Genetic Regulation of Cell Extrusion in *Caenorhabditis elegans*

By

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Abstract

Programmed elimination of cells occurs during animal development and homeostasis to maintain appropriate cell numbers. One evolutionarily conserved method by which organisms eliminate cells in a programmed manner is by cell-autonomous activation of the caspase-mediated apoptosis pathway, which produces a corpse that is engulfed and degraded by phagocytic cells. Cell elimination can also occur by a different method, called cell extrusion, in which the cell to be eliminated is squeezed out from a layer of cells, such as an epithelium. Cell extrusion is also an evolutionarily conserved form of cell elimination and has been observed in organisms ranging from *Drosophila* to mammals. It is the primary method of cell elimination in mammalian epithelial tissues, such as the small intestinal epithelium.

A specific set of cells is eliminated by cell extrusion by caspase-deficient *C. elegans* embryos. Remarkably, these cells show morphological and cytological features of apoptosis in the complete absence of caspases. To identify the genes required for cell extrusion, I performed a genome-scale RNAi screen for worms that express a phenotype arising from defective extrusion. This RNAi screen revealed that genes required for entry into the cell cycle and G1/S-phase transition are required for cell extrusion. From subsequent live-imaging experiments using confocal microscopy, I discovered that cells fated for extrusion earlier entered the cell cycle and arrested in S phase. I found that extruded cells are the much smaller sisters generated by unequal cell divisions. Taken together, my findings indicate that generation from an unequal cell division, entry into the cell cycle, and the S-phase arrest that likely results from these processes are all coupled to the cell extrusion fate, as genetically perturbing any of these processes blocks cell extrusion. In short, I have identified the genetic factors that lead to the arrested cell-cycle state that drives caspase-independent apoptotic cell extrusion by *C. elegans*. Studies of mammalian epithelial cells using hydroxyurea (performed in collaboration with the Jody Rosenblatt Laboratory) indicate that S-phase arrest drives cell extrusion from mammalian epithelia. These findings demonstrate that cell extrusion driven by S-phase arrest is evolutionarily conserved and suggest implications for development, physiology and disease.

Thesis Advisor: H. Robert Horvitz
Title: Professor of Biology

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Chapter One

Introduction

Overview

Apoptotic cell extrusion eliminates the same cells in the absence of caspases that are otherwise eliminated by caspase-mediated apoptosis and engulfment (Denning *et al.*, 2012). I found that cell cycle entry and S-phase arrest are required for cell extrusion. In this introductory chapter, I briefly describe:

i) Apoptosis and the known regulators of apoptotic cell death in *C. elegans* and their mammalian homologs,

ii) Regulation of cell extrusion by vertebrates, *D. melanogaster* and *C. elegans*, and

iii) Regulation of cell cycle entry, transition into S-phase and S-phase arrest.

Apoptotic programmed cell death

1. Apoptosis – a collection of morphological and cytological features

The first account of developmental cell death gave a description of disappearing cells during the development of the toad *Alytes obstetricans* (Vogt, 1842, as cited in Clarke and Clarke, 1996). Subsequent studies described the visible changes cells transitioned through as they died during organismal development (reviewed by Clarke and Clarke, 1996). Morphological changes such as condensation of chromatin, nuclear and cytoplasmic shrinkage, and membrane protrusions that bud off from the dying cell were commonly observed in developmental cell deaths (Glücksmann, 1951; Kerr *et al.*, 1972; Wyllie *et al.*, 1980). In contrast, cells undergoing necrosis, a form of pathological cell death caused by injury, were observed to have a loss of membrane integrity and swelling of organelles, neither of which were observed in developmental deaths (Wyllie *et al.*, 1980). The developmental deaths, initially termed "cellular degenerations" and "shrinkage necrosis," eventually began to be referred to as programmed cell deaths because of their temporal and spatial reproducibility, their likely involvement of a signaling system that triggers these deaths, and their occurrence as a part of the "developmental program" (Glücksmann, 1951; Kerr, 1971; Lockshin and Williams, 1964). The Greek word apoptosis, which translates to "falling off" in English, was adopted to distinguish this form of developmental cell death from necrotic deaths (Kerr *et al.*, 1972). Other features of developmental deaths that were discovered more recently, including DNA fragmentation and externalization of the inner membrane surface lipid

phosphatidylserine (PS), have also been added to the collection of morphological changes that define apoptosis (Arends *et al.*, 1990; Martin *et al.*, 1996; Naito *et al.*, 1997). Both apoptotic DNA fragmentation and PS exposure are detected biochemically, with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and fluorescently labeled protein Annexin V used for detecting DNA fragmentation and externalized PS, respectively (Gavrieli *et al.*, 1992; Koopman *et al.*, 1994; Vermes *et al.*, 1995).

As discussed below, caspases were discovered to be the main drivers of apoptosis. Activation of caspases is broadly considered to be synonymous with apoptosis. However, recent work in *C. elegans* has demonstrated that apoptosis can occur in the complete absence of caspases (Denning *et al.*, 2012; Denning *et al.*, 2013). The identity of the molecular drivers of apoptosis in these contexts – including cell extrusion, the focus of this thesis – remains to be established.

2. Apoptotic programmed cell death in *C. elegans*

A. Morphological and cytological features of apoptotic programmed cell deaths in *C. elegans*

C. elegans has an almost invariant cell lineage: each hermaphroditic animal generates 1090 somatic cells of which 131 undergo programmed cell death and 959 cells remain (Sulston and Horvitz, 1977; Sulston *et al.*, 1983) . Of these 131 deaths, 113 cells die during embryonic development and 18 cells die during the L1 and L2 stages of larval development. The invariance in origins and fates exhibited by other cells is also true of cells that undergo programmed cell

death – the identity, the location and the time of each cell death are established and reproducible among animals. Dying cells can be easily identified from their refractile "button-like" appearance under Nomarski optics. This appearance differs significantly from the slightly indented appearance of cells that do not die.

During the cell death process in *C. elegans*, cells undergo different phases of morphological and ultra-structural changes. Morphological changes that are observable by Nomarski optics were first described for ectodermal cell deaths that occur during postembryonic development (Sulston and Horvitz, 1977). The sequential morphological changes reported for these deaths are: i) appearance of blobs at the nuclear perimeter, ii) a decrease in granularity the nucleoplasm, which starts to appear somewhat refractile, iii) a marked increase in the refractility of the nucleus that gives it a "flattened" appearance, iv) a slight decrease in the internal refractility that leaves a refractile shell at the surface of the nucleus, and v) the shrinkage and subsequent disappearance of the nucleus and cell. Correlated changes in morphological and ultra-structural features, as observed by Nomarski optics and electron microscopy (EM), respectively, have been described for cell deaths occurring in the ventral nerve cord (Robertson and Thomson, 1982). At their generation, these cells are usually smaller than their sisters and a period of decreased refractility is initially observed. Micrographs obtained by EM of this phase show thin parallel processes of the engulfing cell gradually surrounding the cell fated to die. EM micrographs of the next phase show the beginning of chromatin aggregate formation, expansion of the nuclear membrane, shrinkage of cytoplasm, and budding off of membrane bound

fragments into the engulfing cell as the death process starts. In this phase, an increase in nuclear refractility can be observed with Nomarski optics. In the subsequent phase, the entire cell becomes refractile in appearance, and EM micrographs from this phase show a dense nucleus, little cytoplasm and whorling of internal and plasma membranes. In the final phase, the dying cell shrinks and disappears from observation using light microscopy. EM micrographs from this phase show complete breakdown of nuclear membrane with traces of the dying cell fragments within vacuolar structures of the engulfing cell. Observations of both the ectodermal and ventral nerve cord cell deaths note similar morphological changes that include an increase in refractility that is followed by shrinkage and disappearance of the cell. Cell deaths that occur during *C. elegans* embryonic development also follow the same death process (Sulston *et al.*, 1983).

Features of apoptosis observed using EM, such as cytoplasmic condensation, nuclear chromatin aggregation and budding of membrane-bound vesicles are common between developmental programmed cell death in *C. elegans* and apoptotic cell death in other organisms (Robertson and Thomson, 1982). Other features, such as membrane whorls, are not observed during apoptosis in other organisms.

Apoptotic cell deaths in *C. elegans* are also characterized by DNA fragmentation and PS exposure. Similar to mammalian cells, TUNEL staining can be used to observe DNA fragmentation in apoptotic cells in *C. elegans* (Gavrieli *et al.*, 1992; Wu *et al.*, 2000). Transgenic expression of a translational fusion of

GFP with MFG-E8, a protein identified in mice that binds to PS, is used for detecting PS exposure on the surface of apoptotic cells in *C. elegans* (Hanayama *et al.*, 2004; Venegas and Zhou, 2007).

B. Genetic regulators of apoptotic programmed cell death in *C. elegans*

The reproducibility of *C. elegans* programmed cell deaths in terms of location, time and cellular identity produced a unique opportunity for researchers to identify the program that causes cell death. Interestingly, it was a mutagenesis screen for embryos displaying any interesting phenotype with Nomarski optics that identified cell death defective (Ced) mutants with mutations in the engulfment genes *ced-1* and *ced-2* (Hedgecock *et al.*, 1983). These mutants are characterized by an accumulation of excess corpses caused by their failed engulfment. A mutant screen for the suppression of corpse accumulation in an engulfment-defective background led to the identification of *ced-3*, a gene that was found to be generally required for programmed cell death (Ellis and Horvitz, 1986). Screens for the survival of specific cells led to the identification of the genes *ced-4* and *egl-1*, which are required for cell death, and also *ced-9*, which generally functions as an antagonist to the cell death process (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986; Hengartner *et al.*, 1992; Yuan and Horvitz, 1992). The genes *egl-1*, *ced-9*, *ced-4* and *ced-3* define a core programmed cell death pathway that is conserved in most animals. The functions of these genes in programmed cell death and their mammalian orthologs are described below.

i. *ced-3* is required for most cell deaths

Loss-of-function mutations in *ced-3* were isolated from suppressor screens for the accumulation of cell corpses in *ced-1(lf)* animals (Ellis and Horvitz, 1986). The *ced-3* mutations identified lead to a loss of almost all cell corpses. Cells that normally undergo programmed cell death remain alive in these mutants and instead differentiate with fates similar to lineally related cells, such as those of "sister" cells, "aunts," or "nieces," or of homologs that don't undergo death in similar lineages. For example, the M4 sister cell normally undergoes an apoptotic death during embryonic development in wild-type animals. However, in *ced-3* mutants, the M4 sister cell escapes the death fate and expresses the transcriptional reporter $P_{ceh\text{-}28}$*::GFP*, a fluorescent marker of the M4 neuron (Hirose *et al.*, 2010). The V5.paapp cell that dies in wild-type animals adopts the dopaminergic fate of its lineal "aunt" V5.paaa in *ced-3* mutants (Ellis and Horvitz, 1986). The sister of the HSN/PHB precursor cell adopts the fate of either "niece" (HSN or PHB neuron) at a low frequency in *ced-3* mutants (Guenther and Garriga, 1996). The 6 undead Pn.aap cells (belonging to the P1, P2 and P9-P12 lineages) in *ced-3* mutants adopt the neuronal fate of the homologous Pn.aap cells (belonging to P3-P8 lineages) that do not die. Undead cells can sometimes functionally substitute for the lineal relatives whose fate the undead cells adopt (Avery and Horvitz, 1987).

Comparisons of the protein sequence of CED-3 found it to be similar to that of the mammalian interleukin-1β-converting enzyme (ICE), a member of the class of cysteine proteases, later called caspases, that cleave proteins after an

aspartic acid residue (Yuan *et al.*, 1993). Overexpression of a mammalian ICE protein is sufficient to induce apoptosis in mammalian cells, and certain conserved amino acids that prevent cell death when mutated in *C. elegans* are also important in ICE for inducing cell death in mammalian cells (Miura *et al.*, 1993). Similarly, overexpression of CED-3 in cells that normally survive in *C. elegans* can cause a cell death that shares morphological features with programmed cell deaths (Shaham and Horvitz, 1996a).

The *C. elegans* genome encodes a total of 3 caspases – CED-3, CSP-1 and CSP-2, and one caspase-like protein CSP-3. Apart from *ced-3*, only *csp-1* loss of function produces a Ced phenotype and that too only in a sensitized background (Denning *et al.*, 2013). An estimated 800 proteins encoded by the *C. elegans* genome are thought to be targets of CED-3 cleavage (Taylor *et al.*, 2007). Multiple caspases exist in mammals, and many of these such as Caspases 3, 6, 7, 8, 9 and 10 have known roles in apoptotic cell death (Jiang and Wang, 2000; Riedl and Shi; 2004; Yuan and Akey, 2013). Activation of caspases is believed to be the final stage in the regulation of apoptotic cell death.

A small number of cells undergo cell death in *ced-3*-deficient *C. elegans* animals. These include the male linker cell, which does not undergo an apoptotic death (Ellis and Horvitz, 1986, Abraham *et al.*, 2007), a few stochastic apoptotic cell deaths that sometimes occur in *ced-3* mutants (Shaham *et al.* 1999; Denning *et al.* 2013), and a set of cells that undergo apoptotic cell extrusion by caspase-deficient embryos (Denning *et al.*, 2012). The regulation of cell extrusion by *ced-*

3 mutants is the topic of this thesis. This phenomenon is explained in more detail in a later section of this chapter.

ii. *egl-1*, *ced-9* and *ced-4* regulate *ced-3* activation

The genes *ced-4*, *ced-9* and *egl-1* were initially identified from screens that scored the survival of specific cells but have since been found to function generally in programmed cell death (Ellis and Horvitz, 1986; Yuan and Horvitz, 1992; Hengartner *et al.*, 1992; Conradt and Horvitz, 1998). Genetic analysis has shown that these genes function upstream of *ced-3,* such that in cells fated to die, *egl-1* prevents *ced-9*'s inhibition of *ced-4*, which activates *ced-3* function (Hengartner *et al.*, 1992; Shaham and Horvitz, 1996a; Conradt and Horvitz, 1998).

Biochemical experiments have shown that the CED-4 protein physically binds to CED-3 to activate it (Chinnaiyan *et al.*, 1997; Sheshagiri and Miller, 1997; Wu *et al.*, 1997; Yang *et al.*, 1998). The mammalian homolog of CED-4, Apaf-1, was identified from biochemical purification assays for fractions that cleave Caspase-3 *in vitro* (Zou *et al.*, 1997). Apaf-1-deficient mice show signs of reduced apoptosis and resistance to apoptotic stimuli (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998).

CED-9 protein is believed to localize to mitochondria and bind to CED-4 dimers, sequestering them from interacting with and activating CED-3 caspase (Chen *et al.*, 2000; Yan *et al.*, 2005). However, one study conducted in the *C. elegans* germline argues against the co-localization of CED-4 and CED-9 on mitochondria and suggests that while CED-9 does localize to mitochondria, CED-

4 instead is localized to the peri-nuclear periphery (Pourkarimi *et al.*, 2012). The differences in the findings of this study could be because of a focus on radiation-induced germline cell deaths rather than embryonic cell deaths, use of CED-4::GFP transgene for many experiments instead of endogenous protein, and no clear distinction between CED-4 isoforms that have been shown to have opposing functions with regard to apoptosis (Shaham *et al.*, 1996b). CED-9 shows homology to the mammalian Bcl-2 protein family, named after the Bcl-2 protein that is overexpressed in B cells in B-cell lymphomas as a result of chromosomal translocation (Bakhshi *et al.*, 1985; Cleary *et al.*, 1986; Tsujimoto and Croce, 1986). Overexpression of Bcl-2 can prevent apoptosis in mammalian cells (Hockenbery *et al.*, 1990; Vaux *et al.*, 1988; Vaux *et al.*, 1992), and Bcl-2 can functionally replace CED-9 in *C. elegans* (Hengartner and Horvitz, 1994).

EGL-1 is a BH3-only protein, which contain only the third Bcl-2 homology domain. EGL-1 is largely regulated at a transcriptional level and translational level for expression specific to cells fated to undergo apoptotic death (Conradt and Horvitz, 1999; Sherrard *et al.* 2017). Large regulatory regions both 5' and 3' of the *egl-1* coding sequence function to achieve this kind of specificity of expression (Hirose *et al.*, 2010; Hirose *et al.*, 2013; Liu *et al.*, 2006; Nehme *et al.*, 2010; Thellmann *et al.*, 2003). Additionally, the *mir-35* and *mir-58* microRNA families bind to the 3'UTR of *egl-1* transcripts to prevent *egl-1* translation in cells not fated to die (Sherrard *et al.*, 2017). Upon expression, EGL-1 binds CED-9 so as to disrupt CED-9's binding of CED-4, which releases CED-4 and facilitates the activation of CED-3 (del Peso *et al.*, 1998). Pro-apoptotic BH3-only proteins in

mammals include Bim, Bmf, Bid, Bik, Bad, Hrk, Puma and Noxa (Boyd et al., 1995; Inohara et al., 1997; Luo et al., 1998; Nakano and Vousden, 2001; Oda et al., 2000; O'Connor et al., 1998; Puthalakath et al., 2001; Yang et al., 1995). Similar to the transcriptional regulation of *egl-1* in *C. elegans*, transcriptional control of the pro-apoptotic BH3-only proteins Puma and Noxa has been described in mammals (Han *et al.*, 2001; Nakano and Vousden, 2001; Oda *et al.*, 2000; Yu *et al.*, 2001).

iii. Small cell size and apoptotic fate are correlated

Cells that are fated to undergo programmed cell death are generated as smaller sisters from unequal cell divisions of their mothers (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Studies of the unequal divisions of Q.a and Q.p cells show common genetic requirements for the unequal division of these cells as well as the apoptotic fate of their respective daughter cells Q.aa and Q.pp (Chien *et al.*, 2013; Cordes *et al.*, 2006; Gurling *et al.*, 2014; Singhvi *et al.*, 2011; Teuliere *et al.*, 2014). Similar observations have been made for many other neuronal lineages, including the NSM neuroblast lineage (Frank *et al.*, 2005; Guenther and Garriga, 1996; Hatzold and Conradt, 2008). These studies indicate a correlation between the small size of cells fated for apoptosis and their caspase-mediated apoptotic fate. Interestingly, physical perturbation of such unequal divisions also leads to the survival of the daughter cell that is otherwise fated to die, which suggests that their small size likely facilitates the caspase-mediated cell death process (Ou *et al.* 2010).

The genes required for both unequal cell division of the mother cell and the caspase-mediated apoptosis of the daughter cell in the Q lineage define at least three separate pathways – the *pig-1* pathway, the *arf* pathway and the *toe-2* pathway. The *pig-1* pathway is a kinase cascade, in which the genes *par-4*, *strd-1* and *mop-25.1/mop-25.2* encode members of a kinase complex that activates the kinase PIG-1 by phosphorylating it (Chien *et al.*, 2013; Cordes *et al.*, 2006). The genes *par-4, strd-1* and *mop-25.1/mop-25.2* encode proteins homologous to mammalian LKB1, STRADα and MO25 proteins, respectively, which form a complex. PIG-1 is the *C. elegans* homolog of the mammalian AMPK-like kinase MELK. PIG-1 likely controls the spindle positioning and/or asymmetric myosin localization to promote unequal cell division (Cordes *et al.*, 2006; Ou, *et al.*, 2010). The *arf* pathway consists of the genes *arf-1.2*, *arf-3*, and *arf-6*, which encode homologs of ARF GTPases; *grp-1*, which encodes an ARF guanine nucleotide exchange factor (GEF); and *cnt-2*, which encodes an ARF GTPase activating protein (GAP) (Singhvi *et al.*, 2011; Teuliere *et al.*, 2014). These genes might regulate unequal cell division through their known role in membrane trafficking (D'Souza-Schorey and Chavrier, 2006). *toe-2* encodes a protein with a domain common to the proteins Dishevelled, EGL-10 and Pleckstrin, or the DEP domain. TOE-2 might function downstream of the *C. elegans* p38 MAPK homolog MPK-1 in regulating the unequal division of the Q.a cell (Gurling *et al.*, 2014).

Interestingly, most genes of the *pig-1* and *arf* pathways, and *toe-2* are required for cell extrusion by caspase-deficient *C. elegans* embryos (Denning *et*

al., 2012; this thesis). I have found that the genes *pig-1*, *grp-1* and *cnt-2* are required for both the unequal cell division that generates the cell ABplpappap, as well as its cell extrusion that occurs later (Chapter 3). These findings suggest a correlation between cell size and cell extrusion. However, unlike the neurons of Q lineage and the NSM lineage, ABplpappap still undergoes caspase-mediated apoptosis in single mutants of the genes *pig-1* and *grp-1* (Denning *et al.*, 2012). These findings suggest that cell size and the caspase-mediated apoptotic fate are not correlated for ABplpappap.

Cell Extrusion

The main focus of this thesis is the genetic regulation of cell extrusion by *C. elegans*. Cell extrusion is a phenomenon that observed in many organisms and has been studied in detail using vertebrates and *D. melanogaster*. The best-studied instances of cell extrusion by these organisms are reviewed below. Previously established findings about cell extrusion by *C. elegans* are also mentioned.

1. Cell Extrusion by Vertebrates

Cell extrusion functions to remove cells without disrupting the continuous barrier formed by epithelial layers in vertebrates. The contexts in which extrusion occurs in vertebrates and the underlying molecular mechanisms are reviewed below.

A. Apoptotic cell extrusion by vertebrate epithelia

Studies of chicks and mice show that epithelia in vertebrates remove apoptotic cells mainly by apical cell extrusion to maintain epithelial barrier function (Rosenblatt *et al.*, 2001). Exposure of Madine Darby canine kidney (MDCK) cell layers to ultraviolet (UV) light produces apical cell extrusion of cells undergoing apoptosis. Extruding dying cells show increased actin and myosin staining at their interface with live cells, forming a ring-like structure called an "apoptotic actin ring." Preventing actin polymerization with drugs such as Latrunculin A or Cytochalasin D or blocking myosin contraction with inhibitors of myosin regulators Rho Kinase or Myosin Light Chain Kinase prevents the extrusion of apoptotic cells. Although the apoptotic actin ring forms in both the

apoptotic cell and its living neighbors, Rho-mediated actin-myosin contraction in living neighbors is required for extrusion to occur.

The dying cell produces the signal for the actin-myosin contraction in its neighbors (Rosenblatt *et al.*, 2001). This signal is the bioactive lipid sphingosine-1-phosphate (S1P). The S1P binds to and activates $S1P_2$ receptor on the living neighbor cells (Gu *et al.*, 2011). The S1P-$S1P_2$ then activates Rho through p115RhoGEF. In these cells, the p115RhoGEF is recruited basally by microtubules that grow towards the basolateral surface, where p115RhoGEF activates Rho, which in turn assembles and activates myosin to cause contraction and apical extrusion of the dying cell (Slattum *et al.*, 2009). Reducing cell extrusion by inhibiting S1P signaling produces epithelial cell masses that are many layers thick both in MDCK cultures and in zebrafish epidermis (Gu *et al.*, 2015). These cells show a resistance to apoptosis because of a block in cell extrusion, indicating that extrusion promotes apoptosis in epithelial cells. Highly aggressive tumors such as pancreatic ductal adenocarcinomas (PDACs) have reduced $S1P_2$ receptor expression along with reduced levels of apical extrusion. Restoring S1P signaling by exogenous expression of the $S1P_2$ receptor not only restores apical extrusion, but also reduces the ability of PDAC cell lines to form orthotopic tumors and metastases.

These findings indicate that cell extrusion plays a critical role in promoting apoptosis, removing apoptotic cells and preventing cancer in vertebrate epithelia.

B. Live cell extrusion by vertebrate epithelia

Overcrowding in vertebrate epithelia leads to the extrusion of live cells (Eisenhoffer *et al.*, 2012). This extrusion of live cells can be observed in normal mammalian intestinal epithelium, zebrafish epidermis, and in crowded MDCK monolayers (Eisenhoffer *et al.*, 2012). Cell densities that promote live cell extrusion lead to accumulation of the stretch-activated channel Piezo1 in the form of cytoplasmic aggregates (Gudipaty *et al.*, 2017). The aggregated Piezo likely functions to activate the same S1P-$S1P_2$-Rho pathway that functions in apopotic cell extrusion to promote live cell extrusion (Eisenhoffer *et al.*, 2012; Gudipaty *et al.*, 2017). The extruded live cells eventually die after expulsion by caspase-mediated cell death induced by a loss of adhesion contacts, called anoikis. This finding demonstrates that cell extrusion is an important means of restoring homeostasis in crowded regions of vertebrate epithelia.

C. Cell extrusion in genetically abnormal conditions

A few examples of cell extrusion or changes in cell extrusion caused by genetic alterations are described below. These examples show how mutations in certain genes can affect cell extrusion, thus defining a role of these genes in cell extrusion by vertebrates.

i. APC

Amongst its many other roles (reviewed by Aoki and Taketo, 2007), the tumor suppressor APC also regulates microtubule dynamics by binding to and stabilizing microtubules (Munemitsu *et al.*, 1994; Nathke *et al.*, 1996; Smith *et al.*, 1994). Wild-type APC functions in the cell undergoing extrusion to drive apical

extrusion, and does so by controlling actin and microtubules at the site of contraction near the basal surface (Marshall *et al.*, 2011).

Germline mutations that produce truncations in APC give rise to the pathological condition familial adenomatous polyposis (FAP), which is characterized by high frequency of polyp formation in the intestines (Groden *et al.*, 1991; Kinzler *et al.*, 1991). Similar mutations in the APC murine homolog drive intestinal tumorigenesis in mouse models (Fodde *et al.*, 1994, Oshima *et al.*, 1995; Su *et al.*, 1992). Expression of truncated versions of APC found in tumors that mostly lack the microtubule-binding domain drastically reduces apical cell extrusion and increases basal cell extrusion (Marshall *et al.*, 2011). These findings show that a decrease in apical extrusion and an increase in basal extrusion likely contribute to tumorigenesis in vertebrates and that the tumor suppressor APC prevents this tumorigenic process.

ii. Oncogene-driven extrusion

Oncogenic mutations in the Ras family GTPase K-Ras, including K-Ras^{G12V}, drive many aggressive cancers (reviewed by Prior *et al.*, 2012). Expression of K-Ras^{G12V} drives basal extrusion of cells in a cell-autonomous manner (Slattum *et al.*, 2014). These basally extruding K-Ras^{G12V} cells have abnormally high levels of autophagy that leads to the breakdown of S1P, the signaling molecule that is required for apical extrusion. Another study using the K-Ras^{G12D}-induced pancreatic cancer mouse model and examining human premalignant and malignant pancreatic lesions has suggested that loss of p120 catenin inhibits basal cell extrusion during the earliest stages of tumor invasion

and that the loss of p120 catenin initiates the down-regulation of S1P signaling via activation of NF-κB, which then promotes basal cell extrusion and invasion (Hendley *et al.*, 2016).

Expression of oncogenic versions of the gene ERBB2 in single cells inside growth-arrested acinar structures formed by MCF10A breast-cancer cells causes clonal outgrowth and subsequent MAPK-driven translocation (or extrusion) into the lumen of these acinar structures (Leung and Brugge, 2012). Such an outgrowth and translocation into the lumen could be a primary step in metastasis. ERBB2 is frequently amplified in breast cancers (Slamon *et al.*, 1987) and functions upstream of the MAPK pathway. The acinar structures formed by MCF10A cells are three-dimensional and are believed to better recapitulate many of the cell dynamics of tissues than monolayer cell culture.

These observations suggest that oncogene activation might drive tumorigenesis and metastasis by promoting basal cell extrusion and driving translocation of cells into blood vessel lumens.

iii. Altered adhesion

Cells in which the adhesion molecule E-cadherin is cleaved are extruded from epithelial monolayers (Grieve and Rabouille, 2014). The formation of adhesion contacts with the new neighbors brought together by extrusion of such cells in turn facilitates this extrusion and also keeps epithelial barrier function intact. The neighboring cells that function to close the gap formed by extrusion require functional E-cadherin for this function (Lubkov and Bar-Sagi, 2014).

Many pathological conditions of the epithelium exhibit altered expression levels of or mutations in adhesion molecules such as E-cadherin. These conditions include many types of carcinomas (reviewed by Cavallaro and Christofori, 2004) and inflammatory bowel conditions like celiac and Crohn's disease (Schuermann *et al.*, 1993; Zeissig *et al.*, 2007). The pathology of these conditions with altered adhesion could possibly be explained by defective regulation of cell extrusion.

iv. Cell competition

Cell competition is a cell-cell interaction that leads to the active elimination of less fit cells by fitter neighbors (reviewed by Vincent *et al.*, 2013). During cell competition, neighboring "winner" cells can eliminate "loser" cells by cell extrusion. In the examples described below, these loser cells carry either gain-of-function mutations in oncogenes such as *Ras* or *Src*, or loss-of-function mutations in tumor suppressor genes such as the polarity gene *scrib*. Most examples of extrusion caused by cell competition have been shown in MDCK cells.

Mosaic expression of H-Ras^{G12V}, an oncogenic form of the Ras family GTPase H-Ras, drives the apical extrusion of only the cells expressing this oncogene in MDCK cell culture (Hogan *et al.*, 2009). Expression of H-Ras^{G12V} causes upregulation of the tumor suppressor EPLIN1, which activates multiple downstream molecules in both the extruding cell and its neighbors to cause this extrusion (Kajita *et al.*, 2014; Ohoka *et al.*, 2015).

