drugs can target the cell cycle regulators and/or Wnt/β-catenin signaling. The specificity can be examined by chemical genet-
ics in Wnt/β-catenin mutants or cell cycle mutants. Therefore,
diverse C. elegans mutants for Wnt/β-catenin signaling will provide a great opportunity to identify drugs that can potentially treat Wnt/β-catenin signaling-associated human diseases such as colon cancer.

**ONCOGENIC PATHWAYS: NOTCH SIGNALING**

**Overview of Notch signaling**

In most multicellular organisms, the Notch signaling pathway is a highly conserved signaling pathway that controls proliferation, differentiation, cell fate specification, and apoptosis (Artavanis-Tsakonas et al., 1999). Notch and most of its ligands (DSL: Delta/Serrate/LAG-2) are trans-membrane proteins. Notch ligands are expressed in the cells that are adjacent to the Notch expressing cells. In absence of Notch ligand, CSL (CBF1, Suppressor of Hairless, LAG-1) transcription factors are associated with co-repressors (Fig. 3A). This complex inhibits the expression of Notch target genes (Fig. 3A). Once the Notch interacts with a ligand, an ADAM-family metalloprotease cleaves the outside of Notch receptor (Fig. 3B). After this cleavage, γ-secretase cleaves the remaining part of the Notch receptor inside the inner leaflet of the cell membrane (Fig. 3B). This releases the intracellular domain of the Notch (NICD), which then translocates to the nucleus (Fig. 3B). In the nucleus, the NICD forms a complex with CSL (CBF1, Suppressor of Hairless, LAG-1) transcription factors and mastermind-like protein (MAML-1) to activate the expression of target genes (Fig. 3B). Importantly, an aberrant Notch signaling pathway potentially contributes to cancer development in several different ways (Allenspach et al., 2002) or to loss of the specific cell type (Liu et al., 2010). Therefore, the Notch signaling pathway represents a novel target for cancer therapeutic intervention and regenerative medicine.

**Notch signaling and cancer stem cells**

Aberrant activation of Notch signaling has been detected in a variety of human cancers including pancreatic cancer (Ristorcelli and Lombardo, 2010; Avila and Kissil, 2013), colon cancer (Miyamoto and Rosenberg, 2011), osteosarcoma (Hughes, 2009), glioblastoma (Lino et al., 2010), lung cancer (Galluzzo and Bocchetta, 2011), head and neck cancer (Sun et al., 2014), and breast cancer (Reedijk, 2012). Notably, activation mutations of human Notch1 are reported in approximately 50% of T-ALL (acute lymphoblastic leukemia) cases (Ferrando, 2009). The role of Notch in stem cell regulations has been thoroughly studied in several model systems to date. Moreover, the role of the Notch signaling in the initiation and maintenance of cancer stem cells has recently become a subject of study in a number of diverse model organisms. For example, Notch signaling is upregulated in pancreatic cancer stem cells (Abel et al., 2014). Reduction of the Notch signaling by either genetic inhibition (e.g. Hes1 shRNA) or drug treatment (e.g., γ-secretase inhibitor) in pancreatic cancer decreased cancer stem cell population and tumorsphere formation (Abel et al., 2014). In addition, the inhibition of Notch signaling by treatment with GSI-18 (γ-secretase inhibitor) depleted the stem cell-like subpopulation derived from medulloblastoma cell lines and abolished xenograft formation (Fan et al., 2006). By contrast, the activation of the Notch signaling pathway with DSL peptide stimulated tumorsphere formation and increased cancer stem cell population (Abel et al., 2014).

Bao et al. also reported that Notch-1 activation induced cancer stem cell self-renewal capacity through epithelial-mesenchymal transition (EMT) (Bao et al., 2011). Therefore, the Notch signaling pathway is important in maintaining cancer stem cell population, and targeting its pathway in cancer has promising therapeutic potential.

