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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 researches 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

CAENORHABDITIS ELEGANS AS A MODEL SYSTEM

The nematode *Caenorhabditis elegans* (*C. elegans*) is a multicellular organism that has become a popular model for biological and basic medical research. It has also been widely used as a model system to explore fundamental questions in multiple aspects of biology, including evolution, development, cell fate specification, stem cell regulation, tumorigenesis, and aging. The *C. elegans* has also been considered as an ideal model system for live animal high-throughput drug screening, as 1) their tissues are transparent at all developmental stages, 2) tissue-specific fluorescent transgenic markers to study physiological and cellular processes *in vivo* are well established, 3) a large number of mutant strains are available in Caenorhabditis Genetics Center (CGC) [http://www.cbs.umn.edu/research/resources/cgc], 4) whole genome sequencing has been completed, 5) they have a short lifespan (2 to 3 weeks) and a strong genetic power, and 6) the aspects of mammalian diseases can be successfully modeled in the *C. elegans* (O'Reilly *et al*., 2014).

The *C. elegans* life cycle includes embryogenesis (~12 h), four larval stages (L1-L4; total of ~3 days) and adulthood (~10

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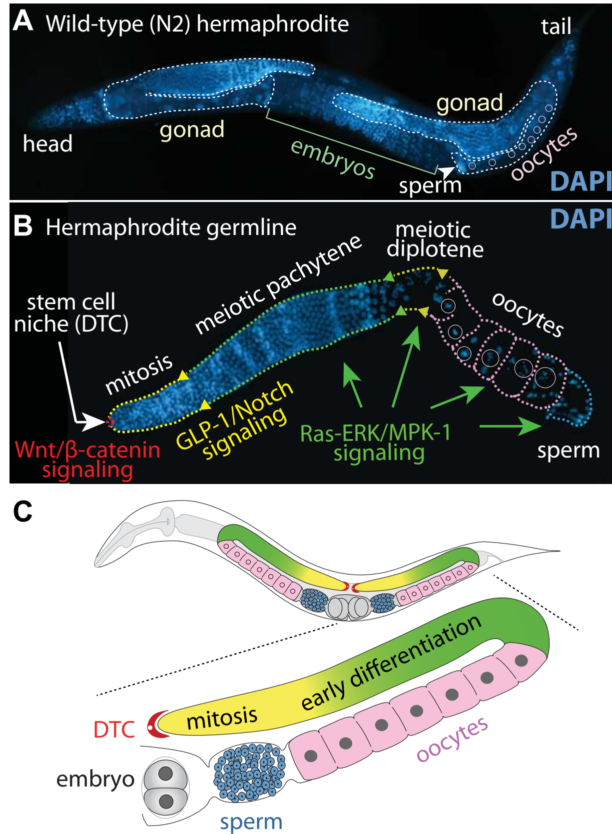

Fig. 1. *C. elegans* and conserved signaling pathways. (A) An adult wild-type (N2) hermaphrodite stained with DAPI (4',6-diamidino-2-phenylindole). The hermaphrodite has two gonadal tubes. They produce both sperm and oocytes, and are therefore self-fertile (see embryos). (B) A dissected adult hermaphrodite 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 meiotic cell 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).

days) (Kimble and Crittenden, 2005). *C. elegans* exists as either hermaphrodites or males. Wild-type hermaphrodites can produce sperm during larval development (L3-L4) and then switch to oogenesis in adulthood (Fig. 1A, 1C). However, males make sperm continuously throughout their lifespan. In addition, the germline is organized in a simple linear fashion that progresses from germline stem cells at one end to maturing gametes at the other (Fig. 1B, 1C). *C. elegans* germline development is tightly regulated by conserved external signaling pathways, including Wnt, Notch and Ras, (Fig. 1B) as well as intrinsic regulators, including gene expression regulators and cell cycle regulators (Kimble and Crittenden, 2007). Aberrant control of these signaling pathways can cause the loss of the somatic distal tip cells (DTCs, which function as the germline stem cell niche) and germline stem cells as well as extra DTC formation, uncontrolled germline proliferation, and abnormal germ cell fate specification that are all associated with sterility and germline tumors. Therefore, these features of *C. elegans* germline make it a very suitable organism for the phenotype-based high-throughput screening of drugs that target oncogenic signaling pathways and the identification of therapeutic targets.