Similar to apical extrusion induced by oncogenic H-Ras^{G12V}, oncogenic v-Src also induces apical extrusion from MDCK cells when expressed in a mosaic fashion (Kajita *et al.*, 2010). Upregulation of focal adhesion kinase (FAK) activity that results in the activation of MAPK signaling and upregulation of myosin, both are responsible for apical cell extrusion of v-Src-expressing cells.

MDCK cells expressing a constitutively active (CA) form of the proto-oncoprotein YAP lose in cell competition and are apically extruded by surrounding wild-type neighboring cells (Chiba *et al.*, 2016). Vimentin and filamin in wild-type neighboring cells promotes the extrusion of CA-YAP cells. Using co-culture systems, Chiba *et al.* determined that the fitness order in cell competition is wild type (WT) > CA-YAP overexpressing > K-Ras^{V12} overexpressing > v-Src overexpressing.

MDCK cells with silencing of the polarity factor *scribble* (*scrib*^{KD}) are also eliminated by cell competition induced apical extrusion (Wagstaff *et al.*, 2016). These *scrib*^{KD} cells undergo mechanical compaction when surrounded by wild-type neighboring cells. These cells with already elevated levels of the tumor suppressor p53 have a further increase in p53 levels after compaction, which is mediated by Rho Kinase and p38 MAPK pathways. This increased level of p53 is sufficient to make cells lose cell competition and be apically extruded.

These examples demonstrate that cells in epithelia compete with their neighbors and cells that carry potentially harmful mutations are less fit to compete with their normal healthy counterparts. Cell extrusion is one of the processes by which such cells are eliminated; other processes include lineage

displacement, a process by which stem cells are induced to differentiate by cell competition and are no longer able to contribute cells to a tissue, and cell death (reviewed by Clavería and Torres, 2016). This kind of competition is thought to be a tumor-suppressive mechanism and has been termed epithelial defense against cancer or EDAC (reviewed by Kajita and Fujita, 2015).

D. Summary and relevance to cell extrusion by *C. elegans*

As discussed above, apoptotic cells are removed by cell extrusion from epithelial layers in homeostatic conditions, whereas crowding can cause extrusion of live cells from such layers so as to maintain a constant cell density. Cell extrusion can also occur under genetically abnormal conditions. Under normal conditions, vertebrate epithelial layers extrude cells in the apical direction. However, under pathological conditions, basal cell extrusion can occur and is believed to be a primary step in cell invasion, such as that observed in invasive tumors. The S1P-$S1P_2$ pathway is central to physiologically occurring cell extrusion from mammalian tissues. Cell extrusion or changes in cell extrusion caused by genetic abnormalities highlight the role of other genes and pathways in this process.

I did not identify homologs of the genes required for S1P production in the unbiased large-scale RNAi screen for disruption of cell extrusion by *C. elegans* embryos (Chapter 2). A clear homolog of the S1P2 receptor has not been identified in *C. elegans*. However, a homolog of the S1P-$S1P_2$ pathway member Rho GTPase, *rho-1*, was identified from the RNAi screen, suggesting a

conserved role of actin-myosin dynamics in cell extrusion by vertebrates and *C. elegans* (see Chapter 2 and Appendix). Additionally, even though I did not identify any of the genes that cause or modify cell extrusion when mutated in vertebrates from the RNAi screen, a role of the E-cadherin homolog HMR-1 has been demonstrated by Denning *et al.* (2012) in cell extrusion by *C. elegans*, suggesting that altered adhesion might also be common to cell extrusion by vertebrates and *C. elegans*. A candidate screen that tests the genes involved in vertebrate cell extrusion in a more focused way might find a role for other genes that act in vertebrate extrusion in the analogous process in *C. elegans*.

2. Cell Extrusion in *Drosophila melanogaster*

Cell extrusion occurs in *D. melanogaster* during normal development as well as under genetically abnormal conditions. The different contexts in which cell extrusion occurs in this organism and the molecular mechanisms involved are reviewed below.

A. Cell extrusion in development

i. Dorsal closure

Drosophila embryos undergo dorsal closure, during which an epithelial embryonic tissue called the amnioserosa gets internalized to promote the fusion of adjacent layers of epidermis (reviewed by Kiehart *et al.*, 2017). A subset of the cells in the amnioserosa undergo apoptotic basal cell extrusion to generate the force required to bring the lateral epidermis layers together for fusion (Toyama *et al.*, 2008). Reducing apoptosis in these cells by the expression of

anti-apoptotic *p35* and enhancing apoptosis by expressing pro-apoptotic *grim* decreased and increased the rate of dorsal closure, respectively (Toyama *et al.*, 2008). This example demonstrates the role of cell extrusion in generating morphological forces during development.

ii. Leg-joint development

Apoptotic basal cell extrusion also plays a role in epithelial folding in leg-joint development (Monier *et al.*, 2015). The cause of apoptosis in these cells is a sharp signaling boundary of the transforming growth factor-β (TGF-β) homolog Decapentaplegic (Dpp) in the leg-joint tissue, as neighboring cells with similar Dpp signaling do not undergo cell death (Manjon *et al.*, 2007). The dying cells pull the apical surfaces of the surrounding live cells as they undergo cell extrusion and thus create the fold of the leg-joint (Monier *et al.*, 2015). This example demonstrates a sculpting role of cell extrusion during development.

iii. Metamorphosis

During metamorphosis, histoblasts (or pupal epidermal cells) replace larval epidermal cells (LECs) (reviewed by Madhavan and Madhavan, 1980). LECs undergo apoptotic basal cell extrusion at their interface with histoblasts, which is driven by reduction in E-cadherin and contraction of apical actomyosin cables in both the apoptotic cell and its neighbors (Teng *et al.*, 2017). The activation of these processes that facilitate extrusion is dependent on caspase function. Disrupting myosin in the apoptotic cell compromises its extrusion, whereas disrupting it in the neighboring cells causes a delay in extrusion. This

example demonstrates a role of cell extrusion in tissue maturation during development.

iv. Notum midline refinement

The fly notum, the dorsal part of the thoracic segment, undergoes refinement of cell packing before terminal differentiation (Cohen *et al.*, 2010; Zeitlinger & Bohmann, 1999). Crowding at the notum midline causes delamination, basal extrusion and subsequent cell death (Marinari *et al.*, 2012; Levayer *et al.*, 2016). The crowding stochastically induces caspase activation, which is required for basal cell extrusion (Levayer *et al.*, 2016). This example demonstrates the role of cell extrusion in creating an efficient cellular organization in tissues during development.

v. Neuroblast ingression

During *D. melanogaster* embryonic development, neuroblasts (NBs) undergo ingression, a process that largely resembles basal cell extrusion. During ingression, NBs detach from their neighbors, move inside the embryo and subsequently undergo neurogenic divisions (reviewed by Hartenstein and Wodarz, 2013). Pulses of planar-polarized junctional and non-junctional (medial) myosin on the apical surface are required for this ingression (Simones *et al.*, 2017). The role of pulse generation by medial myosin may be more important for the neuroblast ingression (An *et al.*, 2017). These pulses drive both the apical constriction of the neuroblast and the disassembly of adherens junctions. This example demonstrates the role of cell extrusion in coordinating cell fate with cell position.

B. Cell extrusion in genetically abnormal conditions

A few examples of cell extrusion or changes in cell extrusion caused by genetic alterations are described below. These examples show how mutations in certain genes can affect cell extrusion, thus defining a role of these genes in cell extrusion by *D. melanogaster*.

i. Cell competition

Loss of *dCsk* in discrete regions of the imaginal epithelia causes basal cell extrusion of cells in direct contact with wild-type neighboring cells (Vidal *et al.*, 2006). The extruded cells subsequently undergo apoptosis. This effect disappears if the entire imaginal epithelium is *dCsk*$^-$. The basal extrusion process requires function of E-cadherin, p120-catenin, Rho GTPase and the JNK signaling pathway. Disrupting the function of any of these pathways or molecules reduces both apoptosis and basal extrusion of *dCsk*$^-$ cells.

Tumorigenic clones mutant for the polarity gene *scrib* are eliminated from eye imaginal epithelium by basal cell extrusion (Vaughen and Igaki, 2016). Extrusion of *scrib*$^-$ cells occurs before apoptosis and is caused by the upregulation of Slit-Robo2-Ena pathway in these cells arising from increased JNK signaling. The disruption of cadherin-based adhesion and actin-myosin regulation downstream of Ena promote the extrusion of these *scrib*$^-$ cells.

ii. TGF-β signaling

Loss of signaling from TGF-β ligand Dpp leads to cell loss via basal cell extrusion in imaginal disc epithelia (Shen and Dahmann, 2005; Gibson and Perrimon, 2005). This basal extrusion of cells is observed in mutant clones of both the Dpp receptor gene *thickveins* (*tkv*) as well as the gene *mothers against Dpp* (*mad*), which encodes a downstream transcription factor. Dpp signaling organizes the cytoskeleton in the wing epithelial cells. In the absence of Dpp signaling, *tkv*$^{-}$ mutant clones have F-actin localized to the center and a loss of apical microtubule network. Furthermore, these clones lose E-cadherin contacts to normal neighbor cells and form E-cadherin contacts with similar *tkv*$^{-}$ mutant clones.

iii. Abl kinase

Loss of *abl*, which encodes the protein Abelson kinase, produces basal cell extrusion in ventral cells during gastrulation when cells go through coordinated apical constriction (Jodoin *et al.*, 2016). The misexpression of the actin regulator protein Ena in embryos with loss of *abl* disrupts apical-basal polarity, adherens junction positioning and mislocalizes myosin to basolateral surfaces. These perturbations result in the basal extrusion of affected cells. Depletion of *ena* is sufficient to suppress the cell extrusion caused by loss of *abl*.

iv. Epigenetic regulation of non-canonical Wnt signaling

Mutant follicle stem cell (FSC) clones lacking the polycomb group genes *Psc* and *Su(z)2* are extruded basally from the epithelium in *Drosophila* ovaries (Li *et al.*, 2010). The disruption of planar cell polarity pathway genes *starry night*

(*stan*), *four-jointed* (*fj*) and *daschous* (*ds*) prevents extrusion of these cells, indicating that non-canonical Wnt signaling mediates this cell extrusion process.

C. Summary and relevance to cell extrusion by *C. elegans*

Cell extrusion plays a major morphological role during normal embryonic and larval development in *D. melanogaster*. Similar to the case in vertebrates, specific genetic alterations induce cell extrusion in this organism. Unlike cell extrusion by mammals, cell extrusion in *D. melanogaster* occurs almost exclusively in the basal direction. A variety of molecular pathways such as those for TGFβ signaling, caspase-mediated apoptosis, and non-canonical Wnt signaling are involved in the basal cell extrusion in this organism. I have not identified a role of the corresponding players in cell extrusion by *C. elegans* from my unbiased large-scale RNAi screen (discussed in Chapter 2). I did identify the *C. elegans* gene encoding the homolog of the actin-myosin regulator RhoA, *rho-1*, from the genome-scale RNAi screen for candidates required for cell extrusion and a role of the *C. elegans* E-cadherin homolog, HMR-1, has been previously demonstrated by Denning *et al.* (2012). A significant role of actin-myosin dynamics and adhesion in cell extrusions observed in *D. melanogaster* is described in the sections above, and a similar role in vertebrate cell extrusion has been discussed in a previous section. These observations suggest that roles of actin-myosin regulation and adhesion in cell extrusion are likely conserved between vertebrates, *D. melanogaster* and *C. elegans*. A candidate screen that tests in a more focused manner the pathways that modulate the actin-myosin

and adhesion for cell extrusion by *D. melanogaster* could possibly uncover a role of analogous pathways in cell extrusion by *C. elegans*.

3. Cell extrusion by *C. elegans*

A. Features of cell extrusion by *C. elegans*

Cell extrusion by *C. elegans* was first observed during cell lineage tracing studies of *ced-3(lf)* embryos (Ellis and Horvitz, 1986). The cell ABalapapaa, which is otherwise eliminated by caspase-mediated apoptosis, was found to detached from the embryo at 3.5 hours of embryonic development. A few such detached cells were found to be floating between the embryo and the egg shell in *ced-3(lf)* embryos.

A subsequent characterization of this phenomenon revealed that at least 8 cells can be extruded in *ced-3(lf)* embryos and that on average 6 cells are extruded during embryonic development in embryos of this genotype (Denning *et al.*, 2012). Cell extrusion is not restricted to *ced-3(lf)* embryos, but occurs in all mutants in which the activation of the caspase CED-3 does not occur, such as *egl-1(lf)*, *ced-9(gf)* and *ced-4(lf)* mutants. Identification of these cells using a time-lapse imaging technique that traces the ancestry of extruded cells revealed that cells that are extruded in caspase-deficient embryos are eliminated by caspase-mediated apoptosis and subsequent engulfment in wild-type embryos.

Remarkably, extruded cells display morphological and cytological features of apoptosis, even though these cells are completely devoid of caspases. These features include chromosomal condensation, DNA degradation and PS

exposure. Hence, Denning *et al.* (2012) showed that apoptosis can occur in the complete absence of caspases. The regulators of this caspase-independent apoptosis of extruded cells are yet to be found.

B. Previously identified genetic requirement of cell extrusion by *C. elegans*

An EMS mutagenesis screen for regulators of cell extrusion revealed the involvement of the PIG-1 kinase, the *C. elegans* homolog of a mammalian AMPK-like kinase MELK (Denning *et al.*, 2012). In mammals, the master kinase LKB1 functions in a complex with the pseudokinase STRADα and the MO25α proteins to regulate a family of AMPK-like kinases. Epistasis experiments support that an analogous complex (with the homologs of LKB1, STRADα and MO25α encoded by the *C. elegans* genes *par-4*, *strd-1*, and the paralogs *mop-25.1*, *mop-25.2* and *mop-25.3)* functions to regulate cell extrusion by partially activating PIG-1.

Another gene identified from the EMS mutagenesis screen for regulators of cell extrusion is *grp-1* (Denning *et al.*, 2012), which encodes the *C. elegans* homolog of the of the mammalian ADP ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) GRP1. Candidate-based testing of the *C. elegans* ARF homologs encoded by the genes *arf-1.2* and *arf-3* revealed that these genes also regulate cell extrusion.

As extruded cells are found to not express adhesion markers such as the classical cadherin, HMR-1, or the Disc Large homolog DLG-1, the PAR-4 – PIG-1 kinase cascade likely promotes extrusion by inhibiting the expression of these adhesion markers in cells fated to be extruded (Denning *et al.*, 2012). Mutating

pig-1 in a *ced-3(lf)* background produces ectopic expression of adhesion genes in and prevents the extrusion of otherwise-extruded cells, suggesting that increased adherence of otherwise extruded cells in the *pig-1 ced-3* double mutant leads to their survival.

C. Summary

Cell extrusion expels cells from caspase-deficient *C. elegans* embryos that are otherwise killed by caspase action. These extruded cells show many hallmarks of apoptosis in the complete absence of caspases, indicating that a yet undefined pathway causes these cells to undergo apoptosis. The PAR-4(LKB-1) – PIG-1(MELK) kinase cascade is required for the cell extrusion process in *C. elegans*. This kinase cascade likely functions to decrease the level of adhesion proteins on the surface of extruded cells to promote their extrusion.

To comprehensively identify genes required for cell extrusion by *C. elegans*, I performed a genome-scale RNAi screen for defective cell extrusion from which I identified cell-cycle genes that were enriched for regulators of cell-cycle entry and S-phase progression. I subsequently found using cell-cycle reporters that cells that are extruded enter and arrest in S phase of the cell cycle. Additionally, preventing this S-phase arrest by RNAi against cell-cycle genes required for S-phase entry also blocked cell extrusion. In the next section, I have reviewed regulators of cell cycle entry and S-phase progression, including those that were identified by the genome-scale RNAi screen. I have provided a more comprehensive overview of my findings at the end of this chapter.

G1/S-phase transition, replication stress and their role in cell extrusion by *C. elegans*

A cell cycle is the sequence of steps that generally results in the division of a cell. Such general cell cycles can be divided into four phases. The process of DNA synthesis duplicates the DNA content of a cell in S phase of the cell cycle before mitotic cell division in the M phase of the cell cycle. In most cell cycles, S and M phases of the cell cycle are separated by the two gap phases G1 and G2, with the G1 phase occurring between M phase and S phase and the G2 phase occurring between the S phase and M phase. Cells can exit the cell cycle temporarily or permanently. In case of a temporary exit from the cell cycle, a cell exists in a resting or "quiescent" state that is also known as the G0 phase. A cell that has exited the cell cycle permanently is said to exist in a "senescent" state. The decision to commit to a cell cycle and divide, or to exit and become quiescent is made in the G1 phase. During the G2 gap phase, a cell prepares for mitosis.

Cell cycles in *C. elegans* follow the same relative order of phases as those observed from yeast to mammals (reviewed by Kipreos and van den Heuvel, 2019). Minor lineage-specific variations in the length of these phases exist and the two gap phases G1 and G2 are absent in early embryonic cell cycles, similar to other metazoans. Mutants of cell-cycle genes display mostly post-embryonic abnormalities, as maternal inheritance of such regulators is sufficient for embryonic cell cycles but not post-embryonic cell cycles. RNAi-mediated targeting of cell-cycle genes in adult parents significantly reduces their maternal

inheritance in the progeny, and therefore has been integral in discovering the roles of cell-cycle regulators in embryonic cell cycles.

I performed an unbiased genome-scale RNAi screen for defective cell extrusion. This screen revealed a role of genes required for G1/S-phase transition. I also found that cells that extrude arrest in S phase likely because they experience replication stress. In this section, I will briefly review the molecular regulation and control of the transition from G1 phase to S phase and the replication stress response as understood from studies of yeast and metazoans. I will also provide the *C. elegans* homologs of the regulators of these processes and will list which of those homologs were identified for a role in cell extrusion.

1. G1 phase

A. Role of G1 phase in the cell cycle

Coordination of cell growth with cell division is an important feature of the G1 phase of the cell cycle. Only after achieving a critical cell size is a cell cycle initiated in late G1, indicating that cell growth in the G1 phase is important for maintaining cell size (Hartwell *et al.*, 1974; Johnston *et al.*, 1977; Jorgensen and Tyers, 2004). Altering cell sizes results in an alteration of the subsequent G1 phase duration in multiple cell types from a variety of organisms (Dolznig *et al.*, 2004). The connection of growth with cell cycle entry seems to be regulated by limiting the levels of cyclins (discussed in the next section) required for cell cycle

entry. For example, the overexpression of a G1 cyclin leads to a reduction of cell size in *S. cerevisiae* (Cross, 1988; Jorgensen *et al.*, 2002; Nash *et al.*, 1988).

The G1 phase also integrates information about the internal state of the cell and its external conditions to decide whether or not to continue the cell cycle, giving rise to a G1 checkpoint called the restriction point or R point (Hartwell *et al.*, 1973; Pardee, 1974; Blagosklonny and Pardee, 2002). Before this point, if conditions are not appropriate, or if the cell receives signals to adopt an alternative fate, the cell withdraws from the cell cycle. However, once a cell passes through this checkpoint and commits to the cell cycle, cells continue through the cycle even if, for example, nutrients are withdrawn (Zetterberg and Larsson, 1985).

As discussed in a later section, a true G1 phase seems to be absent in *C. elegans* early embryonic cell cycles, as a role of all the genes that regulate this phase (discussed in the next section) does not appear until late embryonic development. However, I have found that cells that are extruded enter the S phase and require a subset of G1-phase regulators for entry into S phase. Reduction of function of these G1-phase regulators can prevent entry into S phase and can instead produce a quiescence-like phase that is coupled to the survival (non-extrusion) of a cell that is otherwise extruded. These observations suggest that a G1-like phase exists in cells that are fated to be extruded.

B. Molecular regulation of G1 phase

During G1 phase a cell evaluates whether it can and should undergo division (Pardee, 1974). After each cell division, extracellular signals that activate

mitogenic signaling pathways, such as the Ras pathway, lead to the production of G1-specific cyclins that bind with cyclin-dependent kinases (CDKs), which then phosphorylate targets to promote G1 progression (Aktas *et al.*, 1997; Albanese *et al.*, 1995; Filmus *et al.*, 1994; Kerkhoff and Rapp, 1997; Lavoie *et al.*, 1996; Weber *et al.*, 1997; Winston *et al.*, 1996). Progression through G1 phase is controlled by successive expression of the cyclins D and E, which associate with CDK4/6 and CDK2, respectively. Initially, cyclin D partners with CDK4/6 to phosphorylate the Retinoblastoma (Rb) protein, which binds to and inhibits E2F family transcription factors that are critical for entry into S phase and its progression (Ezhevsky *et al.*, 2001). This initial phosphorylation of Rb by cyclin D/CDK4/6 kinase keeps E2F bound to Rb but releases histone deacetylases bound to it (Harbour *et al.*, 1999). The released histone deacetylases promote the expression of cyclin E, which upon expression binds to its partner CDK2. The cyclin E/CDK2 complex is initially inhibited by the protein p27Kip1 (Soos *et al.*, 1996). In contrast to this inhibitory function, p27Kip1 promotes the otherwise-inefficient binding of cyclin D with CDK4/6, and thereby the activation of cyclin D/CDK4/6 complexes (Alt *et al.*, 2002; Cheng *et al.*, 1999; LaBaer *et al.*, 1997; Muraoka *et al.*, 2002). The increase in the levels of cyclin D during G1 eventually causes sequestration of p27Kip1 for the formation of cyclin D/CDK4/6 complexes. This sequestration results in an increased activity of cyclin E/CDK2 complex kinases, which then phosphorylate the remaining p27kip1 and trigger its subsequent ubiquitination and degradation (Sheaff *et al.*, 1997, Vlach *et al.*, 1997). The activated cyclin E/CDK2 complexes further phosphorylate Rb, which

then releases E2F transcription factors that transcribe genes important for S phase entry and progression (Lundberg and Weinberg, 1998). These genes encode the DNA replication protein polymerase α; nucleotide synthesis enzymes thymidine kinase and dihydrofolate reductase; DNA replication proteins ORC1, CDC6, and MCM2-7; cyclins A and E; and the CDK-activating phosphatase CDC25A (Blake and Azizkhan, 1989; Botz *et al.*, 1996; Dou *et al.*, 1992; Geng *et al.*, 1996; Hateboer *et al.*, 1998; Leone *et al.*, 1998; Ohtani *et al.*, 1995; Ohtani *et al.*, 1996; Ohtani *et al.*, 1998; Pearson *et al.*, 1991; Schulze *et al.*, 1995; Vigo *et al.*, 1999; Yan *et al.*, 1998). These proteins play an essential role in the transition from G1 phase into S phase, as discussed in the next section.

C. *C. elegans* homologs of regulators of G1 phase and their role in cell extrusion

The *C. elegans* homologs of the mammalian G1 and S phase cyclins D, E are encoded by *cyd-1* and *cye-1*, respectively (Brodigan *et al.*, 2003; Fay and Han, 2000; Park and Krause, 1999). The *C. elegans* homologs of CDK2 and CDK4/6 are encoded by the similarly named genes *cdk-2* and *cdk-4*, respectively (Liu and Kipreos, 2000). The *C. elegans* genes *lin-35*, *efl-1* and *cki-1* encode homologs of Rb, E2F and p27Kip1, respectively, which function mostly to regulate postembryonic cell cycles (Boxem and van den Heuvel, 2002; Hong *et al.*, 1998). Early embryonic cell cycles do not show a requirement for the G1 regulator cyclin D/CDK-4 kinase or G1/S phase transition regulator cyclin E/CDK-2 kinase (Boxem *et al.*, 1999; Boxem and van den Heuvel, 2001). RNAi against *cye-1* allows embryos to develop until the 100-cell stage, after which they arrest

(Fay and Han, 2000), indicating that a G1/S-phase regulator is required only after this stage for cell cycle entry. Homolog of the mammalian protein CDK1 (discussed in the next section), which mostly regulates cell-cycle progression through S, G2 and M phases by mammalian cells, CDK-1, is believed to be the main driver of these early embryonic cell cycles as inferred from experiments with knockdown of redundantly functioning partners of CDK-1, the mitotic B-type cyclins (Boxem *et al.*, 1999; Chase *et al.*, 2000; van der Voet *et al.*, 2009; Deyter *et al.*, 2010).

Of the genes discussed above, I identified *cye-1* and *cdk-2* from the genome-scale RNAi screen for defective cell extrusion from *ced-3(lf)* embryos (see Chapters 2 and 3). RNAi against *cyd-1* or *cdk-4* did not produce an extrusion defect, consistent with its role in post-embryonic cell cycles. These findings suggest that cells that are extruded likely go through a G1-like phase that is controlled by *cye-1* and *cdk-2*. RNAi against these genes both blocks the extrusion of otherwise extruded cells and also produces a quiescent state in these cells (see Chapter 3), indicating that transition through the G1-like phase is required for cell extrusion.

2. G1/S-phase transition

A. Role of G1/S phase transition in the cell cycle

During the S phase of the cell cycle, a cell endeavors to faithfully copy its genome in a rapid fashion. The duplication of DNA content is achieved by bidirectional replication of DNA from multiple starting points in the DNA known as

replication origins, which range from hundreds in yeast to thousands in mammals. A sequence of highly regulated and complex events that occur at the G1/S phase transition set up the replication machinery at each of the replication origins that are phosphorylated by cyclin/CDK complexes for activation. This sequence of events includes inactivation steps such as degradation or exclusion from the nucleus of specific proteins that are required for the assembly of replication machinery. The phosphorylation and inactivation steps together ensure that the replication process starts only when all the preconditions of S-phase entry have been met and that it occurs only once per cell cycle. Both precocious S-phase entry and DNA re-replication cause DNA damage and genomic instability (reviewed by Arias and Walter 2007; Hook *et al.*, 2007; Macheret and Halazonetis, 2018; Sidorova and Breeden, 2002).

B. Molecular regulation of G1/S-phase transition

The transition into S-phase occurs via a series of intricate events described below. Figure 1 shows a pictorial representation of the steps that occur during S-phase initiation.

i. Pre-replication complex loading

DNA replication origins have specific sequences in *S. cerevisiae* and *S. pombe* (Stinchcomb *et al.*, 1979; Struhl *et al.*, 1979; Marahrens and Stillman, 1992; Dubey *et al.*, 1996). Although such sequence specificity is not found in metazoan replication origins, certain conserved features of mammalian origins have been identified (reviewed by Prioleau and MacAlpine, 2016). The assembly of replication complexes at origins is performed by the pre-replication complex,

which includes Origin Recognition Complex (ORC) proteins, CDC6, CDT1, and Mini-Chromosome Maintenance (MCM) proteins 2,3,4,5,6 and 7. Initially, the ORC proteins recognize and bind to origins of replication and recruit replication initiation factors CDC6 and CDT1 (Coleman *et al.*, 1996; Maiorano *et al.*, 2000). CDC6 and CDT1 are required for the subsequent loading of MCM2-7 complex proteins, which function as an integral part of the helicase that unwinds double-stranded DNA ahead of the replication machinery (Coleman *et al.*, 1996; Maiorano *et al.*, 2000; Nishitani *et al.*, 2000; Tanaka and Diffley, 2002). Subsequent to MCM2-7 loading, CDC6 and CDT1 are sequentially released (Fernandez-Cid *et al.* 2013; Coster *et al.* 2014; Kang *et al.* 2014; Ticau *et al.* 2015). A second CDC6 is then recruited by the ORC/MCM complex that remains on the origin, which helps in the CDT1-dependent recruitment of a second MCM complex and leads to the formation of head-to-head MCM double hexamer (DH) formation (Evrin *et al.* 2013, 2014; Fernandez-Cid *et al.* 2013; Sun *et al.* 2013; Ticau *et al.* 2015). The steps involved in pre-replication complex ensure that one round of DNA replication per cell cycle or "replication licensing," which is described in more detail in the next section.

ii. Mechanisms of replication licensing

Replication licensing is a process that ensures DNA replication occurs only once during S phase and not again in the same cell cycle. As a part of this replication licensing process, the ORC proteins are subjected to inactivating phosphorylation by cyclin A/CDK complexes and subsequently undergo ubiquitination and degradation (Mendez *et al.*, 2002; Li and DePamphilis, 2002;

Li *et al.*, 2004). This process ensures that ORC proteins do not initiate replication more than once during a cell cycle. Similarly, CDC6 and CDT1 are both subjected to inactivating phosphorylations by CDKs, which leads to nuclear export of CDC6 and degradation of CDT1 (Delmolino *et al.*, 2001; Fujita *et al.*, 1999; Jiang *et al.*, 1999; Li *et al.*, 2003; Saha *et al.*, 1998; Sugimoto *et al.*, 2004). As high CDK activity prevents pre-replication complex formation by the mechanisms described above, these complexes are formed only in late M and early G1 phases of the cell cycle when CDK activity is low. In metazoans, a small protein called Geminin adds another layer of regulation by interacting with CDT1 to inhibit MCM assembly (McGarry and Kirschner, 1998; Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001; Maiorano *et al.*, 2004).

iii. Pre-initiation complex loading and replication initiation

To initiate replication, proteins that together constitute the pre-initiation complex have to first be loaded on to chromatin with MCM DH complexes and ORC left at origins after removal of CDC6 and CDT1 from pre-replication complexes. This pre-initiation complex loading process requires the proteins MCM10, SLD2 (or RecQL4 in metazoans), SLD3 (or Treslin/TICRR in metazoans), SLD7 (MTBP in metazoans), CDC45, Dbf4/CDC7 kinase (also known as Dbf4-dependent kinase or DDK), polymerase ε, Go-Ichi-Ni-San (GINS) complex proteins (SLD5, PSF1, PSF2 and PSF3) and Dpb11 (or TopBP1 in metazoans). MCM10 facilitates the phosphorylation of MCM DH complexes by DDK during the start of S phase, which leads to the efficient recruitment of SLD3/7 and CDC45 to replication complexes (Bruck and Kaplan, 2015; Deegan

et al. 2016; Fang *et al.* 2016; Herrera *et al.* 2015). S-phase cyclin/CDK complexes phosphorylate SLD2 and SLD3, which allows their subsequent interaction with TopBP1 (Tanaka *et al.* 2007; Zegerman and Diffley, 2007). The phosphorylation of SLD2 allows a complex to form between Dpb11/TopBP1, SLD2, GINS, polymerase ε (Muramatsu *et al.*, 2010). This complex subsequently binds to the CDC45/(SLD3/7)/MCM complex, which at this point is already at replication origins (Tanaka *et al.*, 2007; Zegerman and Diffley, 2007). As a result of this binding, Dpb11/TopBP1, SLD2 and SLD3/7 are released (Kanemaki and Labib 2006; Heller *et al.* 2011; Yeeles *et al.* 2015) and the active CMG helicase is formed from the CDC45, MCM2-7 and GINS complex proteins. The CMG helicase is essential for DNA replication and functions to unwind double-stranded DNA (dsDNA) during replication so that the replication machinery can create the complimentary strands of DNA behind it. Subsequent to CMG helicase assembly, Ctf4, Pol α, Csm3, Tof1, Mrc1, FACT and Topo I are recruited to the replication origins and together with the CMG helicase constitute the replisome progression complex (RPC) (Gambus *et al.*, 2006; Gambus *et al.*, 2009). A further requirement for proteins Pol δ, replication factor C (RFC), proliferating cell nuclear antigen (PCNA), and replication protein A (RPA) along with the RPC has been more recently shown for *in vitro* reconstitution of the replisome (Georgescu *et al.*, 2015).

iv. Replication progression and replication termination

Similar to assembly of replication complexes and replication initiation, the topics of replication progression and termination are complex, complicated and

intricate. As unperturbed DNA replication progression and replication termination are not relevant to my findings about cell extrusion, I refer the reader to reviews that describe these processes in detail (Burgers and Kunkel, 2017; Dewar and Walter, 2017).