**C. elegans Notch signaling**

The Notch signaling pathway and its core components in C. elegans are highly conserved. The C. elegans has two Notch receptors, GLP-1 and LIN-12, which mediate cell-cell interaction during development (Greenwald, 2005). Specifically, GLP-1/Notch signaling in the C. elegans germline is critical for germline stem cell maintenance and continued mitotic division (Kimble and Crittenden, 2007). LIN-12/Notch signaling in the C. elegans somatic cells specifies vulva cell fate during early larval stages (Greenwald, 2005). When LAG-2 (GLP-1/Notch ligand) is expressed in DTCs (Fig. 3C) (Henderson et al., 1994) and interacts with the GLP-1/Notch receptor, proteolytic cleavage of the GLP-1/Notch receptor follows (Fig. 3C). GLP-1/Notch intracellular domain (NICD) is then translocated from the membrane into the nucleus (Fig. 3C). In the nucleus, the NICD forms a tertiary complex with LAG-1/CBF1 DNA binding protein and LAG-3/SEL-8/Mastermind transcription co-activator to activate the expression of target genes: fbf-2 (PUF RNA-binding protein) (Lamont et al., 2004) and lip-1 (MAPK phosphatase) (Berset et al., 2001; Lee et al., 2006), lst-1 (Nanos-like zinc finger domain-containing protein) (Yoo et al., 2004; Kershner et al., 2014), and sygl-1 (Novel protein) (Kershner et al., 2014) (Fig. 3C). Importantly, FBF-2, and LIP-1 inhibit meiosis-promoting regulators (e.g., GLD-1, GLD-2, GLD-3, and MPK-1) in the C. elegans germline (Yoo et al., 2004; Lee et al., 2006; Kimble and Crittenden, 2007; Lee et al., 2007a). Therefore, loss of GLP-1/Notch signaling in germline causes a severe proliferation defect during early meiotic entry, resulting in no germline stem cell maintenance and sterility (Fig. 3D) (Austin and Kimble, 1987), while constitutive activation of this signaling promotes proliferation of germline stem cells and their progenitor cells as well as inhibits entry into meiosis, resulting in germline tumors and sterility (Fig. 3E) (Berry et al., 1997). Therefore, the aberrant regulation of GLP-1/Notch signaling can cause either loss of germline stem cells or overproliferation of a specific cell type, resulting in sterility.

**Phenotype-based drug discovery for Notch signaling using glp-1 mutants**

The C. elegans has multiple Notch mutants, including glp-1(q46) or glp-1(q175) null mutants, glp-1(bn18 or q224) temperature-sensitive (ts), loss-of-function (lf) mutants, and glp-1(ar202) temperature sensitive (ts), gain-of-function (gf) mutant (Table 1). In C. elegans germline, GLP-1/Notch signaling is essential for germline stem cell maintenance and mitotic germ cell proliferation (Kimble and Crittenden, 2007). For example, at 20°C or lower, temperature sensitive, loss-of-function mutants for glp-1, “glp-1(bn18) and glp-1(q224)” produce sperm and oocytes and are therefore fertile (Fig. 3D and 3E left). Conversely, at 25°C, they have a severe proliferation defect and early meiotic entry (Fig. 3D right). However, a temperature sensitive, gain-of-function mutant for glp-1, “glp-1(ar202)” with constitutively active GLP-1/Notch signaling promotes proliferation of germline stem cells and inhibits entry into meiosis, resulting in germline tumors at 25°C (Fig.
Notably, this germline tumor phenotype is rescued by the depletion of GLP-1/Notch signaling component genes, including LAG-3/SEL-8-mastermind (Petcherski and Kimble, 2000). Therefore, glp-1(lf) or glp-1(gf) mutants are useful animals for the identification of drugs that may target GLP-1/Notch signaling positively or negatively (Fig. 3F). The specificity can be tested by measuring the expression of direct GLP-1/Notch signaling target genes (e.g., fbf-2, lip-1, fob-1).
lst-1, or sygl-1) (Lamont et al., 2004; Lee et al., 2006; Kershner et al., 2014) or by analyzing the effect of drugs on GLP-1/Notch-unrelated synthetic mutants with germline tumors (e.g., glt-3, nos-3) (Eckmann et al., 2002). Together, C. elegans GLP-1/Notch signaling pathways and their core components are highly conserved in vertebrates, including humans. Therefore, the identification and characterization of such drugs will provide a tremendous promise for cancer therapy.