ONCOGENIC PATHWAYS: WNT SIGNALING

Overview of Wnt signaling pathway

The Wnt signaling pathway is critical for many aspects of animal development, including stem cell self-renewal, differentiation, cell fate specification, polarity, and cell migration (Katoh, 2008). There are three Wnt signaling pathways: canonical Wnt/β-catenin pathway, non-canonical Wnt/planar cell polarity (PCP) pathway, and non-canonical Wnt/calcium pathway (James *et al*., 2008). All three Wnt signaling pathways are activated by the binding of Wnt ligand to Frizzled family receptor. In absence of its ligand, cytoplasmic β-catenin interacts with APC (polyposis coli) and Axin scaffold proteins, and then is phosphorylated by CKIα kinase and GSK3β (Glycogen Synthase Kinase 3β). The phosphorylated β-catenin is then ubiquitinated and degraded by the proteasome (Fig. 2A). Therefore, in the canonical pathway, CKIα, GSK3β, APC, and Axin act as negative regulators. Upon activation, the formation of APC/Axin/CKIα/GSK3β destruction complex is inhibited, which stabilizes β-catenin and leads to its localization in the nucleus (Fig. 2B). In the nucleus, β-catenin interacts with TCF family transcription factors to activate the expression of target genes such as FGF20, DKK1, WISP1, MYC, and Cyclin D1 (He *et al*., 1998; Pennica *et al*., 1998; Tetsu and McCormick, 1999; Chamorro *et al*., 2005) (Fig. 2B). Importantly, these target genes have been implicated in the development of multiple types of cancer, including colon, breast, ovarian, and thyroid.

Wnt signaling and cancer stem cells

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 (Nusse *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

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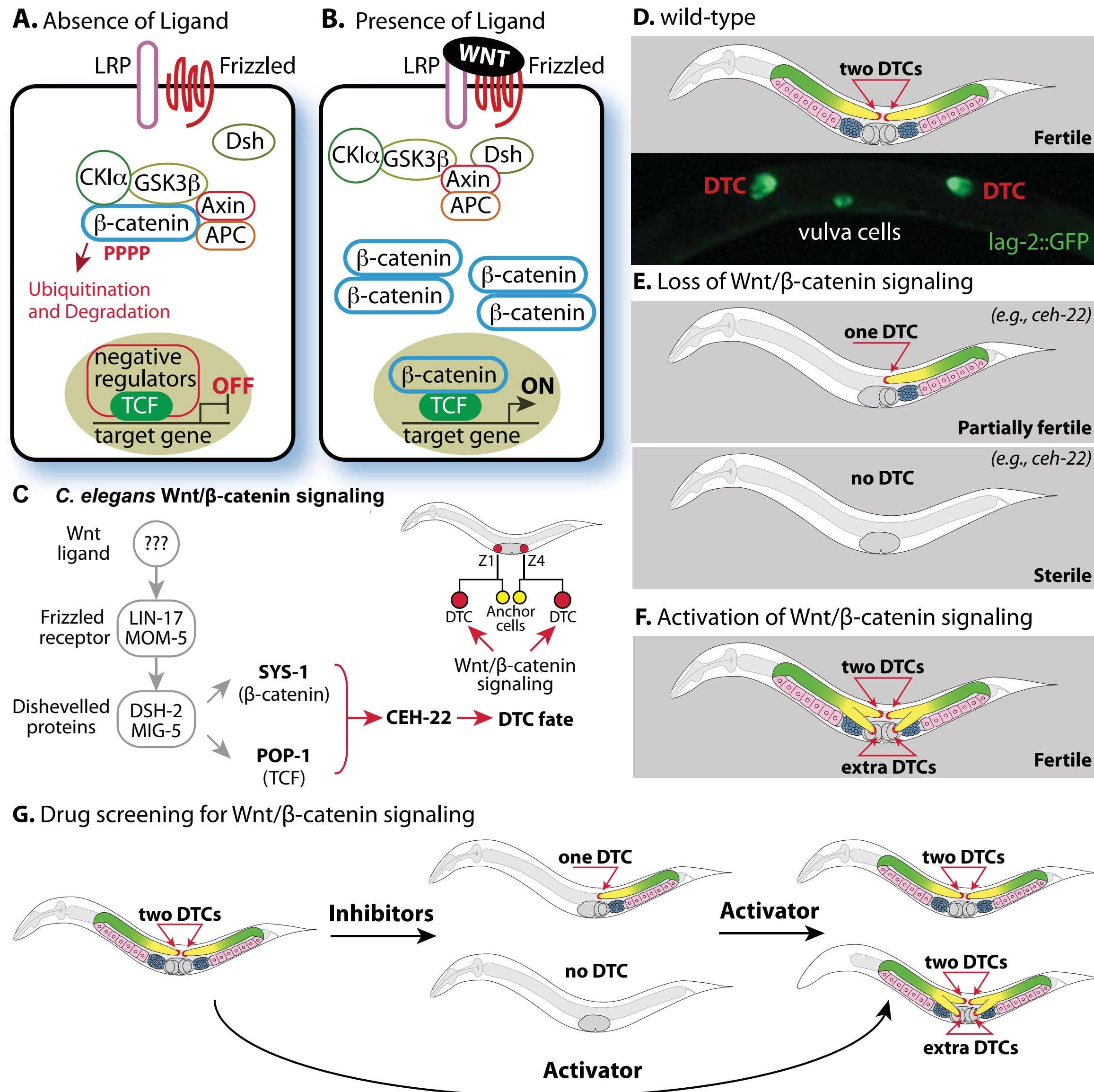