C. *C. elegans* homologs of genes involved in G1/S-phase transition and their role in cell extrusion

The proteins involved in replication licensing and initiation are well conserved between *C. elegans* and other organisms in which DNA replication has been studied. The proteins ORC-1, ORC-2, ORC-3, ORC-4 and ORC-5 constitute the origin recognition complex in *C. elegans* and have been well characterized in their role in replication licensing (Sonneville *et al.*, 2012). Orthologs of other pre-replication complex members CDC6, CDT1 and MCM2-7 exist in *C. elegans* and are encoded by the genes *cdc-6*, *cdt-1* and *mcm-2-7*, respectively. Like other organisms, replication licensing in *C. elegans* larval cells occurs in the G1 phase (Zhong *et al.*, 2003). However, as early embryonic cell cycles do not have gap phases, licensing in early embryonic cells occurs in late M phase so that replication can begin immediately after S phase. Furthermore, instead of degrading CDT1 in such cycles, ORC is excluded from the nucleus of the cell to prevent re-initiation of DNA replication (Sonneville *et al.*, 2012). Homologs of the pre-initiation complex proteins MCM10, SLD2, CDC7, CDC45, GINS complex (SLD5, PSF1, PSF2 and PSF3) and Dpb11/TopBP1 also exist in *C. elegans* and are encoded by the genes *mcm-10*, *sld-2*, *cdc-7*, *cdc-45*, *sld-5*,

psf-1, *psf-2*, *psf-3* and *mus-101*, respectively. Homologs of other proteins such as SLD3 and Dbf4 have not been found and might have diverged in sequence.

Of the *C. elegans* genes involved in the G1/S-phase transition, I identified the genes *psf-1*, *psf-2*, *psf-3* and *mus-101* from the genome-scale RNAi screen for defective extrusion (see Chapters 2 and 3). Consistently, I found using cell-cycle reporters that cells that are extruded entered the S-phase but then arrested in S-phase. This finding suggests that G1/S phase transition and the assembly and firing of replication origins that occurs during this period is required for cell extrusion.

3. Positive and negative control of the G1/S-phase transition

A. Role of G1/S-phase transition control in the cell cycle

The cell cycle is a uni-directional process that starts with cell-cycle entry and normally ends with cell division. The complexity and intricacy of this process can be appreciated by counting just the number of molecules involved in the G1/S-phase transition listed in the previous section. The orchestration of such a complex process and ensuring that it occurs only in one direction requires a tight control of the cell cycle process. A cell achieves such control by regulating cell-cycle proteins at the levels of: i) transcription, ii) phosphorylation, iii) protein-protein interactions, and iv) degradation. Regulating cell-cycle proteins at these levels assists the cell in allowing the cell to control cell-cycle progression both positively and negatively. Cyclin/CDK kinase complexes are examples of control of the cell cycle enforced at the levels of transcription of cyclins, protein-protein

interaction of cyclins and CDKs, and phosphorylation of substrates by cyclin/CDK kinase complex. Degradation of CDT1 is another example of how such control is enforced. This section discusses many such examples of how cell cycle control is achieved during the G1/S-phase transition.

B. Molecules that control G1/S-phase transition

i. CDK inhibitors

Progression through the G1 and S phases occurs through the activity of CDKs directed by their partner cyclins. CDK inhibitors (CKIs) restrict the activity of CDKs and thus add a level of control to make cell cycle progression regulated and precise. There are two classes of CKIs: i) the inhibitors of CDK4 (INK4), which include $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, bind only to and inhibit the catalytic subunits of cyclin D-dependent kinases CDK4 and CDK6 (Chan *et al.* 1995; Guan *et al.* 1994; Hannon and Beach 1994; Hirai *et al.* 1995; Serrano *et al.* 1993), and ii) inhibitors that broadly bind cylin D-, cyclin E- and cyclin A-dependent kinases, which include p21Cip1 (Dulic *et al.*, 1994; El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Noda *et al.*, 1994; Xiong *et al.*, 1993), p27Kip1 (Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994) and p57Kip2 (Lee *et al.*, 1995; Matsuoka *et al.*, 1995).

ii. CDK phosphorylation/dephosphorylation

A second layer is added to the regulation of Cyclin/CDK complexes by their phosphorylation and dephosphorylation. These modifications are carried out by the Wee1/Myt1 kinases and CDC25 dual-specificity phosphatases, respectively. CDKs are held in an phosphorylation-induced inactivated state by

Wee1/Myt1 kinases until the cell is ready to progress into the next phase of the cell cycle (Honda *et al.*, 1992; Liu *et al.*, 1997; McGowan *et al.*,1993; Mueller *et al.*, 1995; Russell and Nurse, 1987). In contrast to Wee1, which can phosphorylate and regulate the activity of both CDK1 and CDK2, Myt1 regulates only the activity of CDK1 (Booher *et al.*, 1997). CDC25 phosphatases promote entry into the next phase of the cell cycle by removing inhibitory phosphorylations and thus activating CDKs (Fantes, 1979; Gautier *et al.*, 1991; Gu *et al.*, 1992; Honda *et al.*, 1993; Jinno *et al.*, 1994; Kumagai and Dunphy, 1991; Lammer *et al.*, 1998; Millar *et al.*, 1991; Sadhu *et al.*, 1990; Strausfeld *et al.*, 1991, 1994).

iii. Ubiquitin-proteasome system

Progression through the cell cycle is also regulated via protein degradation by the ubiquitin-proteasome system. There are two main classes of ubiquitinating complexes that function during the cell cycle: i) the anaphase-promoting complex/cyclosome (APC/C), and ii) Skp1-Cullin-F-box (SCF) E3 ligases. The APC/C core complex functions with two cofactors that regulate its activity – i) CDC20, which associates with APC/C to mediate late mitotic transitions, and ii) Cdh1, which associates with APC/C to restrict levels of mitotic proteins during early G1 phase (reviewed by Visintin *et al.*, 1997). In contrast, the SCF E3 ligase complexes were identified in yeast by their role at the G1/S-phase transition (Feldman *et al.*, 1997; Goebl *et al.*, 1988; Hartwell *et al.*, 1974; Skowyra *et al.*, 1997). The F-box part of the SCF complex provides specificity towards the substrate for ubiquitination, and three main F-box proteins serve to regulate the SCF E3 ligase function during the cell cycle – CDC4/Fbw7 (Hartwell

et al., 1974; Koepp *et al.*, 2001; Strohmaier *et al.*, 2001), β-TrCP (Fuchs *et al.*, 1999; Kroll *et al.*, 1999; Latres *et al.*, 1999; Shirane *et al.*, 1999; Suzuki *et al.*, 2000; Tan *et al.*, 1999; Winston *et al.*, 1999), and Skp2 (Lisztwan *et al.*, 1998; Lyapina *et al.*, 1998). The G1/S-phase specific cyclin E, CDK-inhibiting kinase Wee1, and CKIs are the best-known targets of CDC4/Fbw7-, β-TrCP-, and Skp2-specific SCF E3 ligases, respectively (Koepp *et al.*, 2001; Moberg *et al.*, 2001; Strohmaier *et al.*, 2001; Watanabe *et al.*, 2004; Carrano *et al.*, 1999; Sutterlüty *et al.*, 1999; Tsvetkov *et al.*, 1999). SCF^{Skp2} also targets CDT1, a DNA pre-replication complex assembly factor, which serves to limit DNA replication to once per cell cycle (Li *et al.*, 2003; Liu *et al.*, 2004).

Ubiquitin-mediated proteolysis is a prominent mechanism of the replication stress checkpoint response (described in the next section), which mainly targets CDKs to halt progression of the cell cycle by sequestering or degrading their activating phosphatase proteins of the CDC25 family. For example, during this response, Chk1 kinase phosphorylates CDC25A protein, which is subsequently ubiquitinated and marked for degradation by $SCF^{\beta\text{-}TrCP}$ (Jin *et al.*, 2003; Mailand *et al.*, 2002; Hassepass *et al.*, 2003; Xiao *et al.*, 2003).

SCF E3 ligases belong to the class of cullin-RING ubiquitin ligases (CRLs), named so because of two of their subunits – a cullin protein and a RING-domain protein (reviewed by Petroski and Deshaies, 2005; Willems *et al.*, 2004). Neddylation (or attachment of a Nedd8 protein by subsequent reactions presumably carried out by Nedd8-activating, Nedd8-conjugating and Nedd-8 ligase enzymes) increases the activity of CRLs *in vitro* (Kawakami *et al.*, 2001;

Pan *et al.*, 2004; Wu *et al.*, 2000). The deneddylation of CRLs is carried out by the COP9/signalosome complex, which consists of eight largely conserved protein subunits (CSN1-8). As expected, deneddylation by the COP9/signalosome complex causes CRL activity to return to basal levels *in vitro* (Lyapina *et al.*, 2001; Schwechheimer *et al.*, 2001). However, genetic inactivation of the COP9/signalosome *in vivo* produces phenotypes similar to those of CRL inactivation in multiple biological systems, suggesting that continuous cycles of neddylation and deneddylation are required to maintain CRL activity (Cope *et al.*, 2002; Doronkin *et al.*, 2002; Lykke-Andersen *et al.*, 2003; Pintard *et al.*, 2003; Schwechheimer *et al.*, 2001; Tomoda *et al.*, 2004).

C. *C. elegans* homologs of G1/S-phase transition controllers and their role in cell extrusion

CYD-1/CDK-4 regulates cell cycle entry and G1 progression in late embryonic and post-embryonic cell lineages (Park and Krause, 1999). The genes *lin-35* and *cki-1*, which encode the *C. elegans* mammalian homologs of cell-cycle inhibitors Rb and p27Kip1, respectively, function to negatively regulate the G1 progression mediated by CYD-1/CDK-4 (Boxem and van den Heuvel, 2001; Hong *et al.*, 1998). CYD-1/CDK-4 kinase complex phosphorylates and reduce the transcriptional repression by LIN-35 Rb (Boxem and van den Heuvel, 2001; The *et al.*, 2015). Similar to that in mammals, this transcriptional derepression allows the expression of *cye-1* and *cya-1*. CYE-1 and CYA-1 function with CDK-2 to further phosphorylate and inactivate LIN-35/Rb and promote entry into S-phase.

In addition to *lin-35* and *cki-1*, cell cycle entry is also negatively regulated by *lin-23*, which encodes the mammalian homolog of the β-TrCP F-box protein that participates in SCF E3 ligase complex (with the Skp1 and Cul1 homologs encoded by *skr-1/2* and *cul-1*, respectively). *lin-23* loss-of-function mutant embryos show a hyperplasia phenotype and die with over 850 cells, instead of the 558 cells in wild-type embryos at the time of hatching (Kipreos *et al.*, 2000). The COP9/signalosome complex, which regulates the activity of SCF E3 ligase and other CRLs, is conserved in *C. elegans* and is encoded by the genes *csn-1*, *csn-2*, *csn-3*, *csn-4*, *csn-5*, and *csn-6* (Pintard *et al.*, 2003). Reduction in COP9/signalosome activity in *C. elegans* shows a phenotype similar to that produced by reducing the function of the cullin gene *cul-3*, suggesting that as in other organisms, COP9/signalosome is required to maintain CRL function (Pintard *et al.*, 2003).

As in mammals, LIN-23/β-TrCP targets CDC25 phosphatase homologs encoded by the genes *cdc-25.1* and *cdc-25.2* in the *C. elegans* genome (Hebeisen and Roy, 2008; Segref *et al.*, 2010; Son *et al.*, 2016). These and two other CDC25 homologs (CDC-25.3 and CDC-25.4) likely function by removing inhibitory phosphorylations from CDKs and are involved in multiple aspects of embryonic and post-embryonic development (Ashcroft *et al.*, 1999; Ashcroft and Golden, 2002; Kim *et al.*, 2010; Lee *et al.*, 2016; Oh *et al.*, 2016; Spike *et al.*, 2014, Sung *et al.*, 2017). The Wee family kinases that place inhibitory phosphates on CDKs to prevent cell-cycle progression also exist in *C. elegans*

and are encoded by the *wee-1.1* and *wee-1.3* genes (Burrows *et al.*, 2006; Wilson *et al.*, 1999).

Of the genes mentioned here, I identified *cdc-25.2*, *csn-1, csn-4* and *csn-5* from the genome-scale RNAi screen for defective cell extrusion and *lin-23* from a separate candidate RNAi screen of cell-cycle genes. These findings indicate that controllers of G1/S-phase transition are required for cell extrusion. This is consistent with the finding that extruded cells are in S phase (albeit arrested in S phase) when they are extruded.

4. Replication stress checkpoint

A. Role of the replication stress checkpoint in the cell cycle

Replication stress checkpoint is triggered when the DNA replication machinery encounters a difficulty or stress in replicating DNA. Replicative stress can result from a number of reasons including nucleotide depletion (Anglana *et al.*, 2003; Beck *et al.*, 2012; Bester *et al.*, 2011; Poli *et al.*, 2012) as well as interference by transcriptional machinery, difficult to replicate sequences, or dysfunction of replication machinery components (reviewed by Zeman and Cimprich, 2014). The replication stress checkpoint functions to i) prevent entry into mitosis (Palou *et al.*, 2015; Sanchez *et al.*, 1997; Xiao *et al.*, 2003), ii) increase the synthesis of deoxyribonucleotide triphosphates (dNTPs) (Buisson *et al.*, 2015; Zhao *et al.*, 1998), and iii) prevent further firing of origins (Toledo *et al.*, 2013; Zegerman and Diffley, 2010).

B. Molecular regulators of the replication stress checkpoint

The replication stress checkpoint pathway is depicted in Figure 2, which is a pictorial representation of the process described in the rest of this section. Treatment with hydroxyurea (HU) is commonly used to induce replication stress and identify factors regulating the replication stress response (reviewed by Giannattasio and Branzei, 2017). HU produces replication stress by inhibiting the enzyme ribonucleotide reductase (RNR), which functions to maintain appropriate levels of deoxyribonucleotides during DNA replication (Timson, 1975). When a replication stress is encountered, the CMG helicases continue to unwind dsDNA but the replication machinery producing complimentary DNA strands behind the CMG helicase stalls, producing large stretches of single-stranded DNA (ssDNA) that are subsequently bound by Replication Protein A (RPA) (Byun *et al.*, 2005). RPA-bound ssDNA close to stalled replication machinery serves to recruit the replication stress signaling proteins ataxia-telangiectasia mutated- and rad3-related (ATR) protein kinase through ATR's regulatory subunit ATRIP (MacDougall *et al.*, 2007; Zou and Elledge, 2003a). RPA-bound ssDNA also recruits the clamp loading protein Rad17, which functions to load the Rad9-Rad1-Hus1 (9-1-1) complex onto the DNA (Shiomi *et al.*, 2002; Venclovas *et al.*, 2000, Zou *et al.*, 2002; Zou *et al.*, 2003b). Dpb11/TopBP1, binds the 9-1-1 complex and is phosphorylated by ATR (Kumagai *et al.*, 2006; Yoo *et al.*, 2007). Phosphorylated Dpb11/TopBP1 in turn promotes the complete activation of ATR kinase. Claspin, another target of ATR kinase, is phosphorylated and mediates the interaction of ATR with Chk1 kinase (Chini and Chen, 2003; Kumagai and Dunphy, 2000) As a result of this interaction, ATR phosphorylates and activates

Chk1 kinase (Guo *et al.*, 2000; Liu *et al.*, 2000; Lopez-Girona *et al.*, 2001; Zhao *et al.*, 2001). Chk1 functions downstream of ATR kinase to activate the replication stress response. The proteins Timeless and Tipin function together in a complex and mediate ATR-substrate interactions during replication stress (Chou and Elledge, 2006; Gotter *et al.*, 2007; Unsal-Kaçmaz *et al.*, 2007; Yoshizawa-Sugata *et al.*, 2007).

To prevent cell cycle progression, Chk1 kinase imparts an inactivating phosphorylation on the CDK-activating phosphatase CDC25, which leads to sustained inhibitory phosphorylation on CDKs that results in a suspended cell cycle (Liu *et al.*, 2000; Molinari *et al.*, 2000). A similar mechanism involving CDC25 proteins works to prevent late origin firing during replication stress (Falck *et al.*, 2002; Sørensen *et al.*, 2003). The increase in dNTP synthesis upon replication stress occurs from an increase in the expression of RRM2, a subunit of the RNR enzyme that functions to convert ribonucleotides into deoxyribonucleotides for DNA synthesis during S phase (Buisson *et al.*, 2015).

C. Replication stress checkpoint genes in *C. elegans* and their role in cell extrusion

Homologs of the replication stress checkpoint proteins ATR and Chk1 exist in *C. elegans* and are encoded by the genes *atl-1* and *chk-1* (Brauchle *et al.*, 2003). These genes function in normal embryonic development to introduce cell cycle asynchrony between the AB and P_1 cells of the embryo. These genes also function to delay the cell cycle in embryos treated with agents that induce replication stress (Brauchle *et al.*, 2003, Stevens *et al.*, 2016). Other components

of the replication stress checkpoint response such as TopBP1/Dpb11, Claspin, RPA, Rad9, Rad1, Hus1, Rad17, Timeless and Tipin perform orthologous functions in *C. elegans* and are encoded by the genes *mus-101*, *clsp-1*, *rpa-1*, *hpr-9*, *mrt-2*, *hus-1, hpr-17*, *tim-1* and *tipn-1*, respectively (Stevens *et al.*, 2016). However, no clear homolog of ATRIP is encoded by the *C. elegans* genome.

Of the genes that function in replication stress in *C. elegans*, RNAi against *atl-1*, *chk-1*, mus-*101, clsp-1*, *hpr-9*, *tim-1* and *tipn-1* indicate that these genes likely function in cell extrusion from *ced-3(lf)* embryos (see Chapter 3). This finding suggests that replication stress occurs and is required for cell extrusion by *ced-3(lf)* embryos.

Cell cycle S-phase arrest drives cell extrusion

I performed a genome-scale RNAi screen for defective cell extrusion by *ced-3(lf)* animals and found that cell-cycle genes, specifically those involved in G1-to-S phase transition and S-phase progression are involved in cell extrusion (see Chapters 2 and 3). Using transgenes expressing cell-cycle reporters, I discovered that cells that are extruded entered the cell cycle and arrested in S phase prior to their extrusion. Consistently, RNAi against the cell-cycle genes identified from the screen prevented both cell-cycle entry by and extrusion of cells that are otherwise extruded. RNAi against genes required for S-phase arrest also likely function in cell extrusion.

Another class of genes identified from the genome-scale RNAi screen for defective cell extrusion is required for unequal cell divisions that occur in neuroblast lineages that produce a daughter fated to undergo apoptosis. This group of genes is also required for the unequal cell division(s) that generate extruded cells. RNAi against these genes leads to an increase in the size of cells that are otherwise extruded, which subsequently allows such cells to complete the cell cycle, divide and survive.

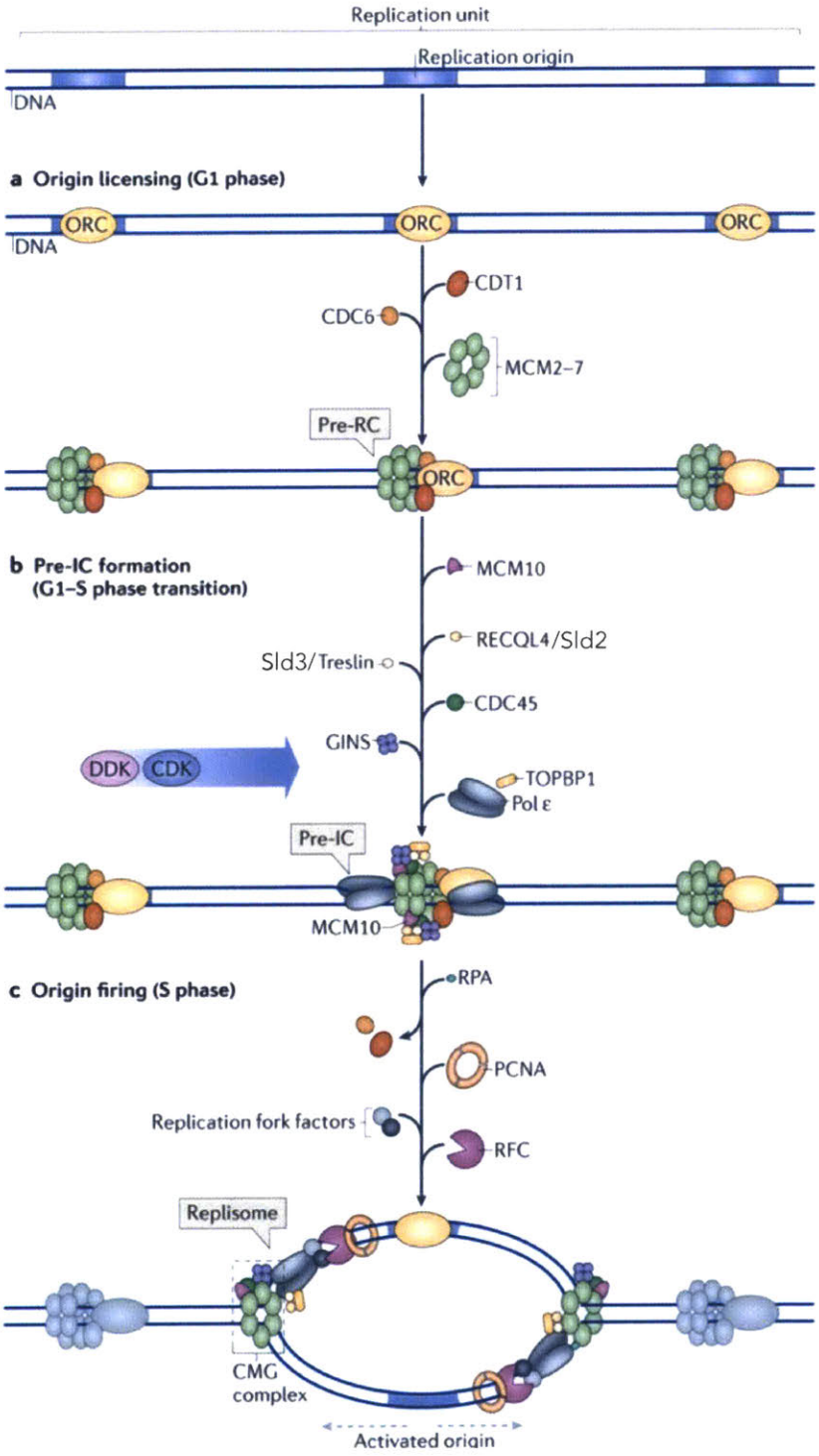
All these findings indicate that S-phase arrest drives cell extrusion by *C. elegans*. I collaborated with the Rosenblatt Laboratory at Kings College London to determine whether this phenomenon was conserved in mammalian cells. Studies of mammalian cell extrusion by the Rosenblatt Laboratory showed that treatment with HU, an agent that causes S-phase arrest, caused a significant

increase in cell extrusion, indicating that S-phase arrest is a conserved driver of cell extrusion.

My studies of cell extrusion by *C. elegans* have led to the discovery that S-phase arrest drives cell extrusion. This discovery establishes a novel and evolutionarily conserved link between two fundamental biological processes – cell cycle and cell extrusion. As both cell cycle and cell extrusion play crucial roles during development, and in physiology and disease, the link that I have discovered could have major implications for all of these processes. My findings and their potential implications are described in Chapters 2 and 3.

Figure 1 – Schematic describing the steps of S-phase initiation. a) During origin licensing, the ORC proteins bind to replication origins and recruit CDC6 and CDT1. The CDC6 and CDT1 proteins facilitate in the assembly of the MCM2-7 complexes. **b)** During pre-initation complex (pre-IC) formation, MCM10 promotes phosphorylation of the MCM2-7 complex, which then recruits SLD3 and CDC45 to the replication origins. S-phase CDKs then phosphorylate SLD2 and SLD3. The phosphorylation of SLD2 allows a complex to form between SLD2, TopBP1, Pol ε, and GINS (not shown in figure). The phosphorylation of SLD3 also increases its interaction with TopBP1, which brings their respective complexes (SLD2/GINS/TopBP1/Pol ε and SLD3/MCM2-7/CDC45) together. The interaction of these two large complexes leads to the release of TopBP1, SLD2 and SLD3, which leaves a functional CMG (CDC45/MCM2-7/GINS) helicase at the replication origin. **c)** The subsequent recruitment of RPA, PCNA, RFC and other replication fork factors leads to origin firing and initiation of DNA replication.

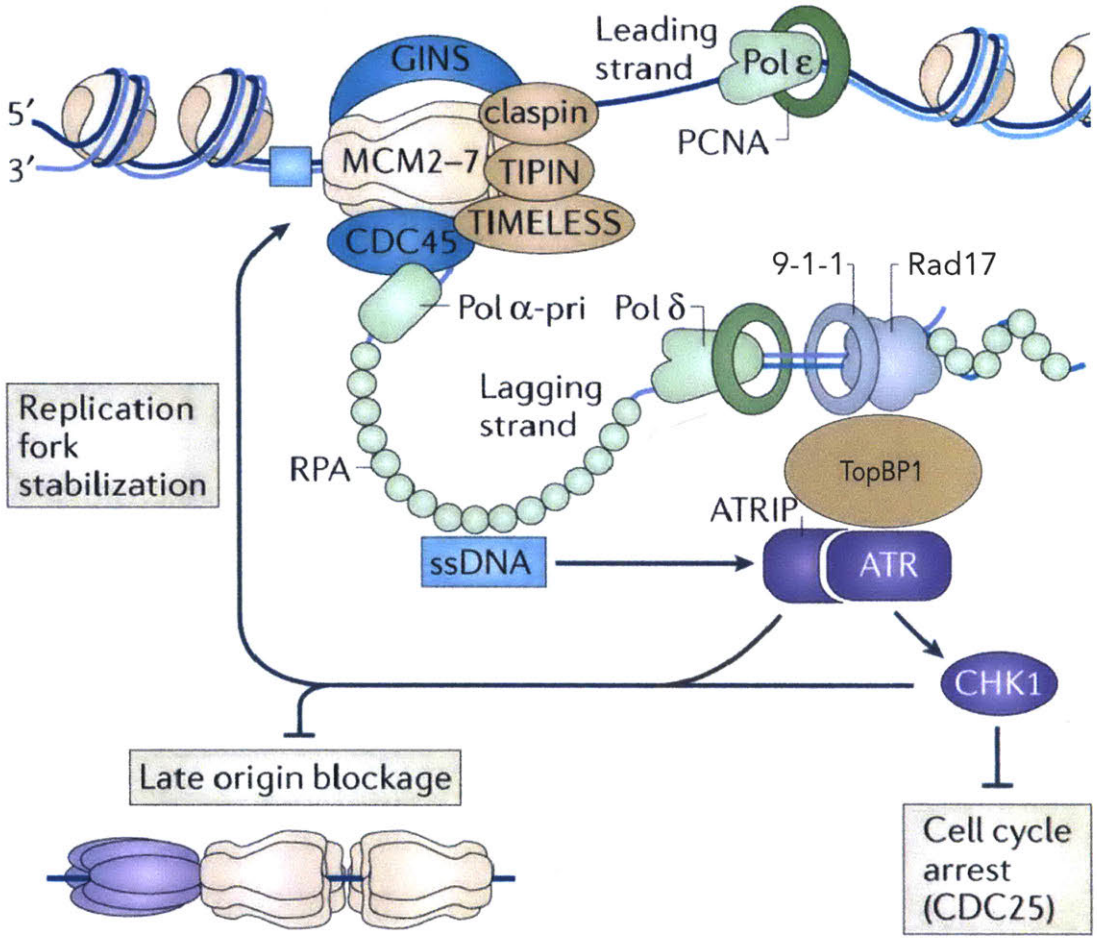
Figure 1



Adapted from Fragkos *et al.*, Nat. Rev. Mol. Cell Biol. (2015)

Figure 2 – The replication stress checkpoint pathway. ssDNA between the CMG helicase and the stalled replication machinery on the lagging strand leads to recruitment of RPA. ATRIP interacts with RPA to recruit ATR kinase to this site of replication stress. RPA also recruits RAD17, which functions to load the Rad9-Rad1-Hus1 (9-1-1) complex at the site of stalled replication. TopBP1 interacts with the 9-1-1 complex and gets phosphorylated by ATR, and in turn promotes the complete activation of ATR. Claspin is phosphorylated by ATR and mediates the activation of Chk1, which functions downstream of ATR to promote cell cycle arrest, block the firing of late S-phase origins and stabilize stalled replication forks. Timeless and Tipin function together to mediate the interactions between ATR and its substrates.