ONCOGENIC PATHWAYS: RAS SIGNALING

Overview of Ras signaling pathway
Ras-ERK MAP (Mitogen Activated Protein) kinase (MAPK) signaling pathways are highly conserved in all eukaryotes and are involved in numerous cellular responses including proliferation, differentiation, cell fate specification, cellular homeostasis, and survival (Fig. 4A) (Marshall, 1995; Whelan et al., 2012). Extracellular stimuli such as growth hormone activate Ras-ERK MAPK signaling through a MAPK signaling cascade. In short, extracellular ligands bind to the epidermal growth factor receptor (EGFR) and activate tyrosine kinase activity of the cytoplasmic domain of the EGFR (Fig. 4A). The EGFR-binding adaptor, Grb2, binds to the phosphotyrosine residues of the activated receptor (Schulze et al., 2005) and to the guanine nucleotide exchange factor, SOS, through two SH3 domains of GRB2. This Grb2/SOS complex when docked to phosphorylated EGFR is activated, which then promotes the activation of Ras proteins (Fig. 4A). Activated Ras phosphorylates and activates RAF kinase, activated RAF phosphatases and activates MEK, and activated MEK phosphorylates and activates ERK MAPK (Fig. 4A). Finally, activated ERK MAPK regulates its downstream targets in the positive or negative manners by phosphorylation (Fig. 4A). The downstream targets include transcription regulators (e.g., GATA-1) (Towatari et al., 2004), translational regulators (e.g., p90 ribosomal S6 kinases: RSK1, 2, 3) (Zhao et al., 1996), cell cycle regulators (e.g., Cyclin D1) (Okabe et al., 2006), and apoptosis regulators (e.g., BCL-2) (Tamura et al., 2004). Therefore, aberrant activation of Ras-ERK MAPK signaling contributes to abnormal gene expression, cell cycle progression, proliferation, and survival. Notably, a constitutively active Ras-ERK MAPK signaling has been shown to lead to the development of all cancers (Saxena et al., 2008). Therefore, the drugs that target Ras-ERK MAPK signaling are potential drugs for treating cancer.

Ras signaling and cancer stem cells
The Ras-ERK MAPK signaling pathway governs many cellular processes in most animals and is deregulated in approximately one-third of all human cancers. Because of its importance in cancer, the Ras-ERK MAPK signaling pathway has been an attractive target for anti-cancer therapy. For example, Moon et al. examined the role of two genes, APC and K-Ras, working in tandem in initiating colorectal cancer progression (Moon et al., 2014). The group’s data showed that a gain-of-function mutation of the oncogenic K-Ras, fixing it in the active, GTP-bound conformation, accelerates the ERK pathway, which in turn activates the Wnt/β-catenin pathway, inducing cancer stem cell marker expression in colorectal cancer cells (Moon et al., 2014). Increased contribution to tumorigenesis and liver metastasis in K-Ras (gf) was observed in the presence of loss-of-function mutations in adenomatous polyposis coli (APC), a negative regulator of β-catenin concentration, another condition characteristic of initial and intermediate stage colorectal cancer (Moon et al., 2014). The study utilized specimens from human colorectal cancer patients and APC<sup>Om6/K-Ras<sup>1,2</sup> mice, which were studied for their ability to form spheroids in vitro and tumors in vivo, respectively. A mouse xenograft model was also utilized using wild-type K-Ras and mutant K-Ras cells to observe cancer stem cell activation as a result of K-Ras mutation. Therefore, Ras activation can initiate the formation of cancer stem cells through Wnt/β-catenin activation.