Fig. 2. Wnt/β-catenin signaling pathways and a strategy for the phenotype-based drug identification using *C. elegans* mutants. (A) Without signaling (absence of ligand), negative regulators (CKIα, GSK3β, Axin, and APC) phosphorylate β-catenin, and then ubiquitinated and degraded by the proteasome. (B) With signaling (presence of ligand), stabilized β-catenin in cytoplasm is translocated into nucleus and activate the expression of target genes. (C) Genetic pathway for control DTC fate specification (see text for explanation). (D) Wild-type hermaphrodite has two DTCs. lag-2::GFP reporter is strongly expressed in DTCs (see green). (E) Loss of Wnt/β-catenin signaling affects DTC fate specification. (F) Activation of Wnt/β-catenin signaling contributes to extra DTC formation. (G) Strategy for the phenotype-based identification of drugs that either inhibit or activate Wnt/β-catenin signaling using *C. elegans* mutants.

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.

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

Table 1. Summary of oncogenic signaling pathways and mutant phenotypes

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(bn18)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)
		Germline tumors at 25°C	
Ras-ERK MAPK signaling	*mpk-1(ga117)*	Pachytene arrest and sterile	(Lackner and Kim, 1998)
	mpk-1(ga111)ts	Pachytene arrest and sterile at 25°C	(Lackner and Kim, 1998)
	let-60(n1046)	Multi-layered small oocytes and	(Hajnal and Berset, 2002)
		Multivulva (Muv) phenotype	(Beitel *et al*., 1990)
	puf-8(q725); lip-1(zh15)	Mog sterile at 20°C	(Morgan *et al*., 2010)
		Germline tumors at 25°C	(Cha *et al*., 2012)

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 MOM-5), Dishevelled proteins (DSH-2 and MIG-5), β-catenin (SYS-1), and TCF transcription factor (POP-1) (Kimble and Crittenden, 2007; Byrd *et al*., 2014) (Fig. 2C). Aberrant regulation of this pathway results in either the loss of DTC fate (Fig. 2E) or the generation of extra DTCs by symmetric division (Fig. 2F). Importantly, POP-1/TCF and SYS-1/β-catenin directly activate the transcription of *ceh-22* (Nkx2.5 homolog in mammals) in DTC potentials (Lam *et al*., 2006) (Fig. 2C). CEH-22/Nkx2.5 is necessary for DTC specification (Lam *et al*., 2006). Therefore, Wnt/β-catenin signaling and its direct targets (e.g., CEH-22) play an essential role in DTC fate specification in the *C. elegans* gonads (Fig. 2C-2F).

Phenotype-based drug screening for Wnt/β-catenin signaling mutants

In *C. elegans*, the DTC functions as a germline stem cell niche, and it employs GLP-1/Notch signaling to promote continued mitotic divisions. The DTC fate is regulated by Wnt/β-catenin signaling and its direct target, CEH-22/Nkx2.5 (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) (Blelloch and Kimble, 1999). The *lag-2* is one of *C. elegans* Notch ligands and expressed in the DTC (Henderson *et al*., 1994; Blelloch *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 ectopic 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, ectopic 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). Moreover, 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 genetics 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/CSL 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.