Figure 2



Adapted from Gaillard *et al.*, Nat. Rev. Cancer (2015)

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Chapter 2

Genome-scale RNAi screen for genes involved in cell extrusion by *C. elegans*

Summary

I performed a genome-scale RNAi screen for the two-excretory cell (Tex) phenotype, and identified both novel and previously known genes required for cell extrusion. In this chapter, I will describe the RNAi screen and discuss the results of this screen.

Introduction

RNA interference (RNAi), a phenomenon discovered from the studies of *C. elegans*, can be used as a tool to inhibit expression of a target gene (Fire *et al.*, 1998). RNAi is caused by RNA oligonucleotides 21-23 bases in length that direct a complex of proteins known as the RNA-induced silencing complex to either cleave or inhibit the translation of an mRNA with a sequence complimentary to that of the RNA oligonucleotides (reviewed in Rana, 2007; reviewed in Zhuang and Hunter, 2012). Silencing of genes by RNAi can be easily performed in *C. elegans* since double-stranded RNA (dsRNA), the agent that triggers RNAi, can be delivered by feeding animals bacteria that produce dsRNA (Timmons and Fire, 1998). This feature of using RNAi for studies of *C. elegans* has allowed the construction of large RNAi libraries and the design and implementation of large-scale RNAi screens that use these libraries (Kamath and Ahringer, 2003; Rual *et al.*, 2004). RNAi is an especially useful tool for studying the role of genes in embryonic processes in *C. elegans*, as mutants of many genes that drive embryonic development do not show embryonic abnormalities because of maternal rescue but then produce post-embryonic lethality or sterility

(Kipreos and van den Heuvel, 2019; Zipperlen *et al.*, 2003; Sönnichsen *et al.*, 2005). The lethality or sterility caused by mutations in these genes prevents analysis of embryonic roles in the subsequent generation.

Daniel (Dan) Denning's characterization of cell extrusion by caspase-deficient *C. elegans* embryos established that the two-excretory cell (Tex) phenotype reliably reflects a lack of cell extrusion and can be used to screen for regulators of cell extrusion on a large scale (Denning *et al.*, 2012). The second excretory cell in the Tex phenotype (normally not found in wild-type and *ced-3(lf)* animals) arises from a block in the extrusion of the cell ABplpappap, which is otherwise generally extruded from *ced-3(lf)* embryos. From EMS mutagenesis screens for the Tex phenotype, Dan identified a number of genes involved in cell extrusion, such as *pig-1*, *grp-1*, etc. and used feeding RNAi as a means to confirm that loss of function of these genes was causal for the Tex phenotype (Denning *et al.*, 2012). However, the nature and function of these genes did not reveal the biological processes involved in cell extrusion. I hypothesized that an approach that identifies regulators of extrusion more systematically and comprehensively might reveal the biology underlying cell extrusion. Additionally, RNAi can identify role for genes that might be missed from EMS mutagenesis screens, such as those that are required to prevent sterility or lethality. As Dan had already validated RNAi as a successful approach for identifying regulators of cell extrusion, I decided to perform a genome-scale RNAi screen to search for the biological pathways that regulate cell extrusion.

Results

Optimizing the screen

To conduct the screen, I used the strain MT20117, with the genotype *nIs433[P_{pgp-12}::4xNLS::GFP]; ced-3(n3692).* This strain is one of the strains that Dan used to conduct the EMS mutagenesis screen for mutants with the Tex phenotype that identified *pig-1* and *grp-1.* The *nIs433* transgene expresses nuclear GFP in excretory cell(s). The *n3692* allele of *ced-3* deletes 650 base pairs, which includes the translational start site and the entire first exon of the *ced-3* gene. RNAi against either *pig-1* or *grp-1* produces a highly penetrant Tex phenotype in this strain.

Most genome-scale RNAi screens that have been performed by feeding have been done in a 96-well format using bacterial cultures (Davis *et al.*, 2017; Goh *et al.*, 2014; Han *et al.*, 2017; Klosin *et al.*, 2017; Lehner *et al.*,2006; Maia *et al.*, 2015; Pereira *et al.*, 2016; Rocha *et al.*, 2018; Roy *et al.*, 2018; Weaver *et al.*, 2014). The 96-well format allows for a simple transfer of bacterial RNAi clones from RNAi library 96-well plates to culture and subsequent screening. A disadvantage of using this method is that the phenotypic screening has to be done in liquid. For this reason, this 96-well format could not be used to screen for the Tex phenotype, as it was difficult to look for one versus two GFP-positive excretory cells in animals in different planes in a small well.

To circumvent the difficulty of screening in liquid cultures, I decided to perform a genome-scale RNAi screen on agar in a 24-well format. Rita Droste, a research specialist in the lab, was indispensable in this effort given her

knowledge from a similar but smaller-scale RNAi screen that she had previously helped perform. I decided to use the *C. elegans* ORFeome RNAi library for the genome-scale screen. This library contains 11,511 RNAi clones targeting 10,953 genes or about 55% of the approximately 20,000 genes encoded by the *C. elegans* genome (Raul *et al.*, 2004). To make the screen faster, I chose the ORFeome library over the larger "Ahringer" RNAi library (Kamath *et al.*, 2003), which targets about 78% of the predicted genes. I posited that even if I identified fewer total genes from a smaller library, I would still be able to identify additional genes by testing other members of the same pathways in candidate RNAi experiments. As a contingency plan, I would test the extra genes targeted by the Ahringer RNAi library in case I was unsuccessful in identifying any pathway controlling cell extrusion from the ORFeome RNAi library.

As a pilot, Rita took 4 24-well plates (corresponding to a 96-well plate from the RNAi library) and randomly seeded the wells, using either a bacterial RNAi clone for *pig-1* as positive control or a bacterial clone carrying the empty vector as negative control. I loaded 2-5 parent L4s into these wells and scored the progeny for the Tex phenotype. I was able to correctly distinguish all wells with the *pig-1* RNAi clone (generating ~60% animals with Tex phenotype) from the wells with empty vector clones (generating 1-4% animals with the Tex phenotype) and found that three L4 parents generated an optimal number of progeny for scoring. This result gave me the confidence to start screening the 11,511 RNAi clones in the ORFeome library.

Screening for genes required for cell extrusion

Rita transferred bacterial RNAi clones from 96-well library plates into 96-well culture blocks with appropriate culture media and then seeded the cultures onto agar in four 24-well plates the following day. Subsequently, I loaded three L4 larvae into each well and screened their progeny at the L3-L4 larval stage for the Tex phenotype. I scored approximately 50 animals in each well, and any well with five or more animals with the Tex phenotype was marked for retesting. Biological or technical replicates were not used in this experiment, and only one experiment per bacterial clone was performed.

I was able to screen 600 to 750 RNAi clones every week by screening four days a week (corresponding to eight 96-well plates with some un-annotated or empty wells). Each set of RNAi clones screened in a day also contained the *pig-1* RNAi clone as a positive control and the empty vector as a negative control. To make the screening completely unbiased, I was unaware of the annotated targets of the RNAi clones during the screening process. I identified the targets of clones that reproducibly generated a Tex phenotype by sequencing these clones.

Analysis of candidates

From the genome-scale RNAi screen of 11,511 clones, I identified 164 candidate RNAi clones that produced a Tex penetrance of greater than 8% (more than four of 50 examined animals) (Table 1). These clones were tested again in triplicates for the Tex phenotype in individual 6 cm plates, and a total of more than 100 animals were examined for each RNAi clone (unless the clone

produced extensive lethality). 28 of the 164 RNAi clones reproducibly generated a Tex penetrance of more than 8% (Table 2). Another two of the 164 clones produced only 5-7% Tex penetrance upon retesting but target the same gene, *mpk-1*, suggesting a significant role of the gene but probably ineffective reduction in levels by RNAi (Table 2). These 30 RNAi clones together correspond to 27 unique genes. These 27 genes belong to five major gene classes – cell cycle regulator, genes required for unequal cell division, regulator of gene expression, cytoskeletal regulator, and gene of unclear relationship in cell extrusion.

I found the potential involvement of cell-cycle genes in cell extrusion to be the most interesting and exciting of my findings. I analyzed the role of cell-cycle genes in cell extrusion in more detail (see chapter 3). Specifically, I imaged live *C. elegans* embryos expressing cell-cycle phase reporters using a confocal microscope. From these studies, I found that extruded cells enter the cell cycle and arrest in S phase. From similar live imaging studies of RNAi-treated embryos, I found that entry into the cell cycle and subsequent S-phase arrest are coupled to and likely required for cells to be extruded. I also found a role for unequal cell division regulators in helping cells to achieve this arrested cell-cycle state that is coupled to cell extrusion. Because of time limitations, I have not characterized the remaining genes in detail. I discuss the potential roles of these genes in regulating cell extrusion below.

Discussion

The transcriptional regulators identified from the RNAi screen are *unc-62*, which encodes a homothorax-like homeodomain protein; *unc-37*, which encodes a Groucho transcriptional corepressor; *lsy-22*, which encodes a short Groucho-like protein; *aptf-2*, which encodes an AP-2-like transcription factor; and *alg-2*, which encodes an Argonaute protein specific to the miRNA-induced silencing complex (miRISC). These genes broadly function in development (Budirahardja *et al.*, 2016; Flowers *et al.*, 2010; Hughes *et al.*, 2013; Jiang *et al.*, 2009; Miller and Okkema, 2011; Peden *et al.*, 2007; Pflugrad *et al.*, 1997; Potts *et al.*, 2009; Sarin *et al.*, 2007; Van Auken *et al.*, 2002; Winnier *et al.*, 1999; Yang *et al.*, 2005). Given their broad roles, it is difficult to predict how these genes function to cause extrusion. One possibility is that *unc-62*, *unc-37* and *lsy-22* control the fate asymmetry between the extruded cells and their surviving sisters, as these genes are known to play such a role in other lineages (Chang *et al.*, 2003; Flowers *et al.*, 2010; Hughes *et al.*, 2013). *aptf-2* has a role in the timing of embryonic cell divisions, which is a crucial component of cell extrusion (as discussed in Chapter 3) (Budirahardja *et al.*, 2016). *alg-2* and another gene encoding a microRNA-induced silencing complex component, *ain-2*, have both been previously identified in screens for the Tex phenotype (Daniel Denning, personal communication). It is unclear how miRISC components function to regulate cell extrusion.

The cytoskeletal regulators I identified from the genome-scale RNAi screen are *rho-1*, which encodes the *C. elegans* homolog of the mammalian

actin-myosin regulator RhoA GTPase, and *mlc-5*, which encodes a myosin light chain protein. Cell extrusion is coupled to and requires epidermal cell movements that result in encasing the *C. elegans* embryo in a single sheet of epidermal cells (Denning *et al.*, 2012). RHO-1 and myosin have a known function in generating the forces required for efficient epidermal enclosure (Fotopoulos *et al.*, 2013; Wernike *et al.*, 2016). Therefore, it is likely that *rho-1* and *mlc-5* function in the force-generation process during epidermal enclosure that leads to the eviction of cells fated to be extruded. As discussed in the cell extrusion section of Chapter 1, Rho-mediated actin-myosin regulation is almost always involved in physiological cell extrusion in vertebrates and *D. melanogaster*. The transcription factor *aptf-2* also has a function in epidermal morphogenesis and could function in cell extrusion by regulating the morphogenesis process (Budirahardja *et al.*, 2016).

I identified a total of five genes from the genome-scale RNAi screen for clones that generate the Tex phenotype that are known for their role in unequal cell divisions of neuroblasts in the Q cell lineage – *grp-1*, *arf-1.2*, *arf-1.3*, *cnt-2* and *toe-2* (Gurling *et al.*, 2014; Singhvi *et al.*, 2011; Teuliere *et al.*, 2014). RNAi against *pig-1*, another gene with a function in Q lineage unequal cell divisions, has been previously shown to produce the Tex phenotype in *ced-3(lf)* animals (Chien *et al.*, 2013; Cordes *et al.*, 2005; Denning *et al.*, 2012). These genes define three different pathways required for unequal cell divisions of Q lineage neuroblasts – the *arf* pathway, the *pig-1* pathway and the *toe-2* pathway (see Chapter 1). Of these genes, I focused on the role of *pig-1*, *grp-1* and *cnt-2* in cell

extrusion because of the high penetrance of Tex phenotype produced by these genes (see Chapter 3).

A number of genes identified from the genome-scale RNAi screen either have a very wide range of known functions or no known functions. In either case, it becomes difficult to comment about how they might regulate cell extrusion. These genes include i) *mpk-1*, which encodes a p38 MAP Kinase protein with broad function in development (Arur *et al.*, 2009; Arur *et al.*, 2011; Bianco *et al.*, 2018; Church *et al.*, 1995; Drake *et al.*, 2014; Kisielnicka *et al.*, 2018; Lackner *et al.*, 1994; Leacock and Reinke, 2006; Lee *et al.*, 2007; Okuyama *et al.*, 2010; Page *et al.*, 2001; Pellegrino *et al.*, 2011; Perrin *et al.*, 2013; Spilker *et al.*, 2009; Yoon *et al.*, 2017); ii) *uba-2*, which functions in the activation of SUMO, a ubiquitin-like protein involved in many facets of *C. elegans* development (reviewed in Broday, 2017); iii) *R05H5.3*, which encodes a homolog of the mammalian nucleoredoxin protein (Kurooka *et al.*, 1997); iv) *C45G7.4*, which encodes a RING-domain protein of unknown function (also identified by Daniel Denning from EMS mutagenesis screens for the Tex phenotype) ; v) *gei-4*, which encodes a protein with no clear mammalian homologs or biochemical function, but with a known function in antagonizing Ras-mediated vulval development in *C. elegans* (Poulin *et al.*, 2005). Of all of these genes, I have attempted to characterize only *C45G7.4*. My preliminary data suggest that *C45G7.4* might be required for unequal cell divisions (see Appendix).

These uncharacterized genes represent an opportunity for further discovery. Characterizing how these genes function to regulate cell extrusion might reveal novel biological aspects of extrusion.

Materials and Methods

Strains and genetics

The *C. elegans* strain MT20117 with the genotype *nIs433[P_{pgp-12}::4xNLS::GFP] I; ced-3(n3692) IV* was used for all RNAi screens and was maintained on Petri plates containing nematode growth medium (NGM) agar (Brenner, 1974). The *E. coli* strain OP50 was used as food.

General microscopy

The RNAi screen for the Tex phenotype was performed using a Nikon SMZ18 fluorescence-dissecting microscope.

RNA interference

Gravid *nIs433; ced-3(n3692)* worms were bleached with 4% NaOCl (sodium hypochlorite bleach) in 0.7 M NaOH solution for 10 min with intermittent vortexing. The recovered eggs were allowed to hatch overnight in M9 media without food to synchronize at the L1 larval stage. The L1s were distributed onto 5 10 cm NGM Petri plates with no food to keep animals for feeding RNAi experiments conducted during that week. Every day, four days a week, L1s from one 10 cm NGM plate without food were transferred onto a 10 cm NGM plate with OP50 bacteria. Two days after the transfer, at the L4 larval stage, the animals were washed off the NGM plates with M9 media containing 0.1% Tween 20 detergent (M9+T20), allowed to starve for 20 min in this media on an orbital shaker and subsequently washed twice with M9+T20. The washed L4 animals

were dropped on a plate with no food. For each 24-well RNAi plate to be loaded with these animals, 24 corresponding 10 µl drops of M9+T20 were deposited on a Parafilm sheet. Three L4 animals were picked from the plate into the 10 µl M9+T20 drops for each of the 24 drops. The 24 drops containing three L4 animals each were transferred to each of the 24 wells using a 10 µl pipette.

24-well RNAi plates were prepared by adding 2 ml of NGM Agar with 1 mM IPTG and 1 mM Ampicillin to each well. These plates were kept at 4°C for experiments to be done up to a month after preparation. Each well of each 24-well plate was seeded with 50 µl of a bacterial culture grown from the corresponding well of a quadrant of an ORFeome RNAi library 96-well plate. These bacterial cultures were grown for at least 12 hours at 37°C and treated with 1 mM IPTG for 1 hr to induce dsRNA production before seeding. dsRNA production was induced in bacterial RNAi clones by IPTG overnight. L4 animals were loaded the next day into the wells of eight 24-well plates, corresponding to two 96-well plates from the ORFeome RNAi library.

Approximately 50 L3 or L4 progeny were scored for the Tex phenotype 3-4 days after loading the L4 parents. Any bacterial RNAi clone producing more than five progeny with the Tex phenotype was marked for further testing.

Experiments to confirm the RNAi clones identified from the large-scale RNAi screen as true positives were done in triplicates in 6 cm Petri plates with appropriate media for RNAi (NGM + 1mM Ampicillin + 1mM IPTG). Each of the set of three plates was seeded with 100 µl of the bacterial culture of the RNAi clone to be tested grown for at least 12 hrs at 37°C. Three L4 animals were

picked onto these plates after appropriate washes. Their L3/L4 progeny were scored for the Tex phenotype after 3-4 days after arresting movement by keeping on ice for 30 min. At least 100 animals were scored for each such experiment, unless extensive lethality occurred, in which case only the escapers of lethality were scored. Sanger sequencing was used to identify the genes targeted by RNAi clones that produced greater than 8% Tex penetrance in the confirmatory experiments.

Table 1 RNAi clones identified from the genome-scale RNAi screen for the Tex phenotype for confirmatory testing. A list of 164 RNAi clones that met the criterion for confirmatory testing, their location in the ORFeome RNAi library, the penetrance of the Tex phenotype produced during confirmatory testing, and the identity of clones that produced a significant Tex penetrance is provided.

Table 1

No.	RNAi target	ORFeome Location	%Tex	n
	control		0	136
1		10001 G9	3	105
2	*cdk-1*	10002 A8	36	75
3	*uba-2*	10003 A7	13	104
4		10008 A4	5	103
5	*R05H5.3*	10008 B2	33	127
6	*arf-3*	10008 B8	15	115
7	*mlc-5*	10008 C1	32	108
8		10008 E8	2	129
9		10009 C9	0	128
10	*cdc-25.2*	10013 D9	22	127
11	*cdc-25.2*	10013 F9	18	136
12	*cdc-25.2*	10013 F11	43	155
13	*unc-62*	10014 C4	11	102
14		10014 C5	1	186
15		10015 D1	2	137
16	*cnt-2*	10015 H9	69	159
17	*rho-1*	10017 E11	66	40
18		10017 F8	1	62
19		10018 B6	2	135
20	*grp-1*	10019 H4	81	110
21	*cye-1*	10020 G9	83	93
22	*unc-37*	10021 G7	26	109
23	*cya-1*	10024 B2	19	145
24		10024 F12	0	123
25		10027 B3	0	116
26		10027 F8	2	92
27		10028 C7	0	104
28	*psf-3*	10037 B12	50	105
29	*arf-1.2*	10051 D11	35	136
30		10056 B5	0	116
31		10056 F3	2	113
32		10069 C6	0	171
33		10070 A6	3	96
34		10070 C5	3	141
35		10114 A7	1	116
36		10144 E2	2	183
37		10154 C1	1	110
38		11001 C10	1	119
39		11002 H2	3	189
40		11005 B3	1	153

Table 1 (cont.)

No.	RNAi target	ORFeome Location	%Tex	n
41		11005 B8	1	150
42		11005 E7	0	109
43	*mpk-1*	11007 B2	5	118
44	*mpk-1*	11007 B6	7	134
45		11007 E3	2	201
46		11008 C3	2	90
47		11008 E4	0	153
48		11008 F5	2	108
49	*gei-4*	11009 C12	14	202
50		11009 D11	2	155
51		11011 B12	3	239
52		11011 E10	0	155
53		11011 F9	3	175
54		11012 A12	5	147
55		11012 C12	1	100
56	*psf-2*	11014 A2	39	156
57		11014 D7	1	124
58		11015 D12	2	165
59		11016 B4	1	133
60		11016 D4	1	164
61	*lsy-22*	11017 C7	20	279
62		11017 C12	1	157
63		11017 D4	0	200
64		11017 E8	1	158
65		11018 C11	0	168
66		11019 A8	1	140
67		11019 C2	0	202
68		11019 G8	1	206
69		11020 B1	2	107
70		11021 F6	2	178
71		11021 F7	0	136
72		11022 D3	2	103
73		11022 F2	2	150
74		11027 E6	0	178
75		11027 F11	1	125
76		11028 B3	0	120
77	*psf-1*	11028 D1	36	116
78		11028 F12	1	154
79		11028 G2	1	137
80		11029 C9	0	135

Table 1 (cont.)

No.	RNAi target	ORFeome Location	%Tex	n
81		11030 A4	0	145
82		11032 B9	0	152
83		11033 C9	1	178
84		11035 G4	1	159
85	*toe-2*	11036 B5	26	208
86		11036 B10	1	193
87		11036 D11	1	165
88		11036 F10	0	152
89		11036 G5	3	221
90	*alg-2*	11037 A7	9	134
91		11037 A10	1	133
92		11037 H4	2	138
93		11038 E6	5	121
94		11039 B1	1	187
95		11039 F3	1	173
96		11040 B11	1	145
97		11040 G8	1	134
98		11042 G6	1	111
99	*aptf-2*	11044 E8	19	166
100		11045 H3	0	114
101		11047 G1	0	152
102		11048 E5	0	120
103		11049 G7	0	116
104	*csn-4*	11049 F3	47	141
105	*csn-5*	11049 F6	39	145
106		11050 C1	1	152
107	*C45G7.4*	11050 C9	38	156
108		11050 C11	1	152
109		11051 F12	1	134
110		11052 A8	10	109
111		11054 A7	0	126
112		11054 A12	0	125
113		11054 B11	9	107
114		11056 E2	0	139
115		11058 H4	0	142
116		11059 A6	0	123
117		11059 D7	0	105
118		11060 B8	0	158
119		11061 F8	2	133
120		11061 F11	1	121

Table 1 (cont.)

No.	RNAi target	ORFeome Location	%Tex	n
121	*csn-1*	11063 G5	43	132
122		11064 B5	0	154
123		11064 F4	0	224
124		11065 E8	2	168
125		11066 A4	3	162
126		11066 A6	1	178
127		11066 H6	1	163
128		11067 G7	3	153
129		11068 D2	0	124
130		11068 H5	1	144
131		11069 A4	2	140
132		11069 B1	1	173
133		11069 C3	0	149
134		11069 E9	4	258
135		11069 G6	1	153
136		11069 H9	0	176
137		11070 C1	1	168
138		11071 B7	0	167
139		11071 G11	0	131
140		11072 E10	1	147
141		11072 H3	2	113
142		11073 G9	1	101
143		11073 G10	2	127
144		11074 E6	0	123
145		11074 F6	0	129
146		11074 F8	0	163
147		11075 A12	0	144
148		11075 H1	0	105
149		11076 B11	0	157
150		11076 C9	1	194
151		11076 E6	1	134
152		11077 C2	0	120
153		11079 B9	0	108
154		11081 D7	0	119
155		11082 D6	1	126
156		11082 E2	1	145
157		11206 E8	0	134
158		11206 F8	0	151
159		11207 H11	4	153
160		11302 A12	0	156
161		11304 C9	0	148
162		11304 F5	1	136
163		11304 G5	2	115
164		11308 D1	3	138

Table 2 Final list of confirmed RNAi clones identified from the genome-scale RNAi screen for the Tex phenotype. A list summarizing the identity, location in the ORFeome library and the penetrance of the Tex phenotype produced during confirmatory testing for bacterial RNAi clones that passed the criterion of confirmatory testing is provided.

Table 2

No.	RNAi target	ORFeome Location	%Tex	n
	control		0	136
1	*cdk-1*	10002 A8	36	75
2	*uba-2*	10003 A7	13	104
3	*R05H5.3*	10008 B2	33	127
4	*arf-3*	10008 B8	15	115
5	*mlc-5*	10008 C1	32	108
6	*cdc-25.2*	10013 D9	22	127
7	*cdc-25.2*	10013 F9	18	136
8	*cdc-25.2*	10013 F11	43	155
9	*unc-62*	10014 C4	11	102
10	*cnt-2*	10015 H9	22	127
11	*rho-1*	10017 E11	66	40
12	*grp-1*	10019 H4	81	110
13	*cye-1*	10020 G9	83	93
14	*unc-37*	10021 G7	26	109
15	*cya-1*	10024 B2	19	145
16	*psf-3*	10037 B12	50	105
17	*arf-1.2*	10051 D11	35	136
18	*mpk-1*	11007 B2	5	118
19	*mpk-1*	11007 B6	7	134
20	*gei-4*	11009 C12	14	202
21	*psf-2*	11014 A2	39	156
22	*lsy-22*	11017 C7	20	279
23	*psf-1*	11028 D1	36	116
24	*toe-2*	11036 B5	26	208
25	*alg-2*	11037 A7	9	134
26	*aptf-2*	11044 E8	19	166
27	*csn-4*	11049 F3	47	141
28	*csn-5*	11049 F6	39	145
29	*C45G7.4*	11050 C9	38	156
30	*csn-1*	11063 G5	43	132

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Chapter 3

Cell cycle S-phase arrest drives cell extrusion

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I conducted all the studies of cell extrusion by *C. elegans* embryos for this chapter. Carlos Pastor, a postdoc in Jody Rosenblatt's lab, conducted the studies of hydroxyurea-treated mammalian epithelial cells based on information from my studies of cell extrusion by *C. elegans*. Rita Droste, a research specialist, was instrumental in the genome-wide RNAi screen. Daniel Denning originally established the project and generated some of the strains that I used for confocal imaging of cell extrusion by *C. elegans* embryos.

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ABSTRACT

Cell extrusion is a conserved process of cell elimination in which a cell is squeezed out of a layer of other cells. Cell extrusion is the primary mode of cell elimination in vertebrate epithelial tissues and the dysregulation of cell extrusion is a hallmark of aggressive mammalian tumors. Despite the importance of cell extrusion to physiology and disease, the mechanisms regulating cell extrusion remain poorly understood. Using a genome-scale RNAi screen and live-imaging studies of *C. elegans* embryos, we have identified and characterized genes required for cell extrusion. These studies led to the surprising discovery that cells that are extruded enter a state of S-phase arrest prior to extrusion and that genetic perturbations that prevent cell extrusion do so by circumventing the S-phase arrest. Consistent with these findings, treatment of MDCK cells with an agent that causes S-phase arrest, hydroxyurea, markedly increased cell extrusion. Our findings show that S-phase arrest is an evolutionarily conserved driver of cell extrusion. We propose that normal processes of cell extrusion involve S-phase arrest. Because the human homologs of genes required for S-phase arrest and extrusion in *C. elegans* are oncogenic, we propose that oncogenes can cause metastatic spread by driving cancer cells into S-phase arrest, which then promotes extrusion from the tumor into surrounding tissue.

INTRODUCTION

Cell extrusion is a process in which a cell is squeezed out of a layer of cells. Cell extrusion has been observed and studied in a wide variety of organisms, including *C. elegans*, *D. melanogaster*, zebrafish and mammals, suggesting that cell extrusion is an evolutionarily conserved mechanism of eliminating unnecessary or harmful cells (Gudipaty and Rosenblatt, 2017; Ohsawa *et al.*, 2018). Vertebrate epithelial tissues use cell extrusion as the primary mode of cell elimination (Gu and Rosenblatt, 2012). Decreased cell extrusion leads to the formation of epithelial cell masses and confers resistance to apoptosis, another mechanism of cell elimination (Eisenhoffer *et al.*, 2012; Gu *et al.*, 2015). Increased cell extrusion is believed to contribute to disorders such as asthma and Crohn's disease (Gudipaty and Rosenblatt, 2017). Disruption and subversion of the cell-extrusion process contributes to the hallmarks of aggressive tumors, such as those found in patients with pancreatic, lung or colon cancers (Gu *et al.*, 2015), and likely promotes the formation of polyps in patients with Peutz-Jeghers syndrome (Eisenhoffer *et al.*, 2012; Hemminki *et al.*, 1998). The molecules and pathways regulating cell extrusion, however, are poorly understood.