C. elegans Ras signaling
The Ras-ERK MAPK signaling pathways governs many cellular processes, including proliferation, differentiation, cell fate specification, homeostasis, and survival in all eukaryotes. The Ras-ERK MAPK signaling pathways in the C. elegans germline are well described by Sundaram (Sundaram, 2006). Notably, core signaling pathways and their components are strikingly conserved (Fig. 4B) (Sundaram, 2006; Whelan et al., 2012). Briefly, two different RTKs, LET-23 (an EGFR homolog) and EGL-15 (a FGFR homolog) stimulate LET-60 (a Ras homolog) and its downstream cascade, consisting of LIN-45 (a Raf homolog), MEK2 (an MEK homolog) and MPK-1 (an ERK homolog) (Fig. 4B). This C. elegans Ras-ERK MAPK signaling controls multiple developmental events, including meiotic cycle progression, oocyte activation, sperm fate specification, spermatogenesis, physiological apoptosis, axon guidance, and vulva development (Lee et al., 2006; Sundaram, 2006; Lee et al., 2007b; Morgan et al., 2010) (Fig. 4B). In wild-type C. elegans hermaphroditic germline, activated MPK-1/ERK was not detected in the distal germline (e.g., germline stem cell region and pre-meiotic region) but became abundant in the proximal part of the pachytene region and in maturing oocytes (Lee et al., 2007a; Lee et al., 2007b) (Fig. 4C). Activated MPK-1/ERK is subject to two redundant modes of downregulation in the germline stem cell region: FBF-1/2 (members of PUF RNA-binding protein family) proteins act post-transcriptionally to repress <i>mpk-1/erkr</i> mRNA and LIP-1 acts post-translationally to inhibit MPK-1/ERK activity (Fig. 4C) (Lee et al., 2007a). This regulation is also conserved in human embryonic stem cells (Lee et al., 2007a; Whelan et al., 2012). Therefore, the dual negative regulation of MAPK/ERK by both PUF repression and MKP (MAPK phosphatase) inhibition may be a conserved mechanism that influences both stem cell maintenance and possibly tumor progression (Whelan et al., 2012). In C. elegans germline, additional regulators have also been identified as inhibitors of C. elegans Ras-ERK MAPK signaling: LARP-1 (La-related protein) and Insulin signaling inhibit Ras-ERK MAPK signaling during oogenesis (Nykamp et al., 2008; Lopez et al., 2013). PUF-8 (a member of PUF protein family) represses <i>let-60/Ras</i> mRNA expression in the germline stem cell region (Vaid et al., 2013). The germline center kinase (GCK1) also represses apoptosis by inhibiting Ras-ERK MAPK signaling (Schouest et al., 2009). Therefore, the Ras-ERK MAPK signaling is regulated positively or negatively by several regulators, including kinases, phosphatases, or RNA regulators.
Fig. 4. Ras signaling pathways and a strategy for the phenotype-based drug identification using *C. elegans* mutants. (A) Conserved Ras-ERK MAPK signaling pathways. (B) *C. elegans* Ras-ERK MAPK signaling pathway. (C) Schematic of adult wild-type hermaphrodite germline. MPK-1/ERK is activated in the proximal pachytene and developing oocytes (see red). In the distal germline, FBF-1/-2 and LIP-1 repress MPK-1/ERK activation at the post-transcriptional and translational levels (Lee et al., 2007a). (D) The germline phenotype of temperature-sensitive *mpk-1*(ga111) loss-of-function mutant. At 20°C, most *mpk-1*(ga111) mutants are fertile, but they are sterile due to pachytene arrest at 25°C. DAPI staining shows normal pachytene cells (left) and arrested pachytene cells (right). (E) The germline phenotype of *puf-8*; *lip-1* homozygote mutant at 20°C (left) and 25°C (right). The *puf-8*; *lip-1* mutants produce only sperm (Mog phenotype) at 20°C, but spermatocytes are dedifferentiated into mitotically dividing cells, resulting in germline tumors at 25°C. (F) Strategy for the phenotype-based identification of drugs that either inhibit or activate Ras-ERK MAPK signaling using *C. elegans* mutants.
Phenotype-based drug discovery for Ras signaling using *mpk-1* or *puf-8; lip-1* mutants

Loss of Ras-ERK MAPK signaling results in germline sterility due to abnormal meiotic progression and gametogenesis (Lee et al., 2007b). Intriguingly, this sterility phenotype was rescued by the inhibition of negative Ras-ERK MAPK regulators (e.g., LIP-1) (Hajnal and Berset, 2002; Lee et al., 2006), in contrast, aberrant activation of Ras-ERK MAPK by removal of *Puf-8* and *Lip-1* promotes sperm fate at 20°C (Morgan et al., 2010) and induces germline tumors at 25°C (Cha et al., 2012). For example, a temperature sensitive, *mpk-1*(*ga111*) loss-of-function mutant is mostly fertile at 20°C, but it is sterile at 25°C due to pachytene arrest (Fig. 4D). This sterile phenotype is rescued by the depletion of MPK-1 inhibitors (e.g., LIP-1) (Hajnal and Berset, 2002; Lee et al., 2006). In addition, activated MPK-1/ERK in *puf-8; lip-1* mutant promotes spermatogenesis without switching into oogenesis (Masculinization of germline (Mog) phenotype) at 20°C and develops germline tumors via dedifferentiation of spermatocytes at 25°C (Fig. 4E) (Morgan et al., 2010; Cha et al., 2012; Datla et al., 2014). Surprisingly, inhibition of Ras-ERK MAPK signaling by either genetic mutation or drug treatment (e.g., U0126) sufficiently rescued *puf-8; lip-1* sterility (Morgan et al., 2010; Cha et al., 2012; Datla et al., 2014). Therefore, *mpk-1(lf)* mutants and *puf-8; lip-1* mutants (Table 1) are useful animals for the identification of drugs that may target Ras-ERK MAPK signaling positively or negatively (Fig. 4F). The specificity can be confirmed using *let-60(n1046)* gain-of-function mutant or a transgenic worm expressing LET-23 chimeras in which the TK domain was replaced with the human mutant TK domain (L858R or T790M-L858R). Both animals with hyperactive Ras-ERK MAPK signaling in somatic vulva precursor cells induce a multivulva (Muv) phenotype. Notably, these Muv phenotypes were rescued by the treatment of Ras-ERK MAPK inhibitors, including AG1478 (an EGFR-TK inhibitor), U0126 (a MEK inhibitor), Gefitinib, Manumycin, and Gliotoxin (Hara and Han, 1995; Bae et al., 2012). Therefore, mutants and transgenic animals are a model system that can be used in signaling or mutation-specific screens for new anti-cancer drugs.