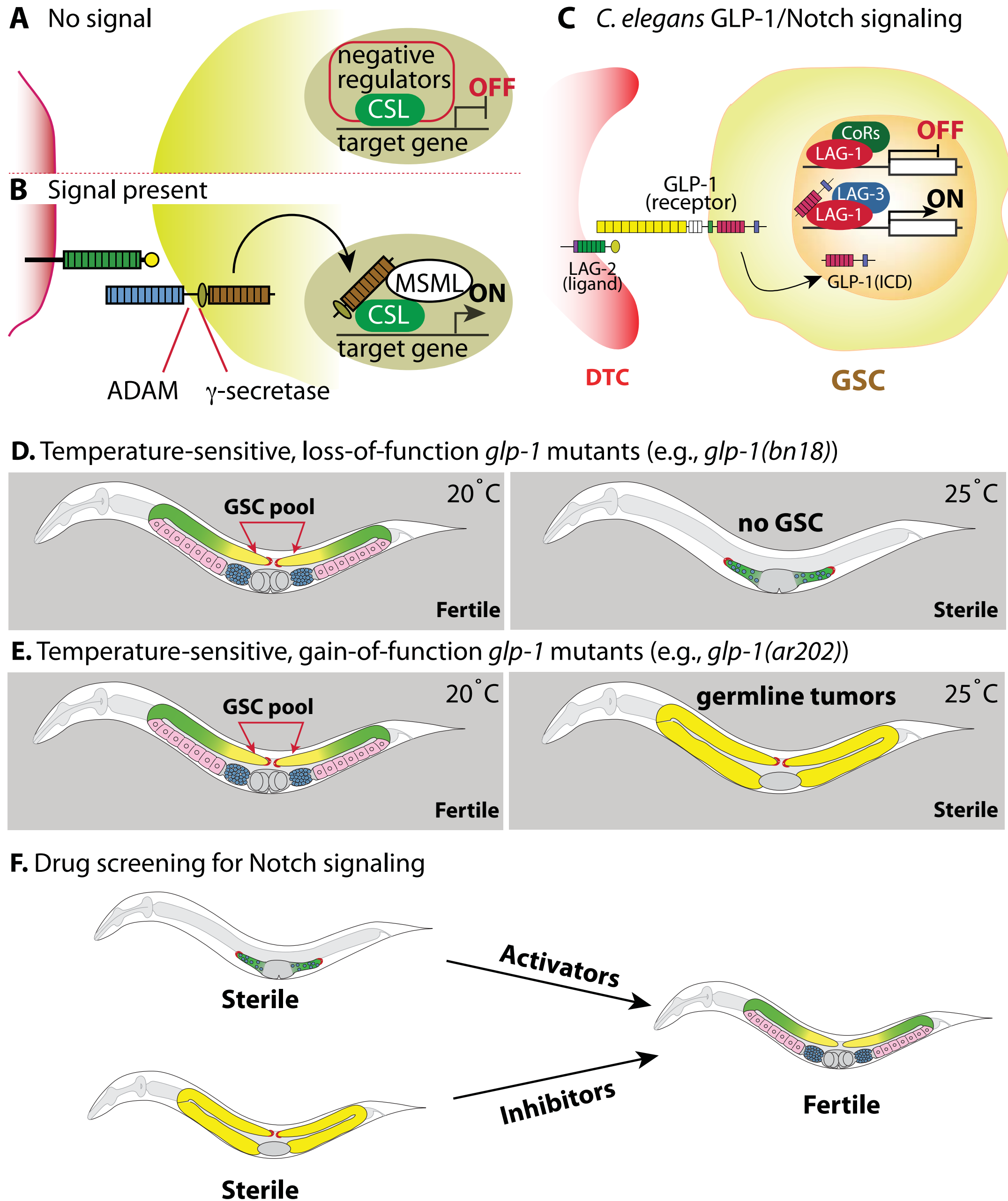

Fig. 3. Notch signaling pathways and a strategy for the phenotype-based drug identification using *C. elegans* mutants. (A) Without signaling, negative regulators inhibit the expression of Notch target genes. (B) With signaling, cleaved NICD transports from membrane to nucleus. In nucleus, NICD form a ternary complex with CSL and co-activator, mastermine-like protein, to activate the expression of target genes. (C) *C. elegans* GLP-1/Notch signaling pathways. The DTC expresses GLP-1/Notch ligands (e.g., LAG-2) and employs GLP-1/Notch signaling to promote continued mitotic divisions. (D) In mutants lacking any of the core elements of the GLP-1/Notch signaling pathways (e.g., *glp-1* loss-of-function mutant), germ cells leave the mitotic cell cycle, enter meiosis, and undergo gametogenesis, resulting in no germline stem cells and sterility. Most temperature-sensitive *glp-1*(*bn18*) loss-of-function mutants are fertile at 20°C, but they are sterile at 25°C. (E) Constitutively active GLP-1/Notch signaling develop germline tumors. Most temperature-sensitive *glp-1*(*ar202*) gain-of-function mutants are fertile at 20°C, but they are sterile due to germline tumors at 25°C. (F) Strategy for the phenotype-based identification of drugs that either inhibit or activate Notch signaling using *C. elegans* mutants.

3E right) (Pepper *et al*., 2003b). 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*,

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., *gld-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 phosphorylates 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*$^{Min/+}$/*K-Ras*^{LA2} 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* hermaphrodite 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 *mpk-1/ERK* 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 *let-60/Ras* 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.