Denning *et al.* (2012) showed that cell extrusion occurs in caspase-deficient *C. elegans* embryos and is regulated by the PAR-4 (mammalian homolog LKB1) – PIG-1 (mammalian homolog MELK) kinase cascade (Denning *et al.*, 2012). The processes of cell extrusion seem similar in *C. elegans* and

mammals: (i) cells extruded from *C. elegans* embryos and mammalian epithelia show characteristics of apoptosis, respectively (Denning *et al.*, 2012; Eisenhoffer *et al.*, 2012; Rosenblatt *et al.*, 2001), and (ii) PAR-4 and its counterpart LKB1 are involved in cell extrusion from mouse and *C. elegans* embryos (Denning *et al.*, 2012; Krawchuk *et al.*, 2015). Interestingly, patients with Peutz-Jeghers syndrome, a disease in which decreased cell extrusion likely contributes to the formation of polyps, carry mutations in the human *LKB1* gene (Eisenhoffer *et al.*, 2012; Hemminki *et al.*, 1998). The exact role of PIG-1 (MELK) in cell extrusion remains unknown.

To identify additional regulators of cell extrusion in *C. elegans*, we performed a genome-wide RNAi screen for defective cell extrusion and used confocal microscopy to analyze the effect of RNAi against the identified genes on the cell extrusion process in caspase-deficient embryos. From this analysis, we found that cell-cycle entry and subsequent S-phase arrest are coupled to cell extrusion and that circumventing S-phase arrest blocks cell extrusion. Additionally, we show that treating mammalian cells with hydroxyurea, which causes S-phase arrest (Timson, 1975; Vesela *et al.*, 2017), promotes cell extrusion. We conclude that S-phase arrest drives cell extrusion and propose that S-phase arrest plays a crucial role in cell extrusions that occur during normal development and in human disease.

RESULTS

Genome-scale RNAi screen identified cell-cycle genes as candidate regulators of cell extrusion

In *C. elegans* mutants deficient in programmed cell death, e.g. carrying a loss-of-function mutation in the caspase gene *ced-3*, cells are extruded from developing embryos (Denning *et al.*, 2012). In wild-type embryos, these cells undergo apoptosis soon after they are generated and then are eliminated by the process of phagocytosis. Of the approximately six cells extruded from *ced-3(lf)* embryos, ABplpappap is the cell most frequently extruded (Denning *et al.*, 2012 and unpublished). If the extrusion process fails, ABplpappap (or a descendant of ABplpappap) can differentiate into a supernumerary excretory cell, producing animals with two excretory cells instead of the one excretory cell in *ced-3(lf)* (and wild-type) animals (Figure 1A). This two-excretory-cell phenotype is referred to as the Tex phenotype. Mutations that reduce ABplpappap extrusion also reduce the extrusion of other cells, so ABplpappap extrusion can be used to identify general regulators of cell extrusion (Denning *et al.*, 2012).

Using the GFP excretory-cell reporter $P_{pgp\text{-}12}$*::4xNLS::GFP*, Denning *et al.* (2012) performed an EMS screen and identified mutants with two GFP-positive cells in *ced-3(lf)* animals, i.e., animals with the Tex phenotype. From this screen Denning *et al.* determined that the PAR-4 (LKB1) – PIG-1 (MELK) kinase cascade is important for cell extrusion. To better establish the mechanistic basis of cell extrusion, we performed a genome-wide RNAi screen of *ced-3(lf)* animals

for the Tex phenotype. We used the ORFeome RNAi library, which includes about 55% of the approximately 20,000 *C. elegans* genes, and performed a feeding RNAi screen (Rual *et al.*, 2004). Briefly, bacteria containing each RNAi clone were grown in an individual well of a 24-well plate, three L4 *ced-3(lf)* animals were added, and the progeny of these animals were scored for the Tex phenotype at the L3-L4 larval stage using a dissecting microscope equipped with fluorescence (Figure 1B). The scorer was blinded to the identity of the RNAi clone. Roughly 50 animals were scored per well, and any well with five or more Tex animals was marked for retesting. We used sequencing to confirm the identity of RNAi clones that reproducibly generated a Tex phenotype fo more than 10% of the animals scored.

Of the 11,511 total clones in the ORFeome library, 30 clones targeting 27 unique genes consistently produced a Tex phenotype. Three of these clones identified genes previously reported to function in cell extrusion, *grp-1*, *arf-1.2* and *arf-3* (Denning *et al.*, 2012), confirming that this RNAi screen could identify genes known to be involved in the Tex biology. Unexpectedly, 10 of these 27 RNAi clones targeted genes that regulate cell-cycle progression (Figure 1C).

To explore a potential role for cell-cycle genes in cell extrusion, we created an RNAi library to target regulators of the *C. elegans* cell cycle (van den Heuvel, 2005). We created this collection from RNAi clones that were mostly available in other large RNAi libraries after verifying their identity (Kamath *et al.*, 2003; Rual *et al.*, 2004), and constructed RNAi clones for genes for which we could not find

suitable clones in these libraries (see Materials and Methods). We screened this library for the Tex phenotype in *ced-3(lf)* animals and found that RNAi against four additional cell-cycle genes produced a Tex phenotype in *ced-3(lf)* animals (Figure 1C, Supplemental Table 1). Interestingly, cell-cycle genes identified from our screens were enriched for regulators of S-phase entry and progression. These genes include *cye-1* and *cya-1*, which encode homologs of S-phase cyclins E and A, respectively; *cdk-1* and *cdk-2*, which encode homologs of CDK1 and CDK2, respectively; and *psf-1*, *psf-2* and *psf-3*, which encode homologs of pre-replicative and replicative complex components PSF1, PSF2 and PSF3, respectively (Figure 1C). Together, our findings revealed that genes that regulate transition into S phase of the cell cycle are necessary to prevent the Tex phenotype and that these genes might play a role in cell extrusion.

We identified multiple components of a number of protein complexes, including the Cyclin E/CDK2 complex, the Cyclin A/CDK1 complex, the GINS complex and the COP9/signalosome (Figure 1C). That RNAi against only certain essential genes produced the Tex phenotype, while knockdown of others - despite producing extensive lethality - did not (Supplemental Table 1) indicates that the expression of the Tex phenotype is not caused by a general reduction in embryonic fitness. These findings revealed that genes that regulate transition into the S phase of the cell cycle are necessary to prevent expression of the Tex phenotype and suggest that these genes play a role in cell extrusion.

RNAi against genes required for entry into S phase prevents cell extrusion

To directly test the involvement of cell-cycle genes in cell extrusion and to determine if transition into the S phase is important for extrusion, we again used the approach of RNAi, which can facilitate the study of essential genes in general and cell-cycle genes in particular (Kemphues, 2005; Kipreos and van den Heuvel, 2019). We focused on the cell ABplpappap, which is extruded in *ced-3(lf)* embryos and occupies a central position on the ventral surface (Denning *et al.*, 2012; Sulston *et al.*, 1983), facilitating direct observation of its extrusion. We asked if RNAi against the genes *cye-1* and *cdk-2*, which produced a highly penetrant Tex phenotype (Figure 1C), blocked the extrusion of ABplpappap. (The cyclin E/CDK2 complex promotes S-phase entry during the cell cycle; Ohtsubo *et al.*, 1995; Tsai *et al.*, 1993.) We used time-lapse confocal microscopy to monitor the location of ABplpappap in embryos from parents treated with RNAi (referred to as "embryos with RNAi" in the rest of the text) against empty vector (referred to as "control" in the rest of the text), *cye-1*, or *cdk-2*, keeping ABplpappap in view by refocusing on it every 30 sec. We found that confocal imaging during a period of about 50 min during which ventral enclosure (migration and meeting of hypodermal cells on the ventral surface of the embryo) occurs was sufficient to determine whether ABplpappap did or did not undergo extrusion. Micrographs obtained using this approach showed that after treatment with RNAi against control, progressively fewer cells were found in the immediate surroundings of ABplpappap, indicating ABplpappap was extruded from the embryo (shown in Figure 2A with 10 min intervals, in Movie 1 with 30 sec intervals). By comparison,

embryos with RNAi against *cye-1* (Figure 2B, Movie 2) or *cdk-2* (Figure 2C, Movie 3) had ABplpappap in the same plane as many other cells in its immediate vicinity throughout the 50-min period, indicating that ABplpappap was not extruded in these embryos.

We also captured images at different focal depths of multiple embryos for each RNAi treatment around the time of completion of ventral enclosure and generated virtual lateral sections of embryos through the plane that intersected ABplpappap. These lateral views show that ABplpappap was extruded ventrally in 10 of 11 embryos with RNAi against control (Figure 2D, Supplemental Figure 1), whereas ABplpappap was not extruded but rather was incorporated into the embryonic body of animals with RNAi against *cye-1* (11 of 11 embryos) (Figure 2E, Supplemental Figure 2) or *cdk-2* (10 of 11 embryos) (Figure 2F, Supplemental Figure 3). These findings demonstrate that RNAi targeting of either *cye-1* or *cdk-2*, mammalian homologs of which together promote S-phase entry, results in a block in ABplpappap cell extrusion.

Entry into S phase is required for and precedes cell extrusion

Since RNAi against genes required for S-phase entry blocked ABplpappap extrusion, we asked if cells that undergo extrusion require entry into S phase for extrusion to occur. To test this hypothesis, we used the transgene *heSi192*, which expresses a truncated human DNA Helicase B (tDHB) - GFP fusion protein (optimized for expression in *C. elegans*) that changes its intracellular location in response to CDK activity (Spencer *et al.*, 2013; van Rijnberk *et al.*, 2017). This

reporter is enriched in the nuclei of quiescent or post-mitotic cells and becomes progressively more enriched in the cytoplasm and depleted from the nucleus of a cell from the start of S phase to mitosis during a cell cycle (Figure 3A). We found that in *ced-3(lf)* embryos with RNAi against control, this fusion protein was mostly absent from the ABplpappap nucleus both before ventral enclosure (Figure 3B) and as it was extruded (Figure 3E) (for 10 of 10 embryos), indicating that ABplpappap enters the S-phase of the cell cycle prior to its extrusion during the period of ventral enclosure. We examined cells extruded at other sites in the embryo and found, as expected, that those cells also displayed a decreased localization of tDHB-GFP in their nuclei (Figures 3I, J, K, L). By contrast, in *ced-3(lf)* embryos with RNAi against *cye-1* (Figures 3C, 3F) or *cdk-2* (Figures 3D, 3G) the ABplpappap nucleus scored positive for the DHB-GFP fusion protein during the period when extrusion normally occurs (10 of 10 embryos each for each RNAi treatment), suggesting that RNAi against these genes prevented the entry of ABplpappap into the S-phase of the cell cycle. Quantification of the nuclear-to-cytoplasmic ratio of DHB-GFP fluorescence intensity in ABplpappap in embryos with RNAi against control, *cye-1* and *cdk-2* and at different stages of ventral enclosure confirmed these observations (Figure 3H). These results show that the genes *cye-1* and *cdk-2* are required for entry into S phase by cells that are extruded and indicate that cell extrusion is blocked when entry of otherwise extruded cells into the cell cycle is prevented. Other cell cycle genes identified from the genome-scale RNAi screen (Figure 1C) likely play a similar role in cell-

cycle entry and progression to the phase that precedes cell extrusion. Some cells were still extruded in embryos with RNAi against *cye-1* or *cdk-2*, likely reflecting an incomplete inhibition. Interestingly, such cells displayed a primarily cytoplasmic localization of DHB-GFP, indicating that those cells had entered the cell cycle (Supplemental Figure 4). This observation provides further support to the hypothesis that cells that undergo extrusion require entry into the cell cycle for extrusion to occur.

To confirm our finding that S-phase entry is required for cell extrusion, we used a second reporter transgene, *isIs17*, which expresses an N-terminal translational fusion of GFP to PCN-1 (Brauchle *et al.*, 2003). PCN-1 is the *C. elegans* homolog of the mammalian protein PCNA, which localizes to replication forks during S phase. Mammalian PCNA-based fluorescent reporters produce changes in brightness and localization as a result of progression through the cell cycle, and the brightness and localization pattern of the PCNA fusion protein can be used to identify the phase of the cell cycle of a cell (Zerjatke *et al.*, 2017). During the embryonic cell cycles of *C. elegans*, GFP::PCN-1 shows a phase-specific brightness and localization pattern similar to that exhibited by mammalian PCNA (Figure 4A; Brauchle *et al.*, 2003). We found that in embryos with RNAi against control, GFP::PCN-1 was localized in bright sub-nuclear foci in ABplpappap just before ventral enclosure began, indicating that this cell was in S phase (5 of 5 embryos) (Figure 4B). By contrast, ABplpappap showed a diffuse nuclear localization of GFP::PCN-1 in embryos with RNAi against *cye-1* (Figure

4C, 4F) or *cdk-2* (Figure 4D, 4G) before (5 of 5 embryos for each RNAi treatment) and also after ventral enclosure (5 of 5 embryos for each RNAi treatment), indicating that this cell was quiescent and failed to enter the cell cycle during ventral enclosure. We conclude that extruded cells enter the S-phase of the cell cycle prior to extrusion and that blocking cell cycle entry prevents cell extrusion.

Cells undergoing extrusion arrest in S phase

Given that extruded cells enter S phase, we asked if they also exit S phase. In *ced-3(lf)* embryos with RNAi against control, GFP::PCN-1 was localized in bright sub-nuclear foci in ABplpappap before (5 of 5 embryos) and after extrusion (5 of 5 embryos) (Figure 4B, 4E), indicating that ABplpappap was in S phase both before and after extrusion. By monitoring the cell cycle progression of ABplpappap, we observed that there was no significant change of GFP::PCN-1 localization in ABplpappap up to and after its extrusion over a period of 35 min (Figure 4H), indicating a lack of S-phase progression. By contrast, we found that other cells on the ventral surface displayed a continuous change of GFP::PCN-1 localization, indicating S-phase progression (Supplemental Figure 5). To determine if S-phase arrest is a general feature of cell extrusion, we examined other extruded cells in the embryo. Indeed, extruded cells displayed an S phase-specific localization pattern of GFP::PCN-1 similar to that of ABplpappap (Figures 4H, 4I, 4J). A small number of extruded cells eventually showed GFP::PCN-1

localization like that normally observed during mitosis after extrusion (Supplemental Figure 6), indicating that these cells were capable of recovering from the arrest and completing S phase, and were likely not lacking molecules critical for cell-cycle progression. However, as all extruding cells exhibited GFP::PCN-1 localization indicating S-phase arrest during ventral enclosure, any recovery likely occurred after the completion of extrusion and ventral enclosure, and therefore could not affect the extrusion fate.

Arrest of DNA replication during S-phase can occur as a result of replication stress, which can arise from many different sources (reviewed by Zeman and Cimprich, 2014), such as a shortage of nucleotides. Replication stress triggers the replication stress response, which functions to stabilize stalled replication forks, prevent cell-cycle progression and prevent further firing of replication origins (reviewed by Zeman and Cimprich, 2014). All these functions of the replication stress response prevent the genomic instability that can arise from collapsed replication forks (Gaillard *et al.*, 2015). Core components of the replication stress response pathway in *C. elegans* and other metazoans include ATR, Chk1, Rad17, Rad9, Rad1, Hus1, Replication Protein A, TopBP1, Timeless, Tipin and Claspin proteins, which are encoded by the *C. elegans* genes *atl-1*, *chk-1*, *hpr-17*, *hpr-9*, *mrt-2*, *hus-1*, *rpa-1*, *mus-101*, *tim-1*, *tipn-1* and *clsp-1*, respectively (Stevens *et al.*, 2016; Yazinski and Zou, 2016). As cells undergoing extrusion displayed an S-phase arrested GFP::PCN-1 localization pattern for the duration of cell extrusion, we asked if triggering the replication stress response is

important for extrusion. RNAi against 7 of the 11 genes encoding core components of the replication-stress checkpoint (*atl-1*, *chk-1*, *hpr-9*, *mus-101*, *tim-1*, *tipn-1* and *clsp-1*) produced a Tex phenotype in *ced-3(lf)* animals (Figure 1C, Figure 4L), indicating an involvement of replication stress response in cell extrusion. These findings suggest that cells that undergo extrusion experience replication stress and trigger the replication stress response, which promotes their extrusion.

Unequal division of ABplpappap's mother cell restricts cell-cycle progression of ABplpappap

Cells that undergo caspase-mediated apoptosis in *C. elegans* are the smaller of the two sister cells generated from unequal cell divisions (Sulston *et al.*, 1983; Cordes *et al.*, 2006; Hatzold and Conradt, 2008; Ou *et al.*, 2010). ABplpappap is smaller than its sister, ABplpappaa in both wild-type embryos, in which it dies (Sulston *et al.*, 1983), and *ced-3(lf)* embryos, from which it is extruded (Figure 5B, 5F and 5G). The genes *pig-1* (homolog of the mammalian kinase gene *MELK*) and *grp-1* (homolog of the mammalian ARF GEF gene *CYTH3*) are required for cell extrusion (Denning *et al.*, 2012). In other cell lineages, such as the Q neuroblast cell lineage, these genes are required for unequal cell divisions that generate apoptotic cells (Cordes *et al.*, 2006; Teuliere *et al.*, 2014). From our genome-wide RNAi screen for clones that resulted in the Tex phenotype, we identified a third gene, *cnt-2* (homolog of the mammalian ARF

GAP gene *CENTG1*), which is involved in unequal cell divisions that generate apoptotic cells in the Q neuroblast cell lineage (Figure 5A; Singhvi *et al.*, 2011).

We wondered if the cell cycle progression of ABplpappap, and perhaps of other extruded cells, is restricted to S-phase entry, arrest and subsequent extrusion because of its generation as the smaller of two sister cells. To explore this hypothesis, we first determined if the size of ABplpappap relative to its sister is increased in *ced-3(lf)* embryos with RNAi against *pig-1, grp-1* or *cnt-2*. Indeed, we found that ABplpappap was significantly larger in embryos with RNAi against *pig-1, grp-1* or *cnt-2* (in 5 of 5 embryos with each RNAi treatment) than in embryos with an RNAi against control (Figures 5B-G). We also observed that RNAi against *cye-1* or *cdk-2*, genes required for cell extrusion but not known to be involved in unequal cell divisions, did not affect the ratio of the size of ABplpappap relative to its sister (in 4 of 4 embryos with each RNAi treatment) (Supplemental Figure 7). We conclude that (i) similar to their role in other cell lineages, the genes *pig-1*, *grp-1* and *cnt-2* are required for the unequal cell division that generates ABplpappap, and (ii) this unequal division occurs independently of other genes required for cell extrusion, such as *cye-1* and *cdk-2*.

Next we monitored the fate of the enlarged ABplpappap in a *ced-3(lf)* embryo with RNAi against *pig-1* using time-lapse confocal microscopy. We found that this cell completed the cell cycle before ventral enclosure (Figure 5H). We constructed virtual lateral sections to assess the fate of ABplpappap around the

time of ventral enclosure in embryos with RNAi against control, *pig-1*, *grp-1* or *cnt-2*. We found that in contrast to being extruded in embryos with RNAi against control, ABplpappap instead divided to generate daughters that were not extruded in embryos with RNAi against *pig-1* (in 5 of 6 embryos), *grp-1* (in 5 of 5 embryos) or *cnt-2* (in 5 of 5 embryos) (Figure 5 I-L). This finding is consistent with the hypothesis that completing the cell cycle prevents cell extrusion and supports the conclusion that cell-cycle arrest in S phase is required for cell extrusion.

The S-phase arrest agent hydroxyurea markedly increases cell extrusion from mammalian MDCK cells

Hydroxyurea (HU) inhibits DNA replication and produces replication stress in and S-phase arrest of cells from a variety of organisms by blocking the enzymatic activity of ribonucleotide reductase, an enzyme required for maintaining appropriate levels of deoxyribonucleotides for DNA synthesis (Timson, 1975; Vesela *et al.*, 2017). We used HU to determine if S-phase arrest could drive cell extrusion from mammalian epithelial monolayers. We treated Madin-Darby Canine Kidney (MDCK) cells with either 2 mM HU or vehicle (negative control) for total 22 hrs and obtained time-lapse micrographs of the monolayers from this period to assess cell extrusion. Cell extrusion was approximately four-fold higher from HU-treated MDCK cells compared to vehicle-treated MDCK cells (Figure 6A, 6B). This finding indicates that S-phase arrest can drive cell extrusion from mammalian epithelial monolayers.

Interestingly, we isolated an allele of the nucleotide metabolism gene *gmpr-1*, *n6070,* from a separate EMS mutagenesis screen for the Tex phenotype in *C. elegans ced-3(lf)* embryos. The *C. elegans* GMPR-1 protein is 69% identical to each of the two human guanosine monophosphate (GMP) reductase enzymes, GMPR1 and GMPR2. GMP reductases function as homo-tetramers to catalyze an irreversible deamination of the nucleotide GMP to the nucleotide inosine monophosphate (IMP), using the cofactor NADPH (Li *et al.*, 2006; Spector *et al.*, 1979). The *n6070* allele causes a missense mutation that converts a highly conserved alanine (Ala132) in the NADPH-binding loop of GMP reductase to valine (Figure 6C), a mutation that likely generates steric clashes with both the NADPH cofactor and a second subunit of GMP reductase (Supplemental Figure 8A-E). In a *ced-3(lf)* background, the *n6070* allele produced a highly penetrant maternal-effect Tex phenotype, which was rescued by extrachromosomal arrays carrying a wild-type copy of the *gmpr-1* gene (Figure 6D). Additionally, RNAi against *gmpr-1* produced a Tex phenotype in *ced-3(lf)* animals (Figure 6D). These data indicate that *n6070* is a loss-of-function allele of *gmpr-1*. Examination of *ced-3(lf)* embryos expressing GFP::PCN-1 with RNAi against *gmpr-1* in which ABplpappap was not extruded revealed unhindered S-phase progression by ABplpappap (Figure 6E) around the time of ventral enclosure in 8 out of 10 embryos. We observed that RNAi against *gmpr-1* did not perturb the unequal division of ABplpappap's mother (Supplemental Figure 7), indicating that *gmpr-1* functions differently from the genes *pig-1*, *grp-1* and *cnt-2* to cause cell extrusion.

Given the role of GMP reductase in nucleotide metabolism, we propose that both HU and GMP reductase promote cell extrusion by similar mechanisms that likely involve generation of nucleotide imbalances resulting in S-phase arrest.

DISCUSSION

We have discovered an evolutionarily conserved biological mechanism of cell extrusion. Cells that are extruded from *C. elegans ced-3(lf)* embryos are the smaller of the two daughters generated by unequal cell divisions, which causes an allocation of a proportionally small share of resources to the smaller daughter. As a result of inheriting a smaller share of resources, such as nucleotides, cells that are extruded experience replication stress and undergo S-phase arrest. These cells then show a lack of expression of adhesion molecules (Denning *et al.*, 2012) likely caused by the S-phase arrest, which allows them to be extruded when experiencing morphological forces such as those occurring during ventral enclosure. Cell extrusion from *ced-3* mutant *C. elegans* embryos can be prevented by a) perturbing the unequal cell divisions that generate the cells that are extruded, which allows cells that otherwise arrest in S phase to proceed through the cell cycle; or b) by blocking the entry of these cells into S phase altogether, which effectively functions to prevent S-phase arrest (Figure 7A). Hydroxyurea, an agent that causes S-phase arrest, drives cell extrusion from mammalian epithelial monolayers. Hence, S-phase arrest is both necessary and sufficient for cell extrusion. Our general model for the process of cell extrusion,

based on the studies of both *C. elegans* and mammalian MDCK cells, is shown in Figure 7B.

Why do the cells fated to be extruded arrest in S phase?

Cells that are extruded in *ced-3(lf)* caspase-deficient *C. elegans* embryos are eliminated by caspase-mediated apoptosis in wild-type embryos. These cells are generated from unequal divisions and are generated smaller than their sisters (this publication; Sulston *et al.*, 1983). In seven of the eight cell lineages that generate extruded cells, the sister of the extruded cell normally undergoes further division(s) (Denning *et al.*, 2012; Sulston *et al.*, 1983). Consistently, we found that in *ced-3(lf)* embryos both ABplpappap and its sister entered the cell cycle but only the larger daughter completed the cell cycle. We postulate that the smaller daughter is deficient in intracellular materials necessary to support the rapid S phase of embryonic cell cycles. For example, the nucleotide requirements for DNA replication are equal for ABplpappap and its much larger sister, ABplpappaa, as they both have the same amount of DNA. The small size of ABplpappap, however, might lead to a faster depletion of nucleotide pools, which is known to induce replication stress and replication arrest (reviewed by Giannattasio and Branzei, 2017). Consistent with a role of nucleotide metabolism in S-phase arrest and cell extrusion, we found that loss-of-function mutation in the nucleotide metabolism gene *gmpr-1* produces a Tex phenotype. Embryonic cell cycles expedite S phase by using a higher number of replication origins -- and thus, replication forks -- compared to the number used later in development

(Kermi *et al.*, 2017). Perhaps reducing the number of active replication forks and thereby reducing the rate at which otherwise insufficient nucleotides or other materials are depleted would allow uninterrupted S phase progression of such cells. Consistent with this hypothesis, we found that RNAi against the replication fork genes *psf-1*, *psf-2* and *psf-3*, resulted in the Tex phenotype.

Why are cells arrested in S phase extruded?

Cells that are extruded do not express the classical E-cadherin HMR-1 and other cell-adhesion molecules, which likely allow them to be extruded as a result of morphological forces generated by hypodermal cells and neighboring neuroblasts during ventral enclosure of the embryo (Chisholm and Hardin, 2005; Denning *et al.*, 2012; Wernike *et al.*, 2016). We found that the S-phase arrest of extruding cells is tightly linked to their ability to be extruded, which suggests that the cell-cycle arrest in cells undergoing extrusion might reduce their adhesiveness. It has been recently demonstrated that CDK1, the master regulator of cell cycle progression, regulates adhesion of cells during interphase and that siRNA-mediated targeting of CDK1 during interphase results in reduced adhesion (Jones *et al.*, 2018). Interestingly, activation of the replication stress checkpoint, which we have implicated in cell extrusion, results in the inhibition of CDK1 activity (Jin *et al.*, 2003; Mailand *et al.*, 2002; Xiao *et al.*, 2003). We propose that this reduction in CDK1 activity as a result of S-phase arrest and S-phase checkpoint activation lowers cell adhesion, which facilitates the extrusion

of cells experiencing appropriate morphological forces such as those generated during tissue development or morphogenesis.

Extrusion of cells arrested in S-phase is likely tumor-suppressive

We have shown that cells fated to be extruded in *C. elegans* embryos enter an S-phase arrest that results in their extrusion into the extra-embryonic fluid, thereby ensuring that they do not remain a part of the developing organism. Similarly, mammalian epithelial cells that are treated with hydroxyurea, an agent that induces S-phase arrest, show significantly increased cell extrusion. S-phase arrest is associated with genomic rearrangements, oncogenesis and cancer progression (reviewed by Gaillard *et al.*, 2015). Therefore, cell extrusion driven by S-phase arrest might act as a tumor-suppressive mechanism. We propose that animals generally eliminate cells that are in S-phase arrest by cell extrusion.

S-phase arrest is a potential mechanism to facilitate cell migration

The extrusion of cells in an arrested cell-cycle state likely reflects a conserved mechanism of decreased cell adhesion that allows for cell detachment in development and physiology. The same detachment that causes cell extrusion might also function to promote cell migration. A requirement for the S-phase checkpoint pathway in embryonic development has been demonstrated for many organisms (Brauchle *et al.*, 2003; reviewed by Kermi *et al.*, 2017). The same pathway might contribute to the detachment and migration of cells that occurs

during embryonic development in *C. elegans* and other more complex organisms (Aman and Piotrowski, 2010; Sulston *et al.*, 1983).