**FERTILITY-BASED IN VIVO HIGH-THROUGHPUT DRUG SCREENING AND DRUG TARGET IDENTIFICATION**

For large-scale high-throughput drug screening, we have recently developed a fertility and image-based, label-free high-throughput workflow (Benson et al., 2014). This automated high-content assay enables effect sorting of *C. elegans* mutants into a 384-well plate using the COPAS™ Worm Sorter and rapidly measuring either fertility or sterility by scoring the number of viable progeny in wells (Benson et al., 2014). For example, homozygote mutants for *puf-8; lip-1*...
are sterile and must be maintained as heterozygotes using a GFP-tagged balancer chromosome for chromosome II (e.g., mll[myo-2::GFP] dpy-10(e128); called mc6g). Heterozygotes for experiments are typically selected by picking GFP-negative mutants using a fluorescence microscope. However, for large-scale animal handling, it would be advantageous to automatically identify homozygote mutants using a fluorescent marker. To this end, we generated a puf-8/mc6g; lip-1/ lip-1; [pmyo-2::mCherry] transgenic mutant strain expressing a red-fluorescent protein, mCherry, in the head (Fig. 5A). The incorporation of the red-head marker, allows effective isolation red-fluorescent protein, mCherry, in the head (Fig. 5A). The sterility by scoring the number of viable progeny in wells using ry), which allows to measure automatically either fertility or signal pathways can be incorporated with the red-head marker (mCherry), allows to measure automatically either fertility or sterility by scoring the number of viable progeny in wells using using an automatic worm sorter, COPASTM BIOSORT. Furthermore, the mCherry head marker also simplifies detection of puf-8; lip-1 progeny that is difficult to identify using bright field optics (Fig. 5A). For fertility and image-based high-throughput drug screening, mutants of Wnt, Notch, or Ras signaling pathways can be incorporated with the red-head marker (mCherry), which allows to measure automatically either fertility or sterility by scoring the number of viable progeny in wells using an automatic worm sorter, COPASTM BIOSORT. Furthermore, the mCherry head marker also simplifies detection of puf-8; lip-1 progeny that is difficult to identify using bright field optics (Fig. 5A). For fertility and image-based high-throughput drug screening, mutants of Wnt, Notch, or Ras signaling pathways can be incorporated with the red-head marker (mCherry), which allows to measure automatically either fertility or sterility by scoring the number of viable progeny in wells using an automatic worm sorter, COPASTM BIOSORT. Furthermore, the mCherry head marker also simplifies detection of puf-8; lip-1 progeny that is difficult to identify using bright field optics (Fig. 5A).