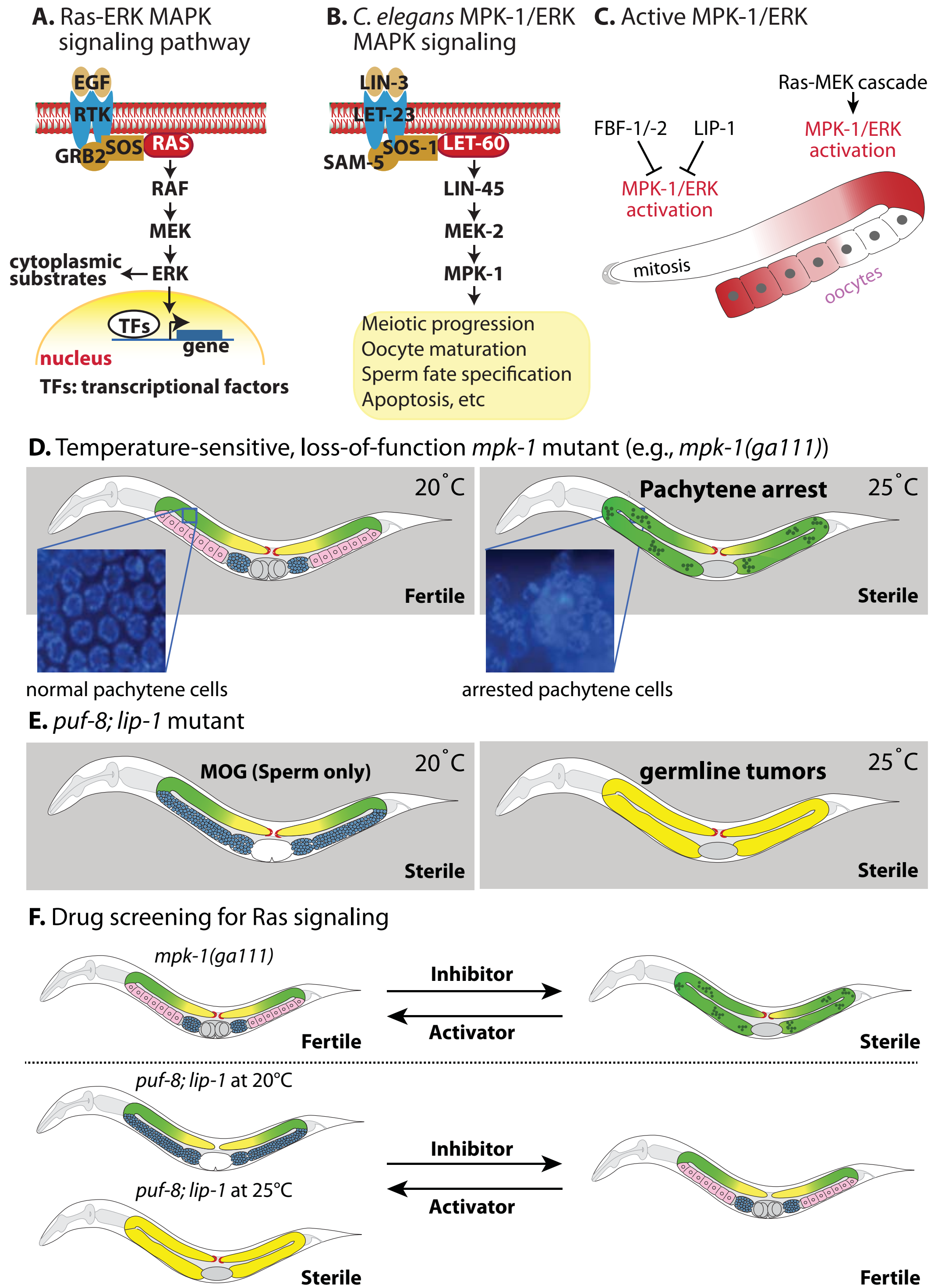

Fig. 4. Ras signaling pathways and a strategy for the phenotype-based drug identification using *C. elegans* mutants. (A) Conserved Ras-ERK MAPK signaling pathways. (B) *C. elegans* Ras-ERK MAPK signaling pathway. (C) Schematic of adult wild-type hermaphrodite germline. MPK-1/ERK is activated in the 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.

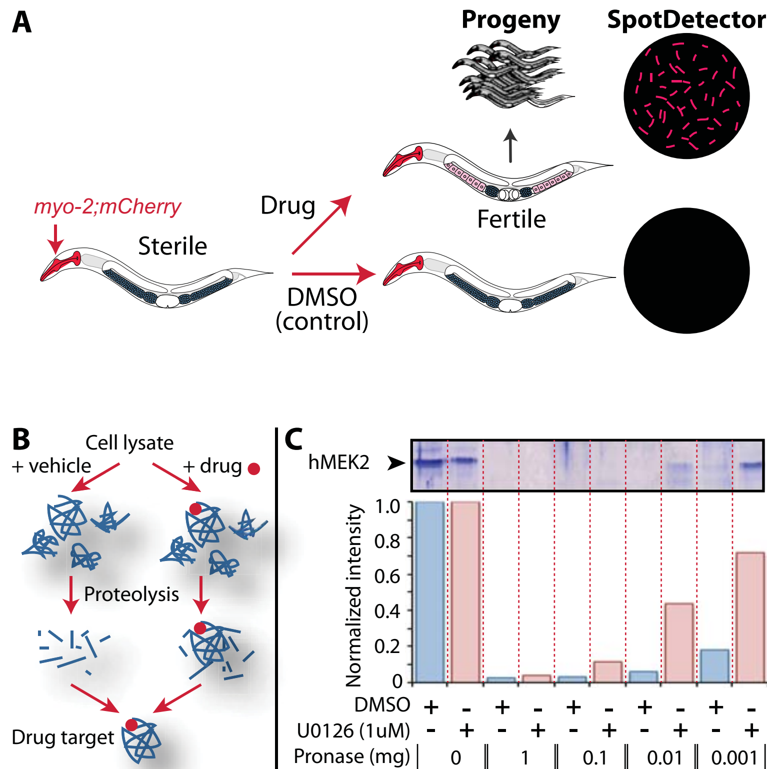

Fig. 5. Fertility and image-based high-throughput screening and *In vitro* DARTS using human MEK2 (hMEK2). (A) Fertility and image-based high-throughput drug screening. Slightly modified from (Benson *et al*., 2014). Fluorescence images can be taken on the Arrayscan V[ti]/SpotDetector can identify numerous progeny in wells treated with drugs and DMSO control. (B) Scheme of DARTS. (C) U0126 protects hMEK2 from pronase degradation. *In vitro* DARTS technique was optimized using a purified GST-tagged human MEK2 (hMEK2) protein with U0126, a well-known MEK2 inhibitor and DMSO. U0126 stabilizes hMEK2 from 0.001 mg pronase.

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., *mIn[mIs14(Pmyo-2::GFP) dpy-10(e128)*, called *mc6g*). Homozygotes 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; [P_{myo-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 of homozygote *puf-8*; *lip-1* mutants [GFP(-) and mCherry(+)] using an automatic worm sorter, COPAS™ *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 Arrayscan V^{ti}/SpotDetector (Benson *et al*., 2014). Detail methods are described in Benson *et al* (Benson *et al*., 2014).

To date, several drugs that target core components of each oncogenic signaling pathways through target-specific *in vitro* screening. Nontheless, 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*., 1998; 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-b-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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