Genes required for S-phase-arrest-mediated cell extrusion are upregulated in invasive cancers

Features such as the inactivation of caspase-mediated apoptosis (Igney and Krammer, 2002; Weinberg 2007), entry but not completion of the cell cycle (Ho and Dowdy, 2002; Weinberg, 2007), and replication stress (Gaillard *et al.*, 2015) are commonly found in tumor cells. Interestingly, these features are also present in cells that undergo extrusion in *ced-3(lf)* embryos. We suggest that cell detachment, which is required for invasion and metastasis of tumor cells into surrounding and distant tissues, might be caused by a subverted cell extrusion process induced by cell-cycle arrest, similar to that caused by loss of the tumor suppressor APC (Marshall *et al.*, 2011). Metastasis of at least some tumor types might not require the epithelial-to-mesenchymal transition (EMT) (Fischer *et al.*, 2015; Zheng *et al.*, 2015), and it is possible that metastasis in these cases is initiated by extrusion of cells arrested in the cell cycle. Interestingly, human homologs of most of the factors we have found to be required for cell extrusion have known oncogenic functions. These include CDC25 (reviewed by Boutros *et al.*, 2007); Cyclin A (reviewed by Yam *et al.*, 2002); Cyclin E (reviewed by Hwang and Clurman, 2005); CDKs 1 and 2 (reviewed by Otto and Sicinski, 2017); GINS complex (reviewed by Seo and Kang, 2018); βTrCP (reviewed by Frescas and

Pagano, 2008); COP9/Signalosome (reviewed by Lee *et al.*, 2011); ATR and Chk1 (reviewed by Lecona and Fernendez-Capetillo, 2018); CENTG1 (reviewed by Jia *et al.*, 2016); CYTH3 (Fu *et al.*, 2014); and MELK (reviewed by Ganguly *et al.*, 2014). We predict that these oncogenes function like their *C. elegans* homologs to drive cells into a cell-cycle arrest that is followed by detachment and metastatic spread. It is noteworthy that region 12q13-q15 of human chromosome 12 includes *CENTG1* (a homolog of the *C. elegans* gene *cnt-2*) and *CDK4* (a gene that promotes cell cycle entry) and is frequently amplified in invasive cancers such as glioblastomas, melanomas, breast cancers and lung cancers (Khatib *et al.*, 1993; Muthusamy *et al.*, 2006; Qi *et al.*, 2017; Reifenberger *et al.*, 1996; Wikman *et al.*, 2005). We speculate that this co-amplification of the *CENTG1* and *CDK4* genes drives cell extrusion in and thereby the invasiveness of these tumor cells.

Materials and Methods

Strains

All *C. elegans* strains were maintained at 22 °C as described previously (Brenner, 1974). We used the Bristol strain N2 as the wild-type strain. The transgenes and mutations used are listed below:

LGI: *nIs433[P_{pgp-12}::4xNLS::GFP::unc-54 3'UTR; unc-76(+)]*

LGII: *heSi192[P_{eft-3}::CDK sensor::eGFP::tbb-2 3'UTR + Cbr-unc119(+)]*

LGIII: *unc-119(ed3)*

LGIV: *unc-30(e191)*, *ced-3(n2427, n3692)*

LGV: *gmpr-1(n6070)*, *ltIs44[P_{pie-1}::mCherry::PH(PLC1delta1) + unc-119(+)]*

LGX: *nIs434[P_{pgp-12}::4xNLS::GFP::unc-54 3'UTR; unc-76(+)]*

Unknown linkage: *stIs10026[P_{his-72}::HIS-72::GFP]*, *isIs17[pGZ295(P_{pie-1}::GFP::pcn-1(W03D2.4)), pDP#MM051 (unc-119(+))]*, *nIs861[pDD111(P_{egl-1}::mCherry::PH::unc-54 3'UTR)]*, *nIs631[pDD111(P_{egl-1}::mCherry::PH::unc-54 3'UTR), pML902 (dlg-1::GFP), unc-76(+)]*, *nEx2560[gmpr-1(+), P_{ges-1}::mCherry]*, *nEx2561[gmpr-1(+) , P_{ges-1}::mCherry]*

nIs631 does not express *dlg-1::GFP*, presumably as a result of silencing. *ced-3(lf)* refers to the *n3692* deletion allele of *ced-3*.

Chemical Mutagenesis Screen

unc-30(e191) ced-3(n2427); nIs434 animals were mutagenized with ethyl methanesulfonate (EMS) as described previously (Brenner, 1974). Using a dissecting microscope equipped with fluorescence optics, we screened F3

progeny corresponding to 30,000 haploid genomes for an extra GFP-positive cell (i.e., displaying the Tex phenotype) near the posterior bulb of the pharynx. Candidates were picked to single Petri plates. Progeny of the singled worms were examined for penetrance of the Tex phenotype. Mutants with a penetrance of the Tex phenotype higher than the *ced-3(n3692); nIs434* strain were outcrossed 3x into *ced-3(n3692); nIs434* and subsequently maintained as homozygotes for the causal mutation, *ced-3(n3692)* and *nIs434*.

Molecular Biology

A 5.6 kb DNA fragment containing the *gmpr-1(+)* genomic locus was used to generate the extra-chromosomal arrays *nEx2560* and *nEx2561* and was amplified from fosmid WRM0624bE09 (Source Bioscience) using the following primers:

Fw – CACTCGAATGCATGGCCAAG

Rv – AAGCTGAATAACGTCCGGGT

RNAi clones were constructed for *atl-1*, *mat-2* and *lin-15B*. Genomic regions of about 1 kb were amplified from wild-type genomic lysates using Q5 Hot Start high-fidelity polymerase (New England Biolabs) with the following primers:

atl-1

F TCGAATTCCTGCAGCTCCTCGAACCCATCATCCCT

R TGACGCGTGGATCCCATGAAGCTGCGTGGTTGTTG

mat-2

F TCGAATTCCTGCAGCCTGGAACTCATCCCATACGC

R TGACGCGTGGATCCCCATTGGAACCTCCAGATGCT

lin-15B

F TCGAATTCCTGCAGCGCTGACACAATTGCGAACAT

R TGACGCGTGGATCCCCGTGTGCATAAAGACCAAGG

These inserts were cloned into the pL4440 vector linearized with *XmaI* (New England Biolabs) using the In-Fusion HD cloning kit (Takara) according to manufacturers instructions. The cloned vector was then transformed into competent HT115 bacterial cells. Correct RNAi clones were identified by Sanger sequencing.

Germline transformation

Transgenic lines were generated using the standard germline transformation procedure (Mello *et al.*, 1991). All DNA was injected at a total concentration of 100 ng/µl, with each individual component at 10 ng/µl and total concentration brought up to 100 ng/µl by addition of 1 kb plus DNA ladder (Invitrogen) at a stock concentration of 1 µg/µl. *nIs861* was a spontaneous integration in a germline cell of an animal injected with pDD111 at 10 ng/µl and 1 kb DNA ladder at 90 ng/µl, and was identified by the 100% transmission of transgene from transformed parent to progeny.

RNAi treatments and Genome-scale RNAi screen

Previously described feeding RNAi constructs and reagents were used to perform RNAi feeding experiments (Fraser *et al.*, 2000; Rual *et al.*, 2004). Briefly, HT115 *Escherichia coli* bacteria carrying RNAi clones in the pL4440 vector were grown for at least 12 hrs in Luria broth (LB) liquid media with 75 mg/L ampicillin

at 37°C. These cultures were seeded onto 6 cm Petri plates with nematode growth medium (NGM) containing 1 mM IPTG and 75 mg/L ampicillin and incubated for 24 hrs at 22°C. For imaging experiments using confocal microscopy, 10 L4 animals were added to each RNAi plate and imaging of progeny embryos was performed on the next day as described in the Microscopy section. For excretory cell counts, five L4 animals were added to each RNAi plate and L3-L4 progeny were scored for number of excretory cells, as described in the Excretory Cell Count section. In case a bacterial clone targeting a certain gene was not available in previously constructed libraries (Kamath *et al.*, 2003; Rual *et al.*, 2004), we generated our own RNAi clone as described in the Molecular Biology section. The ORFeome RNAi library bacterial clone annotated to target *gmpr-1* (Plate 11069 Well F11) was sequenced and found to not target *gmpr-1* and therefore was not identified from our genome-wide RNAi screen.

The ORFeome RNAi library was used for to conduct a genome-wide RNAi screen (Rual *et al.*, 2004). For each day of the RNAi screen, all bacterial colonies from two 96-well plates were cultured for at least 12 hrs at 37°C in LB with 75 mg/L ampicillin. These cultures were then pre-incubated with 1 mM IPTG for 1 hr to maximize induction of dsRNA production. 24-well plates with each well containing 2 ml NGM medium with 1 mM IPTG and 75 mg/L ampicillin were prepared in advance and stored at 4°C until needed; they were brought to room temperature a few hrs before seeding. Each bacterial colony culture was then seeded onto an individual well of a 24-well plate and incubated for 24 hrs at 20°C. Three L4 animals were added to each well and their progeny were

screened 3 days later. Each set of RNAi clones screened also included a *pig-1* RNAi positive control and an empty pL4440 vector negative control. Excretory cell counts were performed as described in the Excretory Cell Counts section.

Microscopy

All RNAi and EMS mutagenesis screens scoring excretory cells were performed using a Nikon SMZ18 fluorescent dissecting microscope. DIC and epifluorescence images were obtained using a 63x objective lens (Zeiss) on an AxioImager Z2 (Zeiss) compound microscope and Zen Blue software (Zeiss). For confocal microscopy, embryos staged at the 200-300 cell stage were picked and mounted onto a glass slide (Corning) with a freshly-prepared 2% agarose pad. Embryos with ventral surfaces facing the objective were selected for imaging. Confocal images were obtained similarly using a 63x objective lens (Zeiss) on a Zeiss LSM800 confocal microscope. Images were processed with Fiji software (NIH), Photoshop CC 2019 (Adobe) and Illustrator CC 2019 (Adobe) software.

Excretory cell counts

Excretory cell counts were performed using a dissecting microscope equipped with fluorescence at a total magnification of 270x. For the genome-scale RNAi screen, roughly 50 animals were examined in each well of a 24-well plate and any well with more than 5 animals with two excretory cells was marked for confirmatory testing. Excretory cell counts in confirmatory RNAi experiments, candidate RNAi experiments and experiments with genetic mutants were conducted using 6 cm Petri plates with appropriate media. Animals were first immobilized by keeping the Petri plates on ice for 30 min. At least 100 animals at

the L3-L4 larval stage were scored for each genotype or RNAi experiment unless there was extensive lethality or growth defect, in which case a lower number or earlier stage animals, respectively, were scored. A cell was scored as an excretory cell if it was located in the anterior half of the animal and its nucleus had strong expression of GFP.

tDHB-GFP fluorescence intensity quantification

ABplpappap nuclear boundary was determined by Nomarski optics and cell membrane boundary was determined by membrane-localized mCherry expressed from the transgene *nIs861*. Mean tDHB-GFP fluorescence intensities inside the nuclear region, entire cell and background were quantified using Fiji software. Mean cytoplasmic tDHB-GFP fluorescence intensity was calculated by the following formula

$$I_{cytoplasm} = \frac{(I_{cell} * cell\ area) - (I_{nucleus} * nucleus\ area)}{cell\ area - nucleus\ area}$$

$I_{cytoplasm}$, I_{cell} and $I_{nucleus}$ denote the mean tDHB fluorescence intensity in the cytoplasm, cell and nucleus, respectively. The ratio of nuclear to cytoplasmic tDHB fluorescence intensity in Figure 3H was adjusted for background fluorescence (measured from a random area outside the embryo boundaries), i.e., the background fluorescence intensity was subtracted from both nuclear and cytoplasmic fluorescence intensity values before calculating the ratios.

Calculation of cell size

Confocal micrographs were obtained for multiple focal planes starting at the ventral surface and ending at the dorsal surface of the embryo, with each plane

separated by a distance of 0.37 μm. The greatest area occupied by a cell in any plane was designated the "maximum area" of a cell.

Statistical analysis

For calculation of statistical significance for ratios, the ratios were first transformed to logarithm values. Two-tailed T-tests were performed on the log-transformed ratios assuming normal distribution of these values. Mann-Whitney test was used to determine statistical significance for extrusion under different chemical treatments.

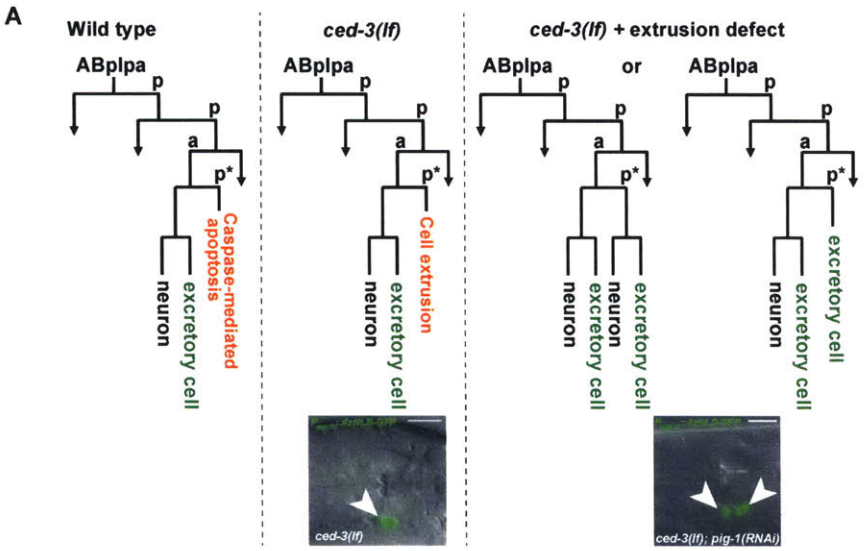
Figure 1 A Genome-scale RNAi screen for genes required for cell extrusion identifies multiple cell-cycle genes

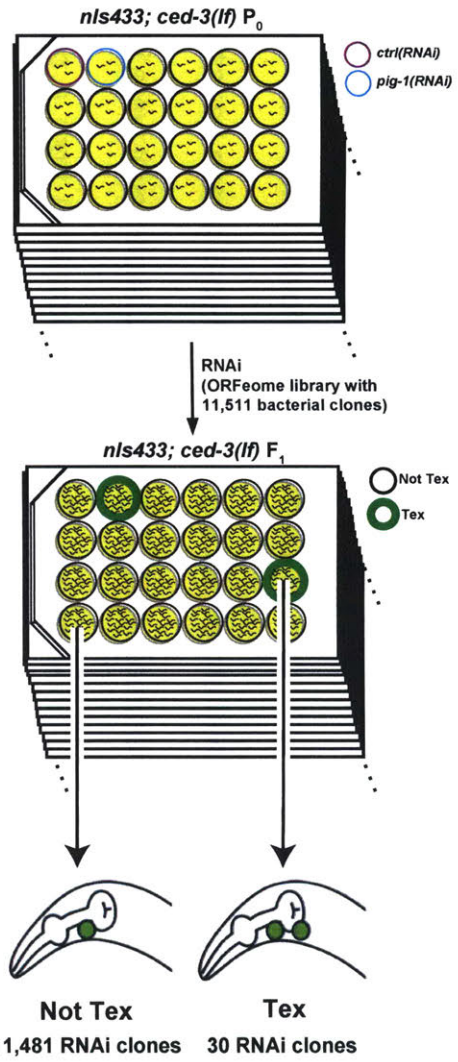
(A) Lineage diagrams show the fate of the cell ABplpappap (marked with an *) in embryos that are wild-type, embryos with a *ced-3(lf)* mutation and embryos with a *ced-3(lf)* mutation and a defect in cell extrusion. ABplpappap is eliminated by caspase-mediated apoptosis in wild-type embryos and by cell extrusion in *ced-3(lf)* embryos. ABplpappap survives in *ced-3(lf)* embryos with extrusion defects and generates an extra excretory cell, giving rise to the Two Excretory cell (Tex) phenotype. A micrograph of the pharyngeal region including the excretory cell(s), which express nuclear GFP and are marked with white arrowhead(s), is shown below the cell-lineage diagrams for representative animal of *ced-3(lf)* genotype with one excretory cell and a representative animal of *ced-3(lf); pig-1(RNAi)* genotype with two excretory cells. Scale bar, 10 μm.

(B) Schematic representation of the genome-wide RNAi screen for the Tex phenotype. Three L4 P_0s carrying the excretory-cell reporter *nIs433* were dropped into each well corresponding to a single RNAi clone in the ORFeome RNAi library, and 50 progeny were scored for the Tex phenotype. Each set of clones screened in one day contained one positive control (*pig-1* RNAi) and one negative control (pL4440 empty vector RNAi). This screen identified 30 clones representing 27 unique genes for which RNAi produced the Tex phenotype, while 11,481 clones did not produce a Tex phenotype.

(C) RNAi clone targets with a function in the cell cycle identified from the genome-scale or candidate-based RNAi screens for the Tex phenotype and the corresponding penetrance of the Tex phenotype. The mammalian homologs of these genes and their functions in mammals are shown. G1/S Role indicates whether an identified RNAi target has a role in G1, G1-to-S phase transition or S-phase progression in mammals. Genes marked by a ‡ superscript were identified from the genome-scale RNAi screen. Others were identified from a candidate RNAi screen of cell-cycle genes for the Tex phenotype (Supplemental Table 1).

Figure 1





C

Genes with known functions in mammalian cell cycle regulation that produce the Tex phenotype when inhibited

RNAi target gene	% Tex (n)	Mammalian homolog	G1/S Role	Role/Function
Control	1 (107)	-	-	-
cdc-25.2‡	33 (280)	CDC25	Yes	Removes inhibitory phosphorylations from CDK2 for G1/S transition and CDK1 for G2/M transition
cya-1‡	12 (363)	Cyclin A	Yes	Binds to CDK2 to regulate S-phase entry and progression and binds to CDK1 to regulate G2 and M phases
cye-1‡	87 (165)	Cyclin E	Yes	Binds to CDK2 to positively regulate transition from G1 to S phase of the cell cycle
cdk-1‡	15 (61)	CDK1	Yes	Master regulator of cell-cycle progression and mitosis
cdk-2	65 (181)	CDK2	Yes	Binds with Cyclins E and A to promote S-phase entry and progression
psf-1‡ *psf-2*‡ *psf-3*‡	36 (116) 39 (156) 50 (105)	GINS Complex	Yes	Complex involved in DNA replication origin firing and replication progression
lin-23	23 (147)	βTrCP	Yes	F-box protein that functions as a part of an SCF E3 ligase to promote G1/S phase transition
csn-1‡ *csn-4*‡ *csn-5*‡	43 (132) 47 (141) 39 (145)	COP9 Signalosome	Maybe	Required for SCF E3 ligase function
atl-1 *chk-1*	9 (444) 9 (220)	ATR Chk1	Yes	S-phase checkpoint, cell-cycle asynchrony

‡Genes identified from the genome-scale RNAi screen

Figure 2 Cell extrusion of ABplpappap requires the function of the cell-cycle genes *cye-1* and *cdk-2*

(A-C) Micrographs of the ventral surface obtained at 10-min intervals over a period of 50 min using time-lapse confocal microscopy show that ABplpappap is progressively extruded from (A) *ced-3(lf)* embryos with RNAi against control, but is not extruded from (B) *ced-3(lf)* embryos with RNAi against *cye-1*, or (C) *ced-3(lf)* embryos with RNAi against *cdk-2*. ABplpappap is marked by an arrowhead. The embryos shown carried the transgenes *stIs10026[his-72::GFP]*, which expresses GFP in the nuclei, and *nIs632[P_{egl-1}::mCherry::PH::unc-54 3'UTR, dlg-1::GFP, unc-76(+)]*, which expresses membrane-localized mCherry in cells that normally express the pro-apoptotic gene *egl-1*. *nIs632* expresses membrane-localized mCherry in ABplpappap and assists in locating this cell on the ventral surface of the embryo. *dlg-1::GFP* is not expressed from *nIs632*. Scale bar, 10 μm.

(D-F) Virtual lateral sections through the ABplpappap cell in *ced-3(lf)* embryos show that ABplpappap is extruded in embryos with RNAi against (D) control, but is not extruded in embryos with RNAi against (E) *cye-1* or (F) *cdk-2*. ABplpappap is marked with an arrowhead in each image. The embryos shown carried the transgenes *stIs10026* and *nIs632*. Scale bar, 10 μm.

Figure 2

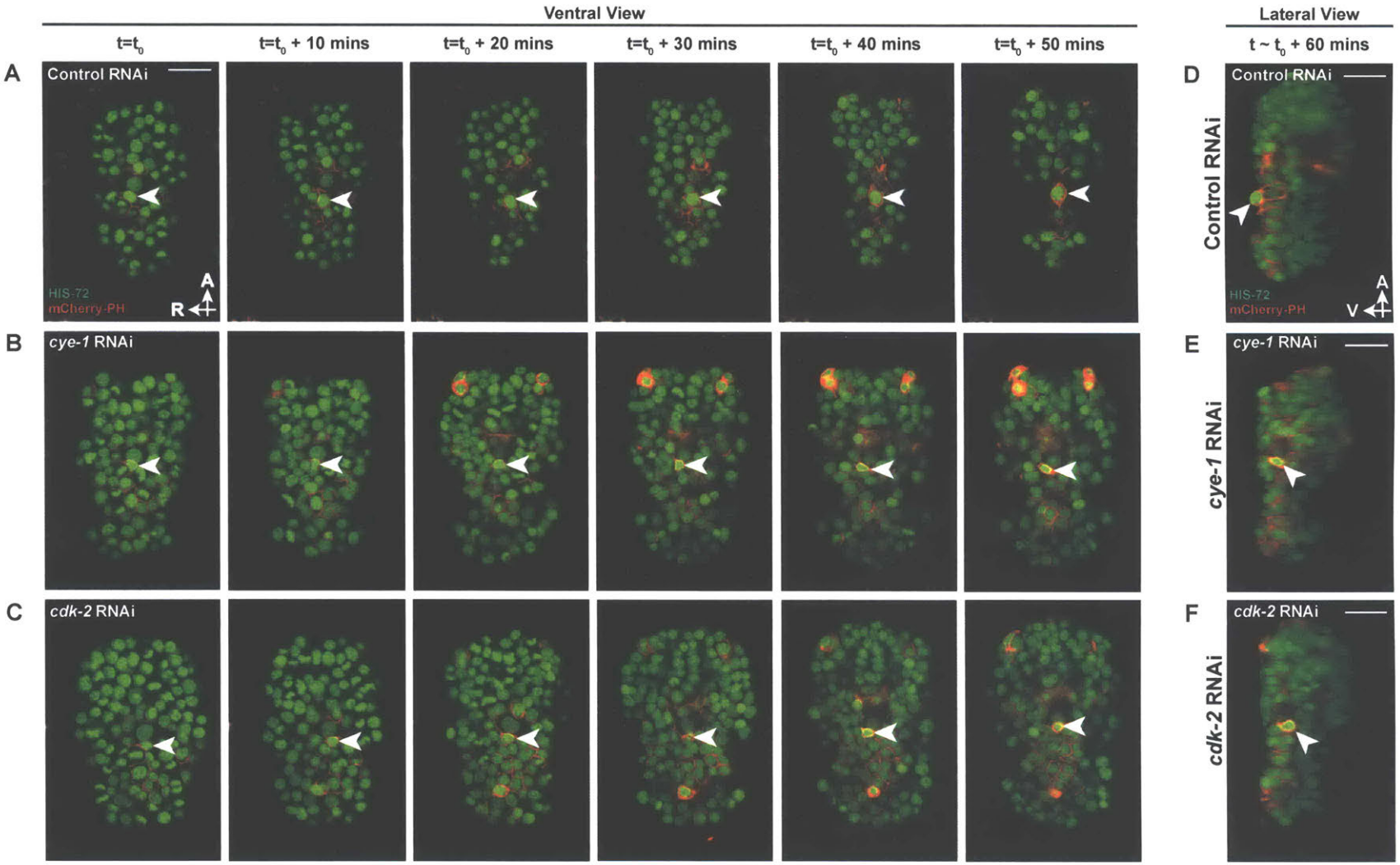

Ventral View
Lateral View
t=t0
t=t0 + 10 mins
t=t0 + 20 mins
t=t0 + 30 mins
t=t0 + 40 mins
t=t0 + 50 mins
t ~ t0 + 60 mins
A
Control RNAi
HIS-72
mCherry-PH
A
R
B
cye-1 RNAi
C
cdk-2 RNAi
D
Control RNAi
Control RNAi
HIS-72
mCherry-PH
A
V
E
cye-1 RNAi
cye-1 RNAi
F
cdk-2 RNAi
cdk-2 RNAi

Figure 3 Cells that are extruded enter the cell cycle in a *cye-1*- and *cdk-2* -dependent manner

(A) Schematic showing that truncated DHB (tDHB) – GFP fusion protein is localized to the nucleus in quiescent/post-mitotic cells and is depleted from the nucleus in cells that are between S phase and mitosis in the cell cycle (van Rijnberk *et al.*, 2017).

(B-D) Micrographs of *ced-3(lf)* embryos expressing tDHB-GFP obtained prior to ventral enclosure using confocal microscopy show nuclear depletion of tDHB-GFP in ABplpappap (marked by an arrowhead) in embryos with (B) RNAi against control, and nuclear enrichment in ABplpappap (marked by an arrowhead) in embryos with RNAi against (C) *cye-1*, or (D) *cdk-2*, indicating that ABplpappap entered the cell cycle in a *cye-1*- and *cdk-2*-dependent manner. The inset in each image shows a magnified view of the ABplpappap cell. The ABplpappap nucleus, as determined by Nomarski optics, is marked by a dotted line in each image. The embryos shown carried the transgenes *heSi192[*$P_{eft\text{-}3}$*::tDHB::GFP::tbb-2 3'UTR]*, which expresses a fusion of GFP with a truncated DHB protein (tDHB), and *nIs861[*$P_{egl\text{-}1}$*::mCherry::PH::unc-54 3'UTR]*, which expresses membrane-localized mCherry in cells that normally express the pro-apoptotic gene *egl-1*. *nIs861* expresses membrane-localized mCherry in ABplpappap and assists in locating this cell on the ventral surface of the embryo. Scale bar, 10 µm.

(E-G) Micrographs of *ced-3(lf)* embryos expressing tDHB-GFP obtained post ventral enclosure using confocal microscopy show an extruded ABplpappap

(marked by an arrowhead) with nuclear depletion of tDHB-GFP from an embryo with (E) RNAi against control, but both ABplpappap survival (marked by an arrowhead) and nuclear localization of tDHB-GFP in embryos with RNAi against (F) *cye-1*, or (G) *cdk-2*. These micrographs suggest that ABplpappap required entry into the cell cycle for its cell extrusion from *ced-3(lf)* embryos and that ABplpappap is not able to complete the cell cycle during its extrusion, as it does not divide in this period. The insets show a magnified view of the ABplpappap cell. The embryos shown carried the transgenes *heSi192* and *nIs861*. Scale bar, 10 μm.

(H) Quantification of the ratio of tDHB-GFP fluorescence intensity in the nucleus to that in the cytoplasm in *ced-3(lf)* embryos expressing tDHB-GFP with RNAi against control, *cye-1*, or *cdk-2* confirms that *cye-1* and *cdk-2* are required for cell-cycle entry by ABplpappap (Figure 6B-G). n=10 embryos per RNAi background. $p<0.01$ (two-tailed T-test of log-transformed ratios) for each of the RNAi treatments against *cye-1* and *cdk-2* as compared to RNAi against control. n=10 embryos for each RNAi treatment.

(I-L) Micrograph of (I) a *ced-3(lf)* embryo expressing tDHB-GFP obtained at the comma stage using confocal microscopy shows nuclear depletion of tDHB-GFP from all (seven) extruded cells (white boxes), suggesting that entry into the cell cycle is associated with cell extrusion. Magnified views are provided for the cells extruded at (J) the anterior sensory depression, (K) the ventral pocket, and (L) the posterior tip of the embryo, with the corresponding regions outlined in (I). The

cell nucleus, as determined by Nomarski optics, is marked by a dotted line for each extruded cell in the magnified images (J), (K) and (L). The embryo shown carried the transgenes *heSi192* and *nIs861*. Scale bar, 10 µm.