To date, several drugs that target core components of each oncogenic signaling pathways through target-specific in vitro screening. Nonetheless, the drugs also alter normal signaling pathways. Moreover, current affinity-based target identification approaches require each drug of interest to be immobilized to a bead or another affinity- or fluorescent- or radioactive-“tag” so that the target protein can be “pulled down”. However, the coupling of compounds to beads and other molecules could lead to alteration or loss of compound bioactivity and binding. Furthermore, these techniques are time-consuming and require extensive biochemistry or medicinal chemistry expertise (Lomenick et al., 2009; Lomenick et al., 2011). Recently, Huang and colleagues reported that the molecular target of the identified drug could be identified using a newly developed technique, called Drug Affinity Responsive Target Stability (DARTS) (Fig. 5B) (Lomenick et al., 2009; Lomenick et al., 2011). DARTS takes advantage of the concept that protease susceptibility of the target protein is reduced upon drug binding (Lomenick et al., 2009; Lomenick et al., 2011). The advantage of this approach is that it is universally applicable as modification of the drug is not necessary and is independent of the mechanism of drug action. In particular, The DARTS is useful for the initial identification of the targets of compounds, but can also be useful for validation of potential protein-ligand interaction (Lomenick et al., 2011). To test whether the DARTS is useful for molecular target identification of the identified drugs, we established an in vitro DARTS technique using a purified GST-tagged human MEK2 (hMEK2) protein with U0126, a well-known MEK2 inhibitor (Duncia et al., 2009; Favata et al., 1998) and DMSO (control) (Fig. 5C). Interestingly, U0126 (1 uM) stabilized MEK2 protein, thereby reducing protease (pronase) sensitivity of the MEK2 protein (Fig. 5C). Although DARTS is a suitable and feasible method to identify the oncogenic target of the drugs, it remains still a major challenge if this method can be applied for all drug targets.

CONCLUSIONS

In this review, we describe three conserved oncogenic signaling pathways that are often associated with the development of cancer stem cells. Importantly, these signaling pathways are highly conserved and critical for germline development in C. elegans. Therefore, aberrant regulations of these signaling pathways cause either partially or completely sterile. Notably, inhibition or activation of these signaling pathways by drug treatment retains fertility (Morgan et al., 2010; Benson et al., 2014). Based on this finding, we here propose possible phenotype-based high-throughput screening methods to identify drugs that may alter oncogenic signaling pathways. The identification of drugs is a powerful tool of biological analysis and therapeutics as well as can lead to the development of new drugs. However, a critical bottleneck is generating useful drug tools is target identification. Current target identification techniques have several limitations. For example, affinity-based target identification is limited by the necessary to modify each individual without losing bioactivity, and non-affinity-based approaches depend on the drug’s ability to induce specific biochemical or cellular readouts (Lomenick et al., 2009; Lomenick et al., 2011). Recently, Huang and colleagues established DARTS as a new technique (Lomenick et al., 2009; Lomenick et al., 2011). This technique is able to identify the targets of drugs based on the principle that when a drug binds to a protein, the interaction stabilizes the target protein’s structure, resulting in proteolytic resistance (Lomenick et al., 2009; Lomenick et al., 2011). If the specific targets of drugs are identified by DARTS technique, the peptides can be identified by mass-spectrometry after trypsin digestion. Therefore, these approaches, described in this review, have great potential for finding new drugs and will also aid research in cancer therapies.

C. elegans has also emerged as an attractive model system for functional analyses of bioactive compounds (e.g., nicotine) and natural components. Previously, Xu and colleagues developed a C. elegans model of nicotine-dependent behavior (Feng et al., 2006). They showed that C. elegans displays acute and chronic behavioral responses through nAChRs (nicotinic acetylcholine receptor, acr-5 and acr-15 in C. elegans) that are known to be critical for nicotine dependence in mammals (Feng et al., 2006; Sellings et al., 2013). In addition, chronic nicotine exposure can alter the expression of microRNAs and genes that are implicated in reproduction, cholinergic signaling and stress responses (Smith et al., 2013; Taki et al., 2014). Kim and colleagues have also reported the effect of the isolated 1,2,3,4,6-penta-O-galloyl-D-glucose (PGG) from Curcuma longa L. on longevity using C. elegans as a model system (Ahn et al., 2013). Notably, they demonstrated that PGG reduced intracellular ROS (Reactive Oxygen Species) accumulation through elevated SOD (Superoxide Dismutase). C. elegans has been used for the discoveries of antimicrobial drugs (Ewbank and Zugasti, 2011; Squiban and Kurz, 2011), antifungal drugs (Anastassopoulou et al., 2011), and Alzheimer’s disease drugs (Lublin and Link, 2013). Therefore, C. elegans provides a tremendous promise for the discovery of new disease or target-specific drugs, and studying the action mechanism of the newly identified or known drugs (or small-molecules) in a live animal.

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