Figure 3

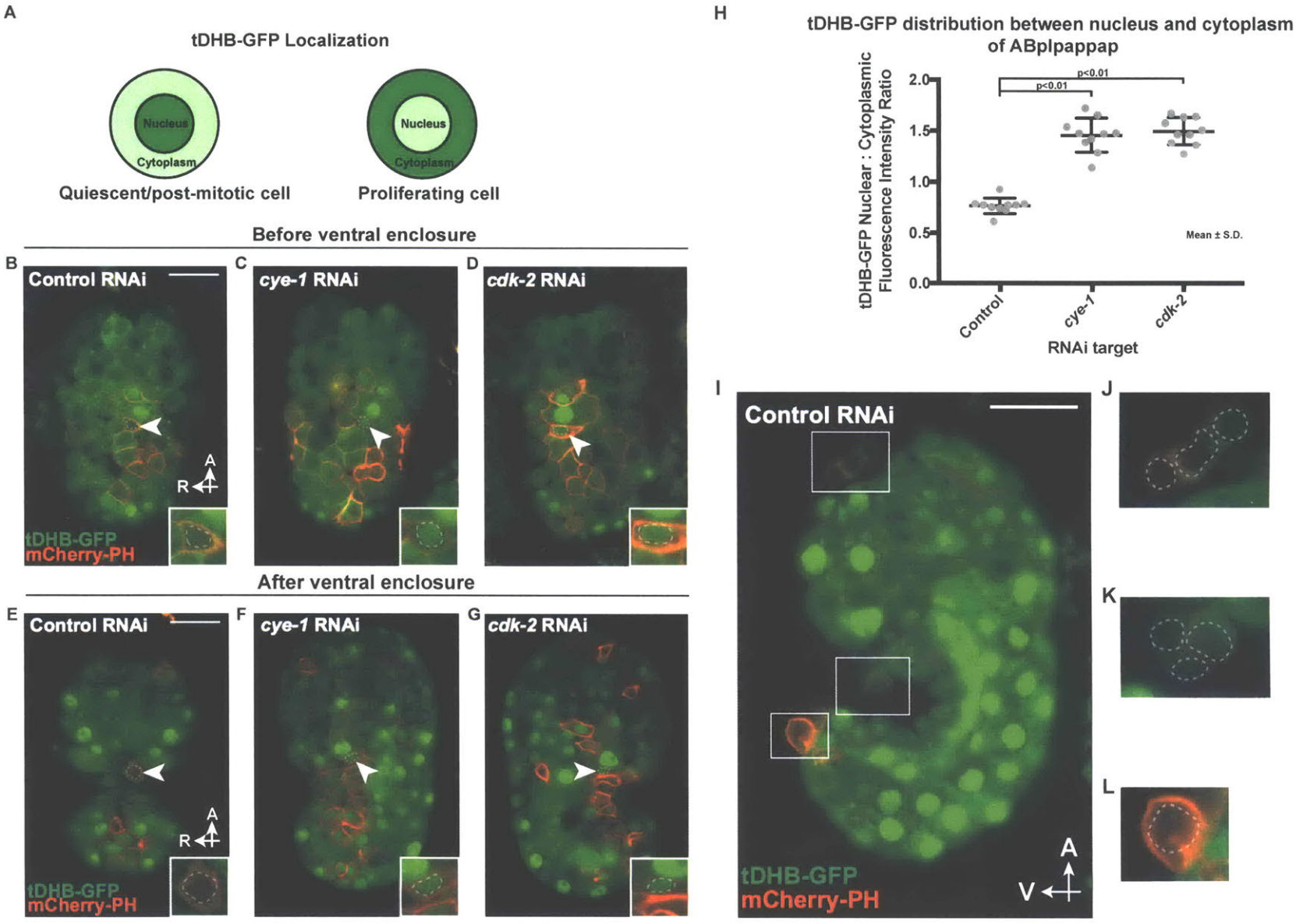

A
tDHB-GFP Localization
Nucleus
Cytoplasm
Quiescent/post-mitotic cell
Nucleus
Cytoplasm
Proliferating cell
Before ventral enclosure
B
Control RNAi
C
cye-1 RNAi
D
cdk-2 RNAi
A
R
tDHB-GFP
mCherry-PH
After ventral enclosure
E
Control RNAi
F
cye-1 RNAi
G
cdk-2 RNAi
H
tDHB-GFP distribution between nucleus and cytoplasm of ABplpappap
tDHB-GFP Nuclear : Cytoplasmic Fluorescence Intensity Ratio
p<0.01
p<0.01
2.0
1.5
1.0
0.5
0.0
Mean ± S.D.
Control
cye-1
cdk-2
RNAi target
I
Control RNAi
J
K
L
A
V
tDHB-GFP
mCherry-PH

Figure 4 Cells undergoing extrusion arrest in S-phase and trigger the S-phase checkpoint

(A) Representative micrographs showing localization patterns of GFP::PCN-1 in the same cell at different phases of the cell cycle or in a different cell in a post-mitotic state. The post-mitotic cell shown is ABplpappaaa (the future RMEV neuron). These cells were from a *ced-3(lf)* embryo, which carried the transgenes *nIs861* and *isIs17[*$P_{pie\text{-}1}$*::GFP::PCN-1]*. Scale bar, 2 μm.

(B-D) Micrographs of *ced-3(lf)* embryos expressing GFP::PCN-1 obtained prior to ventral enclosure using confocal microscopy show a GFP::PCN-1 localization pattern in ABplpappap (marked by an arrowhead) corresponding to S phase in embryos with RNAi against (B) control, and showing a pattern in ABplpappap (marked by an arrowhead) similar to that of a quiescent or post-mitotic cell in embryos with RNAi against (C) *cye-1* or (D) *cdk-2*. These micrographs indicate that ABplpappap enters S phase before ventral enclosure and that it requires *cye-1* and *cdk-2* to enter S phase. The inset in each image shows a magnified view of ABplpappap. The embryos shown carried the transgenes *nIs861* and *isIs17*. Scale bar, 10 μm.

(E-G) Micrographs of *ced-3(lf)* embryos expressing GFP::PCN-1 obtained after ventral enclosure using confocal microscopy show an extruded ABplpappap (marked by an arrowhead) with GFP::PCN-1 localization indicating S-phase in embryos with RNAi against (E) control, and both ABplpappap (marked by an arrowhead) survival and GFP::PCN-1 localization indicating quiescence in

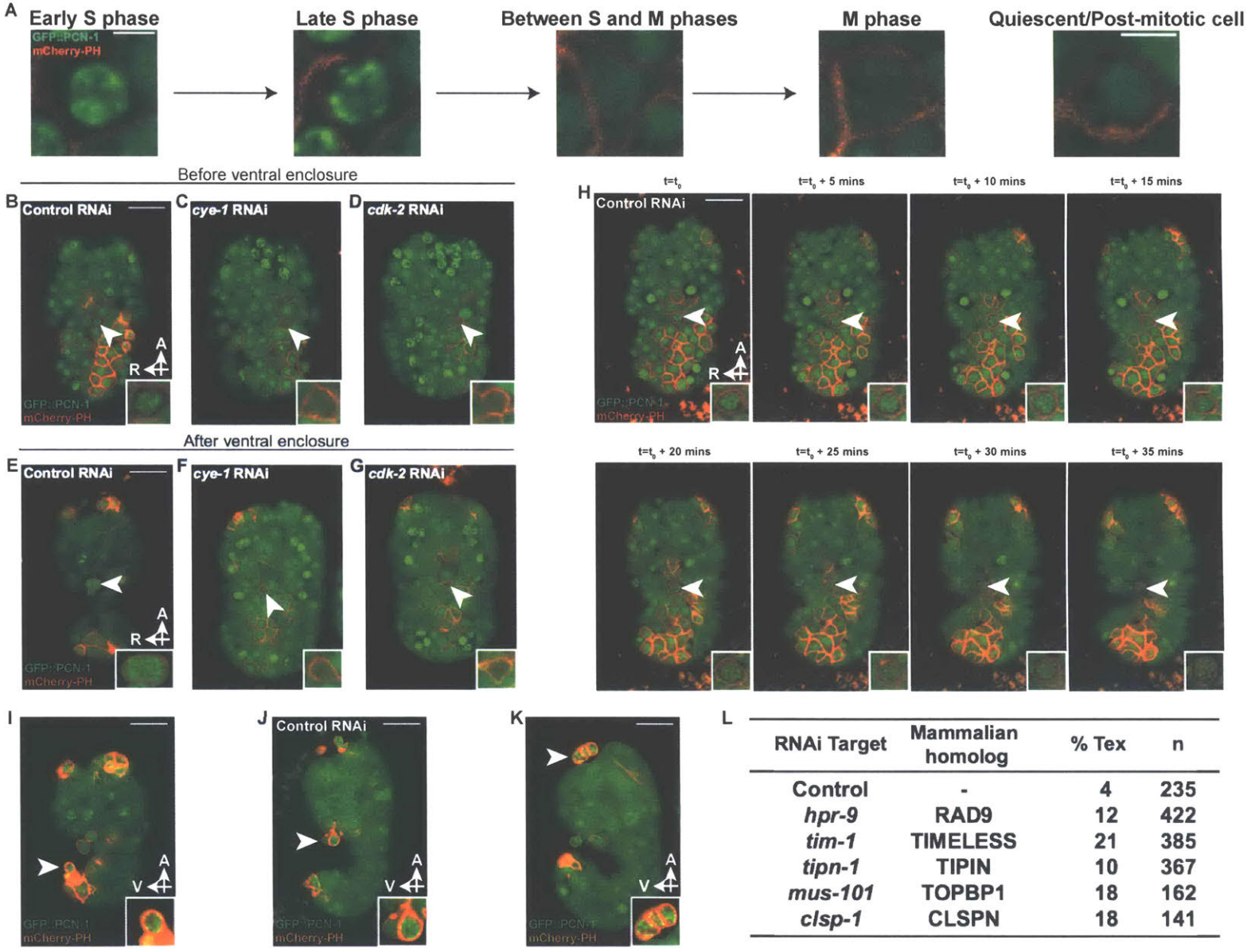
embryos with RNAi against (F) *cye-1* or (G) *cdk-2.* These micrographs indicate that S-phase is associated with ABplpappap cell extrusion. Insets show a magnified view of ABplpappap. The embryos shown carried the transgenes *isIs17* and *nIs861*. Scale bar, 10 µm.

(H) Micrographs obtained at 5-min intervals using time-lapse confocal microscopy show an unchanging GFP::PCN-1 localization pattern, indicating an S-phase arrest in ABplpappap (marked by an arrowhead) as it was progressively extruded over 35 min in a *ced-3(lf)* embryo expressing GFP::PCN-1 and with RNAi against control. The embryo shown carried the transgenes *isIs17* and *nIs861*. Insets show a magnified view of ABplpappap. Scale bar, 10 µm.

(I-K) Micrographs of extruded cells in *ced-3(lf)* embryos expressing GFP::PCN-1 obtained at the comma stage using confocal microscopy show GFP::PCN-1 localization corresponding to S phase in cells at (H) at the posterior tip of the embryo, (I) at the ventral pocket, and (J) at the anterior sensory depression. These micrographs suggest that extruded cells are mostly found in S phase and are likely in an arrested S phase. The inset in each image shows a magnified view of extruded cells marked by an arrowhead. The embryos shown carried the transgenes *isIs17* and *nIs861*. Scale bar, 10 µm.

(L) Penetrances of the Tex phenotype produced by RNAi-mediated targeting of replication-stress checkpoint genes *hpr-9*, *tim-1*, *tipn-1*, *mus-101* and *clsp-1* in *ced-3(lf)* animals. The strain used for scoring the RNAi-induced Tex phenotype carried the transgene *nIs433*, which expresses nuclear GFP in excretory cell(s).

Figure 4



L

RNAi Target	Mammalian homolog	% Tex	n
Control	-	4	235
hpr-9	RAD9	12	422
tim-1	TIMELESS	21	385
tipn-1	TIPIN	10	367
mus-101	TOPBP1	18	162
clsp-1	CLSPN	18	141

Figure 5 *pig-1*, *grp-1* and *cnt-2* are required for the unequal cell division that generates ABplpappap, its S-phase arrest and its extrusion.

(A) Penetrances of the Tex phenotype produced by RNAi-mediated targeting of genes *pig-1*, *grp-1* and *cnt-2* in *ced-3(lf)* animals, which are required for the unequal division of mothers that generate an apoptotic daughter in the Q neuroblast and other cell lineages. The RNAi-treated strain carried the transgene *nIs433*, which expresses GFP in the nucleus of excretory cell(s).

(B-E) Micrographs of *ced-3(lf)* embryos expressing nuclear GFP and membrane-bound mCherry obtained using confocal microscopy showing the relative sizes of ABplpappap (†) and its sister, ABplpappaa (*) in embryos with RNAi against (B) control, (C) *pig-1*, (D) *grp-1* and (E) *cnt-2*. All embryos shown carried the transgenes *stIs10026* and *ltIs44[$P_{pie\text{-}1}$::mCherry::PH]*, which expresses mCherry that localizes to the membranes of all cells. Scale bar, 10 μm.

(F) Ratio of maximum area of ABplpappap to that of its sister shows an increase in *ced-3(lf)* embryos with RNAi against *pig-1*, *gpr-1* or *cnt-2* as compared to the ratio in *ced-3(lf)* embryos with RNAi against control. Error bars, standard deviation. $p<0.01$ for each of the RNAi treatments against *pig-1*, *grp-1* or *cnt-2* as compared to RNAi against control (two-tailed T-test of log-transformed values of ratios). n=5 embryos for each RNAi treatment.

(G) Quantification of the maximum area of ABplpappap shows that ABplpappap was reproducibly larger in *ced-3(lf)* embryos with RNAi against *pig-1*, *grp-1*, or *cnt-2* as compared to the maximum area of ABplpappap in *ced-3(lf)* embryos

with RNAi against control(B-E). Error bars, standard deviation. $p<0.01$ for each of the RNAi treatments against *pig-1*, *grp-1* or *cnt-2* as compared to RNAi against control (two-tailed T-test). n=5 embryos for each RNAi treatment.

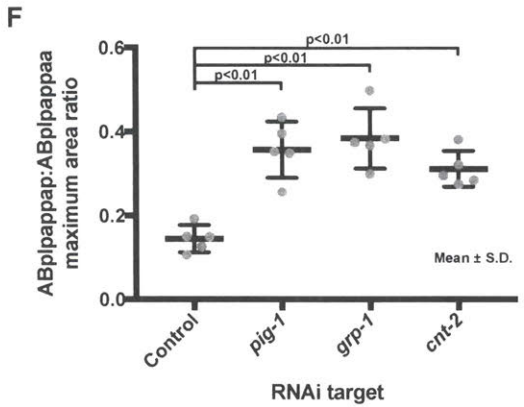
(H) Micrographs of *ced-3(lf)* embryos with RNAi against *pig-1* expressing HIS-72::GFP in all cells and mCherry-PH in *egl-1*-expressing cells (including ABplpappap) obtained at 10-min intervals over a period of 50 min using time-lapse confocal microscopy show that an enlarged ABplpappap divides before ventral enclosure in embryos with RNAi against *pig-1*. The embryo shown carried the transgenes *stIs10026* and *nIs861*. The insets show a magnified view of ABplpappap or its descendants. Scale bar, 10 μm.

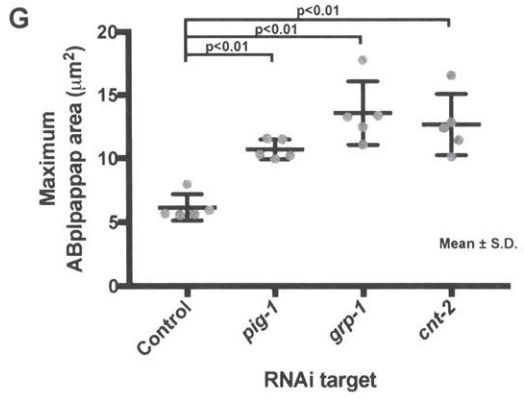
(I-L) Virtual lateral sections of *ced-3(lf)* embryos intersecting (I) ABplpappap in an embryo with RNAi against control, and intersecting (J-L) ABplpappap daughters in embryos with RNAi against (J) *pig-1*, (K) *grp-1*, and (L) *cnt-2* show that ABplpappap is extruded in embryos with RNAi against control and that ABplpappap descendants are not extruded in embryos with RNAi against *pig-1*, *grp-1*, or *cnt-2*. The insets show a magnified view of ABplpappap or its descendants. The embryos shown carried the transgenes *stIs10026* and *nIs861*. Scale bar, 10 μm.

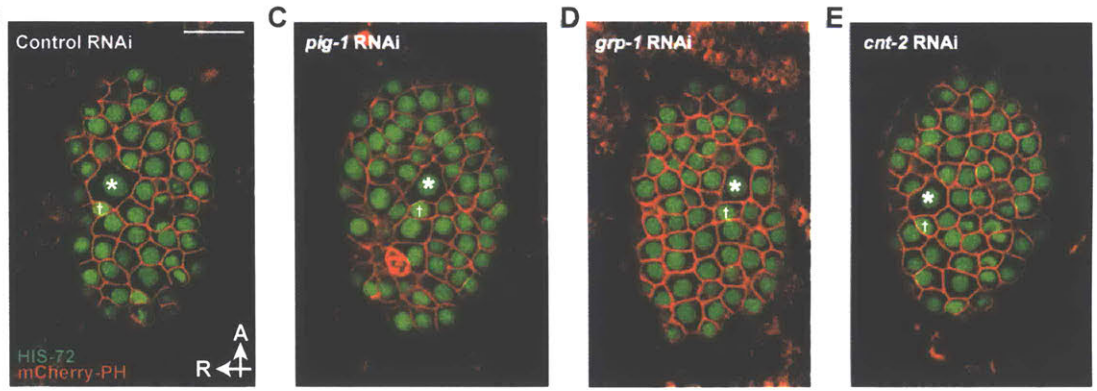
Figure 5

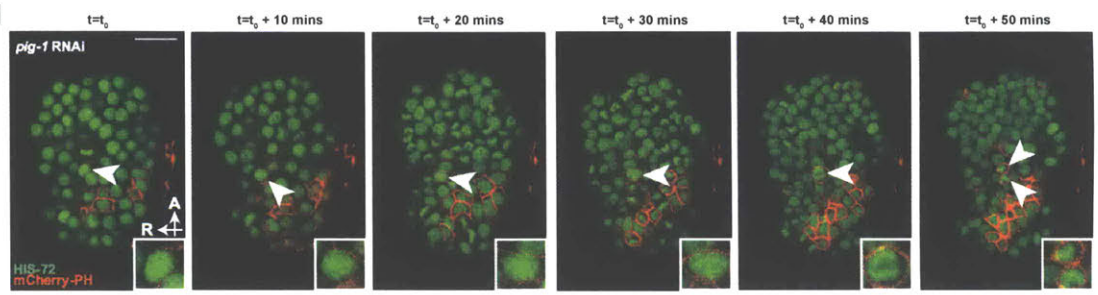
A

RNAi Target	% Tex	n
control	3	139
pig-1	72	126
grp-1	76	199
cnt-2	69	159









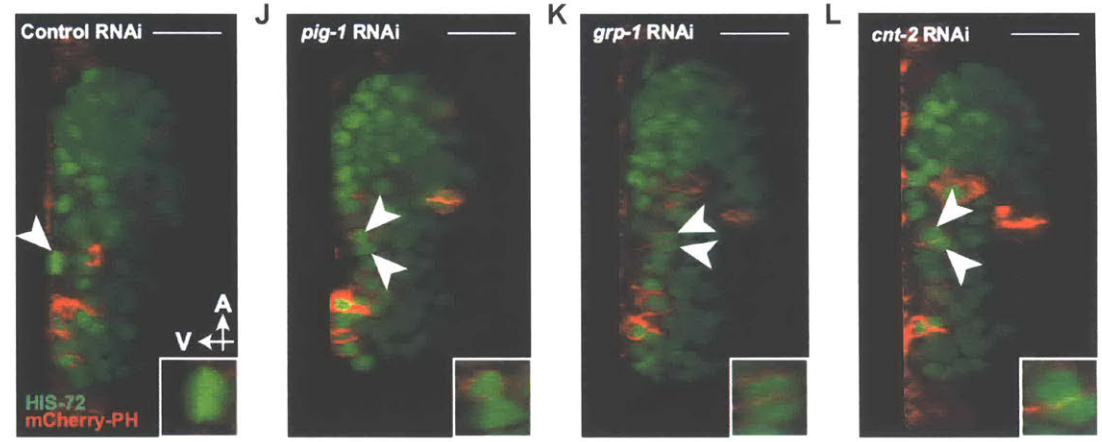


Figure 6 Hydroxyurea and GMP reductase promote cell extrusions from mammalian epithelia and *C. elegans* embryos, respectively

(A) Representative images from time-lapse videos of canine MDCK cell monolayers exposed to either vehicle control or 2 mM hydroxyurea for 22 hrs. Scale bar, 100 μm.

(B) Average number of cell extrusions per hr in vehicle-treated and 2 mM HU-treated MDCK monolayers. Each data point represents a separate experiment conducted for 16 to 22 hrs. Error bars, standard deviation.

$p < 0.01$ (Mann-Whitney test).

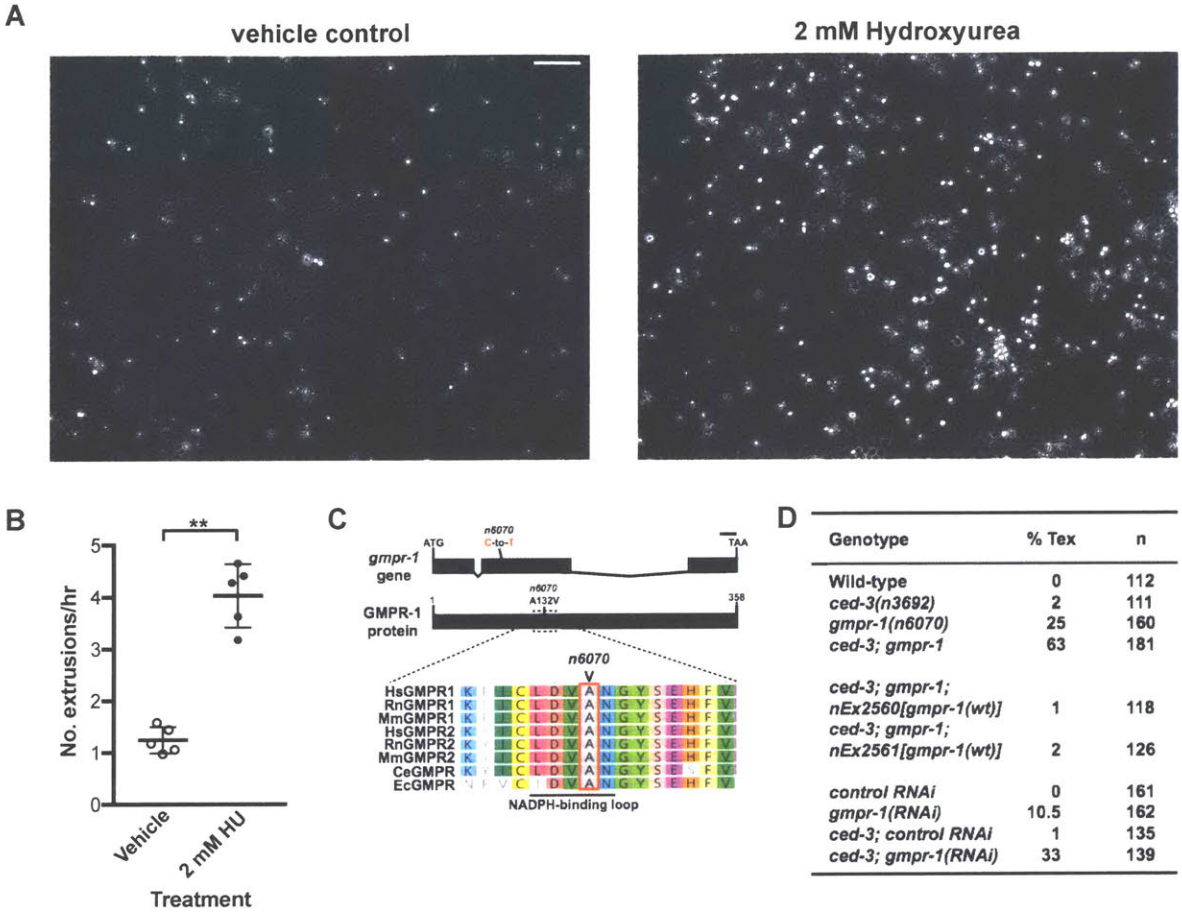
(C) (Top) *gmpr-1* genetic locus and (middle) GMPR-1 protein diagrams show the nature and location of the mutation in the *gmpr-1(n6070)* allele. Scale bar, 100 bases. (Bottom) Sequence alignment of GMP reductases from diverged organisms show a complete conservation among the given species of GMPR-1 alanine 132, which is in the NADPH binding loop. Hs – *Homo sapiens*, Rn – *Rattus norvegicus*, Mm – *Mus musculus*, Ce – *Caenorhabditis elegans*, Ec – *Escherichia coli*.

(D) Penetrances of the Tex phenotype produced by the *gmpr-1(n6070)* allele and RNAi targeting of *gmpr-1* in wild type and in *ced-3(lf)* strains. All strains carried the transgene *nIs433* or *nIs434[*$P_{pgp\text{-}12}$*::4xNLS-GFP]*, both of which express GFP in the nucleus of excretory cell(s).

(E) Micrographs of a *ced-3(lf)* embryo with RNAi against *gmpr-1* showing that the GFP::PCN-1 localization pattern in ABplpappap (marked by an arrowhead)

changes from that corresponding to early S phase before ventral enclosure to that corresponding to late S phase after ventral enclosure. Insets show a magnified view of ABplpappap. The embryos show carried the transgenes *isIs17* and *nIs861*. Scale bar, 10 μm.

Figure 6



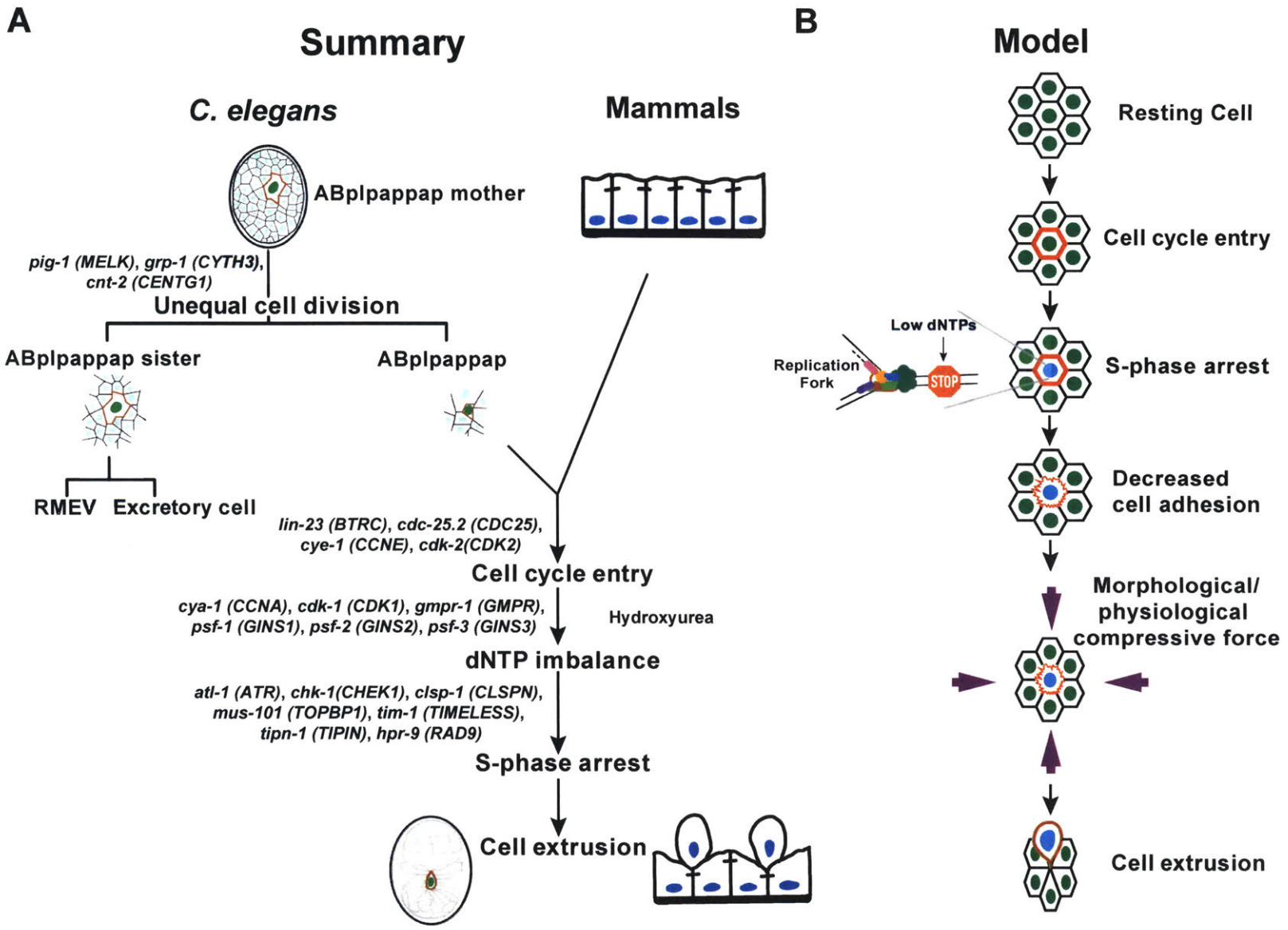
Genotype	% Tex	n
Wild-type	0	112
ced-3(n3692)	2	111
gmpr-1(n6070)	25	160
ced-3; gmpr-1	63	181
ced-3; gmpr-1; nEx2560[gmpr-1(wt)]	1	118
ced-3; gmpr-1; nEx2561[gmpr-1(wt)]	2	126
control RNAi	0	161
gmpr-1(RNAi)	10.5	162
ced-3; control RNAi	1	135
ced-3; gmpr-1(RNAi)	33	139

Figure 7 Summary and Model: S-phase arrest drives cell extrusion

(A) A summary of the genes required for cell extrusion by *C. elegans*, their mammalian homologs (in parentheses) and their associated biological processes that precede the cell extrusion process from *C. elegans* embryos is provided. We have shown that HU increases cell extrusion from mammalian cells. As HU functions by depleting nucleotides, HU likely promotes cell extrusion by the same or similar mechanisms that are required for cell extrusion from *C. elegans* embryos. Relevant cells in the *C. elegans* embryos are outlined in red. Nuclei are in green in *C. elegans* embryos and in blue in mammalian cells. The horizontal hyphen-like lines connecting mammalian cells indicate adhesion junctions.

(B) Model: A cell that enters the cell cycle (marked by red cell boundary) but arrests in S phase (marked by blue nucleus), say as a result of dNTP shortage, will have lowered cell adhesion (marked by wavy red cell boundary). When a cell with lowered cell adhesion caused by S-phase arrest experiences morphological or physiological forces (marked by purple arrows) that cause a squeezing-like effect, the cell gets extruded.

Figure 7


A
Summary
C. elegans
Mammals
ABplpappap mother
pig-1 (MELK), grp-1 (CYTH3),
cnt-2 (CENTG1)
Unequal cell division
ABplpappap sister
ABplpappap
RMEV
Excretory cell
lin-23 (BTRC), cdc-25.2 (CDC25),
cye-1 (CCNE), cdk-2(CDK2)
Cell cycle entry
cya-1 (CCNA), cdk-1 (CDK1), gmpr-1 (GMPR),
psf-1 (GINS1), psf-2 (GINS2), psf-3 (GINS3)
Hydroxyurea
dNTP imbalance
atl-1 (ATR), chk-1(CHEK1), clsp-1 (CLSPN),
mus-101 (TOPBP1), tim-1 (TIMELESS),
tipn-1 (TIPIN), hpr-9 (RAD9)
S-phase arrest
Cell extrusion
B
Model
Resting Cell
Cell cycle entry
Low dNTPs
Replication
Fork
STOP
S-phase arrest
Decreased
cell adhesion
Morphological/
physiological
compressive force
Cell extrusion

Supplemental Table 1 Penetrance of the Tex phenotype produced by RNAi against cell-cycle genes (and non cell-cycle cyclins and CDKs). Tex penetrance produced by each of the RNAi clones in the cell-cycle RNAi library, the number of animals counted for each RNAi clone and if the RNAi clone produced extensive lethality are provided. Genes corresponding to RNAi clones that produced more than 9% penetrance of the Tex phenotype are in bold. *cyb-3* was excluded from this criteria as extensive lethality prevented the counting of sufficient number of animals to assign significance. Some cyclins and CDKs that function outside the cell cycle were included in this library and served as negative controls.

Supplemental Table 1

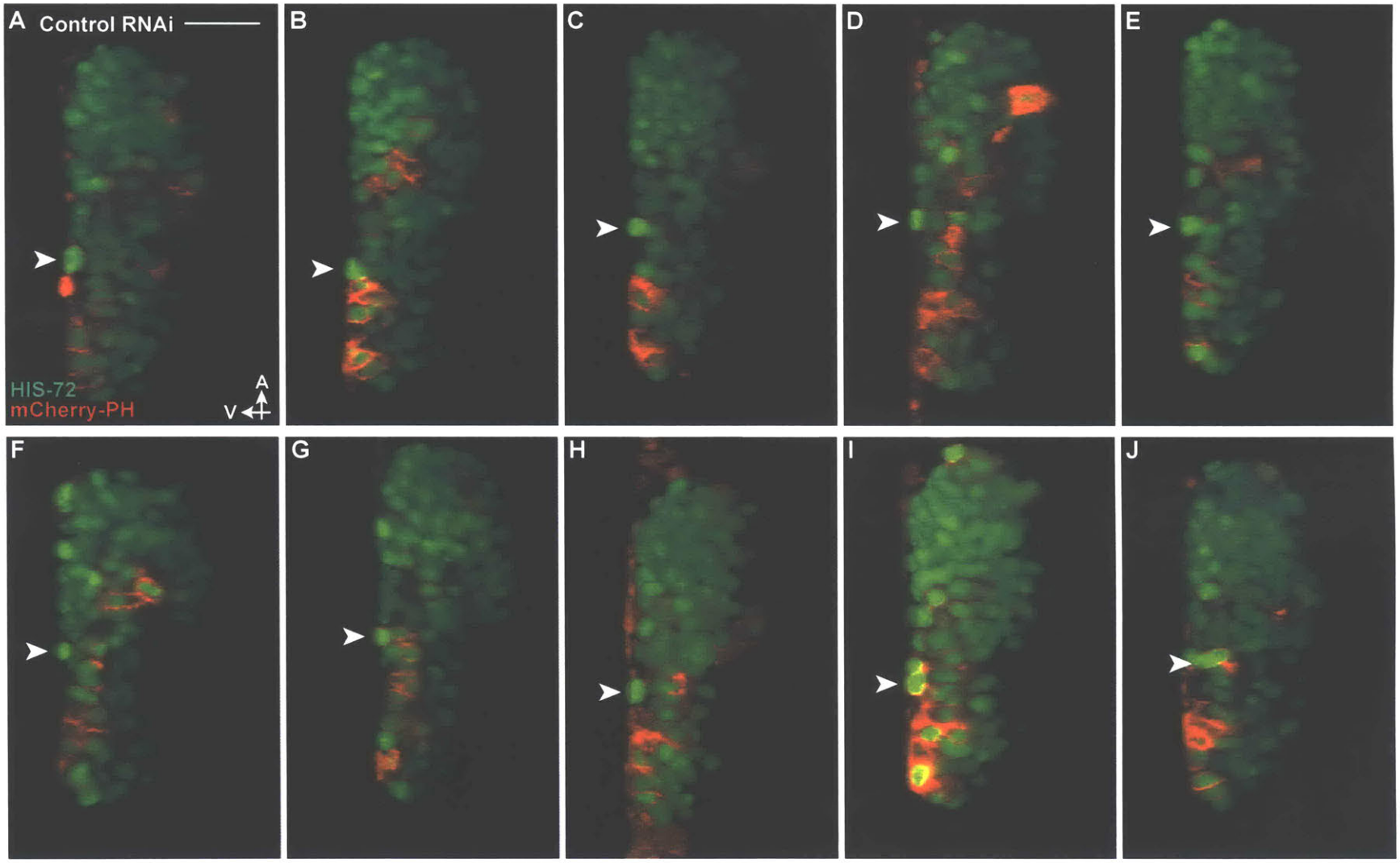
RNAi target	Mammalian Homolog	%Tex	n	extensive lethality?
control	-	1	159	N
atl-1	ATR	10	509	N
cdc-14	CDC14	1	168	N
cdc-25.1	CDC25	0	111	Y
cdc-25.2	CDC25	12	159	N
cdc-25.3	CDC25	2	168	N
cdc-25.4	CDC25	0	183	N
cdk-1	CDK1	15	61	Y
cdk-11.1	CDK11	0	175	N
cdk-11.2	CDK11	1	132	N
cdk-12	CDK12	0	167	N
cdk-2	CDK2	65	181	N
cdk-4	CDK4	1	193	N
cdk-5	CDK5	0	155	N
cdk-7	CDK7	0	130	Y
cdk-8	CDK8	1	186	N
cdk-9	CDK9	1	155	Y
cdt-1	CDT1	1	147	Y
chk-1	CHEK1	10	164	Y
cit-1.1	CCNT1/2	0	105	N
cit-1.2	CCNT1/2	1	244	N
cki-1	CDKN1	0	125	N
cki-2	CDKN1	1	216	N
clk-2	TELO2	1	156	N
cul-1	CUL1	5	81	Y
cul-2	CUL2	0	21	Y
cul-3	CUL3	5	151	Y
cul-4	CUL4	0	141	N
cya-1	CCNA	20	309	N
cyb-1	CCNB1	4	136	N
cyb-2.1	CCNB2	1	102	N
cyb-2.2	CCNB2	0	144	N
cyb-3	CCNB3	14	7	Y

RNAi target	Mammalian Homolog	%Tex	n	extensive lethality?
cic-1	CCNC	1	158	N
cyd-1	CCND	0	111	N
cye-1	CCNE	89	133	Y
cyh-1	CCNH	0	119	Y
cyl-1	CCNL	1	106	Y
cyy-1	CCNY	1	108	N
dpl-1	TFDP1	0	167	N
efl-1	E2F	0	141	N
emb-27	CDC16	0	107	Y
emb-30	ANAPC4	0	130	Y
fzr-1	FZR1	1	146	N
fzy-1	CDC20	0	130	Y
hpr-17	RAD17	5	214	N
hus-1	HUS1	0	132	N
lin-15	-	1	104	N
lin-23	βTrCP	23	147	N
lin-35	Rb	0	134	N
lin-36	-	0	111	N
lin-9	LIN9	1	125	N
mat-1	CDC27	0	80	Y
mat-2	ANAPC1	1	163	Y
mat-3	CDC23	0	108	Y
mdf-1	MAD1L1	3	112	N
mdf-2	MAD2L1	1	111	N
mrt-2	RAD1	0	114	N
rnr-1	RRM1	5	103	Y
san-1	BUB1B	1	145	N
wee-1.1	PKMYT1	1	159	N
wee-1.3	PKMYT1	0	60	Y

Supplemental Figure 1 ABplpappap is extruded by *ced-3(lf)* embryos with RNAi against control

(A-J) Virtual lateral sections of *ced-3(lf)* embryos through the ABplpappap cell (marked by an arrowhead) in 10 embryos (A-J) with RNAi against control show (A-I) ABplpappap extruded in 9 of 10 embryos and (J) not extruded in 1 of 10 embryos. The embryos shown carried the transgenes *stIs10026* and *nIs861*. Scale bar, 10 µm.

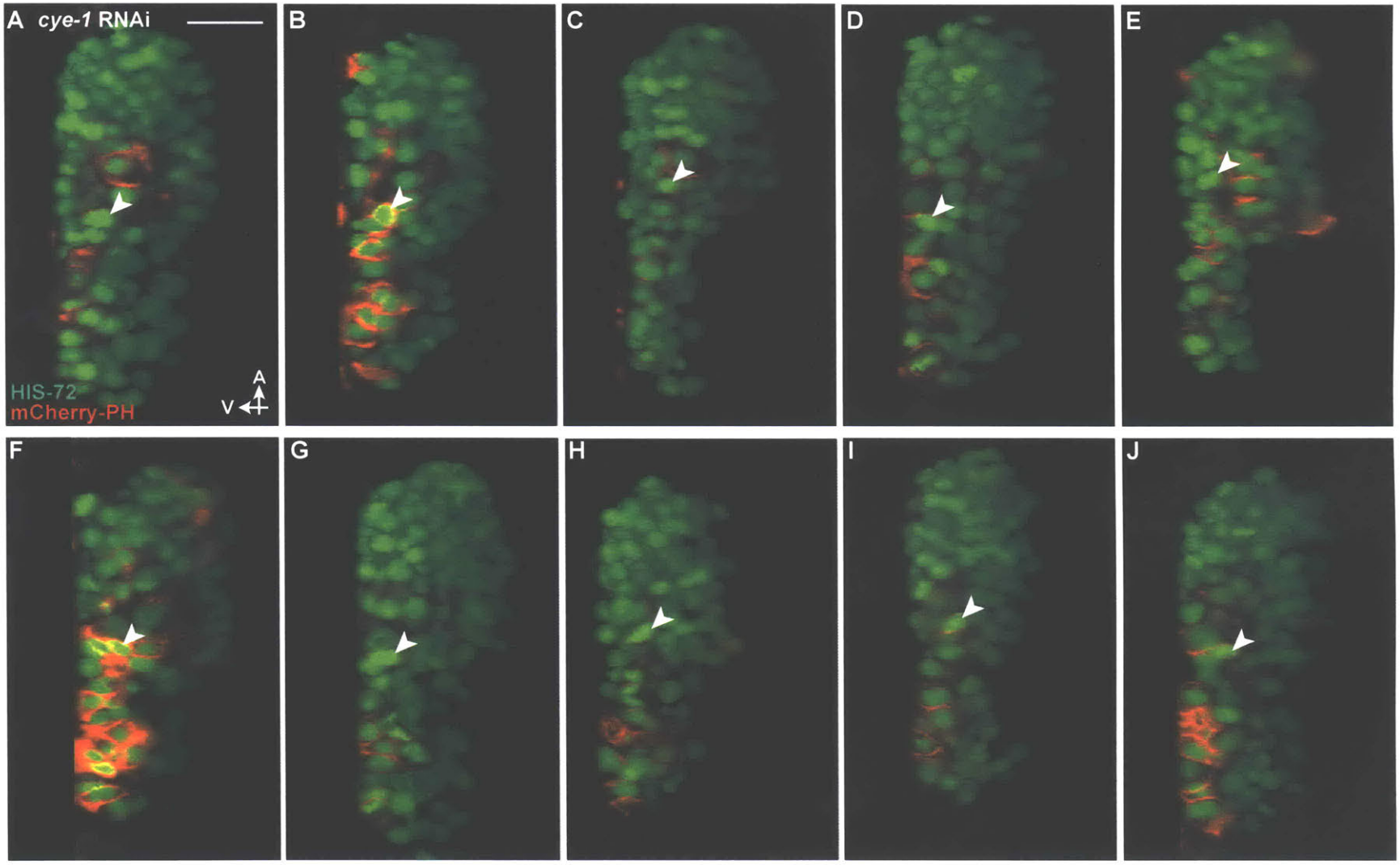
Supplemental Figure 1



Supplemental Figure 2 ABplpappap is extruded by *ced-3(lf)* embryos with RNAi against *cye-1*

(A-J) Virtual lateral sections of *ced-3(lf)* embryos through the ABplpappap cell (marked by an arrowhead) in 10 embryos (A-J) with RNAi against *cye-1* show that ABplpappap is not extruded in 10 of 10 embryos.. The embryos shown carried the transgenes *stIs10026* and *nIs861*. Scale bar, 10 μm.

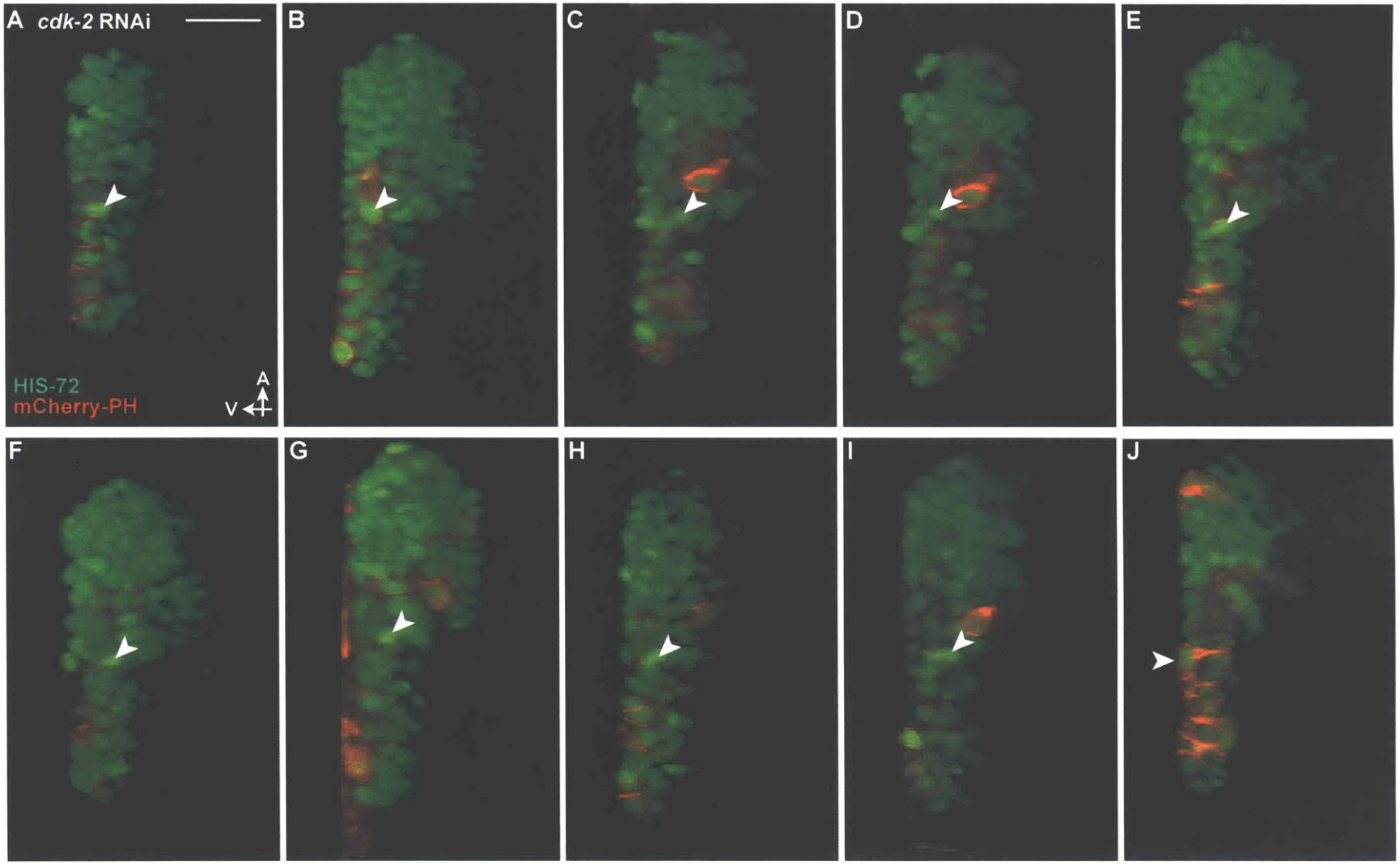
Supplemental Figure 2



Supplemental Figure 3 ABplpappap is extruded by *ced-3(lf)* embryos with RNAi against *cdk-2*

(A-J) Virtual lateral sections of *ced-3(lf)* embryos through the ABplpappap cell (marked by an arrowhead) in 10 embryos (A-J) with RNAi against *cdk-2* show that (A-I) ABplpappap is not extruded in 9 of 10 embryos and (J) is extruded in 1 of 10 embryos.. The embryos shown carried the transgenes *stIs10026* and *nIs861*. Scale bar, 10 μm.

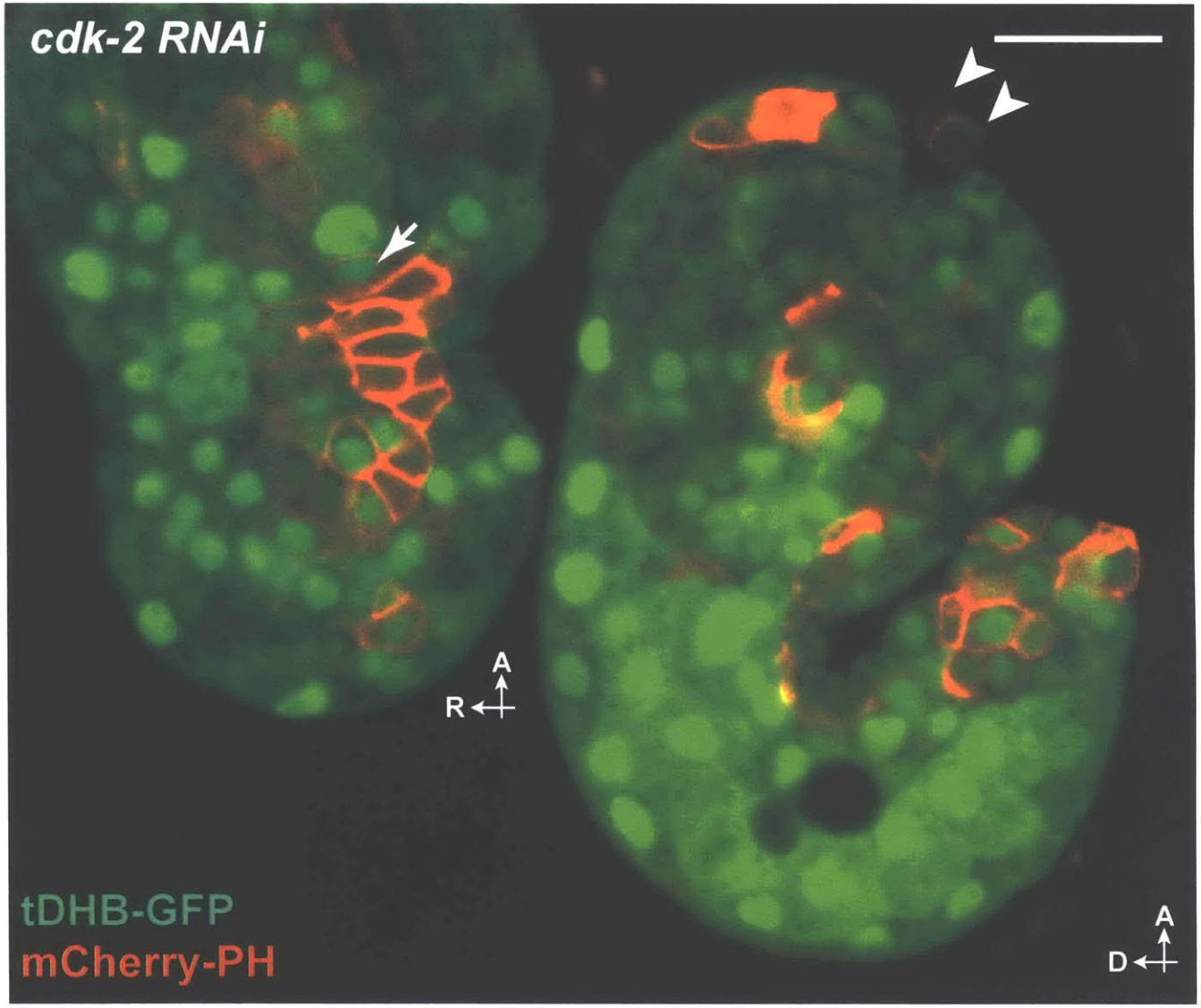
Supplemental Figure 3



Supplemental Figure 4 Cells extruded by *ced-3(lf)* embryos with RNAi against *cdk-2* enter the cell cycle

Micrograph of two *ced-3(lf)* embryos expressing tDHB-GFP and with RNAi against *cdk-2* shows a nuclear-enriched localization of tDHB-GFP in ABplpappap (marked by an arrow) that was not extruded by the first embryo but presumably nuclear-depleted tDHB-GFP in two cells that were extruded from the second embryo (marked by arrowheads). The embryos shown carried the transgenes *heSi192* and *nls861*. Scale bar, 10 μm.

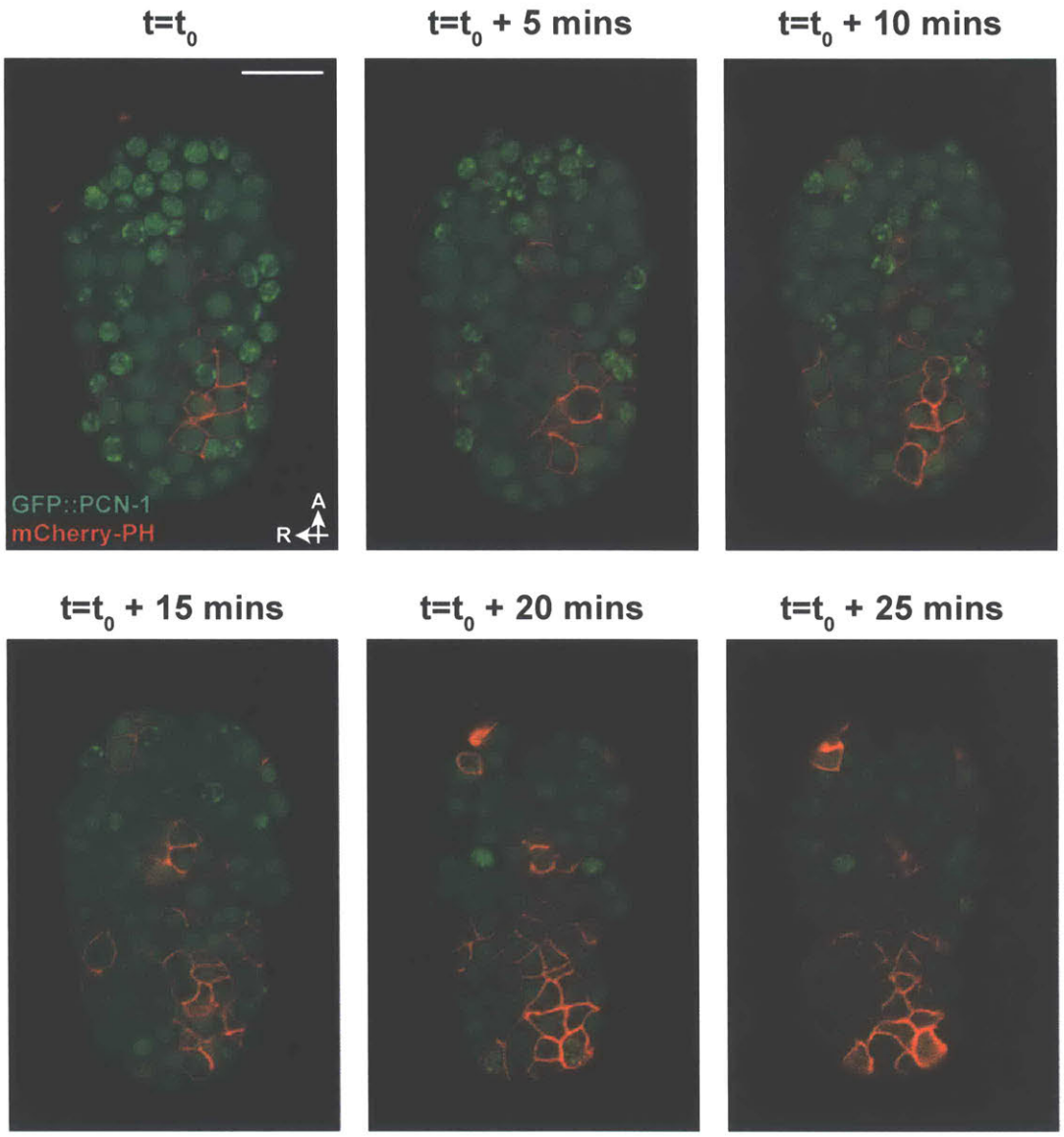
Supplemental Figure 4



Supplemental Figure 5 Cells on the ventral surface of *ced-3(lf)* embryos (other than ABplpappap) progress through and complete S phase

Time-lapse confocal micrographs of a *ced-3(lf)* embryo expressing GFP::PCN-1 obtained at 5-min intervals show GFP::PCN-1 localization reflective of continuous S-phase progression and completion by the cells on the ventral surface of this embryo. The embryo shown carried the transgenes *isIs17* and *nIs861*. Scale bar, 10 μm.

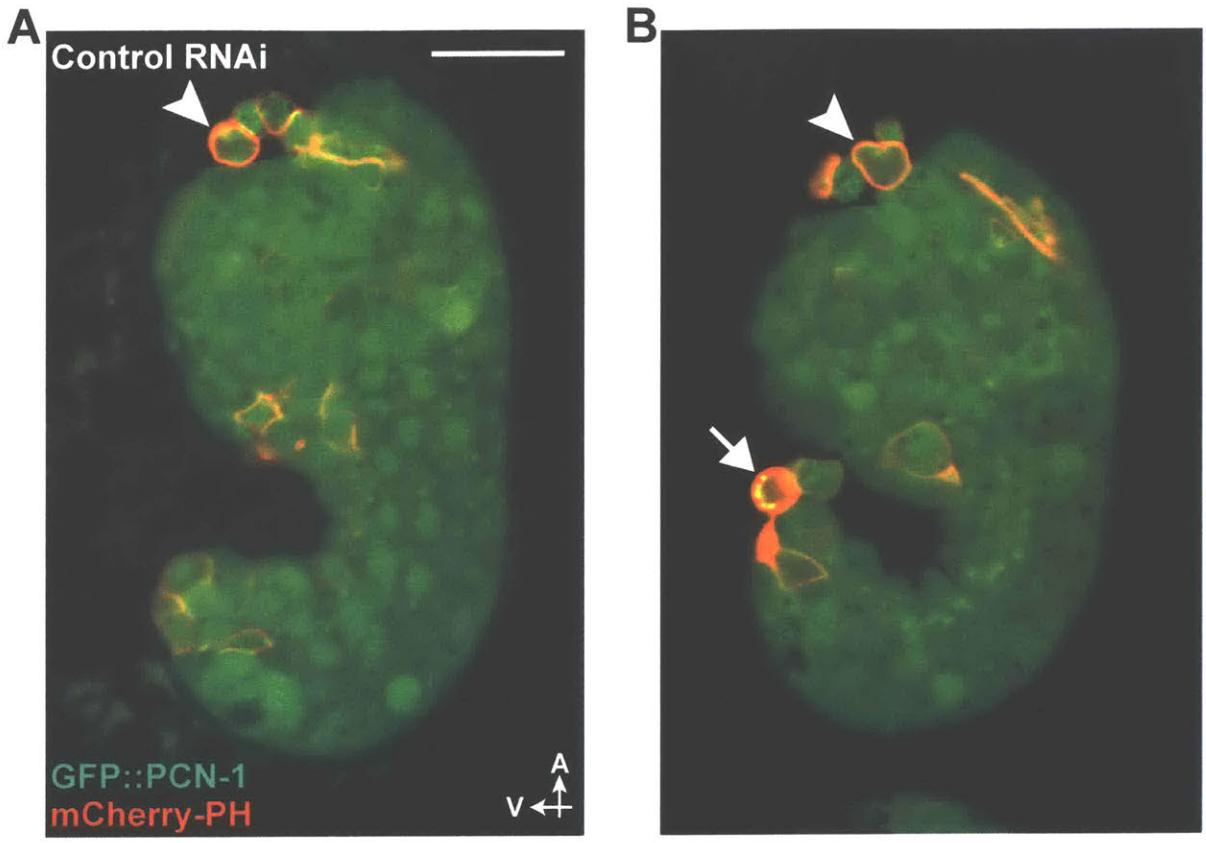
Supplemental Figure 5



Supplemental Figure 6 Some extruded cells can complete S phase after cell extrusion

(A,B) Micrographs of two comma-stage *ced-3(lf)* embryos (more than 1 hr after ventral enclosure) expressing GFP::PCN-1 with RNAi against control show cells with GFP::PCN-1 localization corresponding to M phase (marked by arrowheads; Figure 4A) or corresponding to a late S phase (marked by an arrow; Figure 4A). The embryos shown carried the transgenes *isIs17* and *nIs861*. Scale bar, 10 µm.

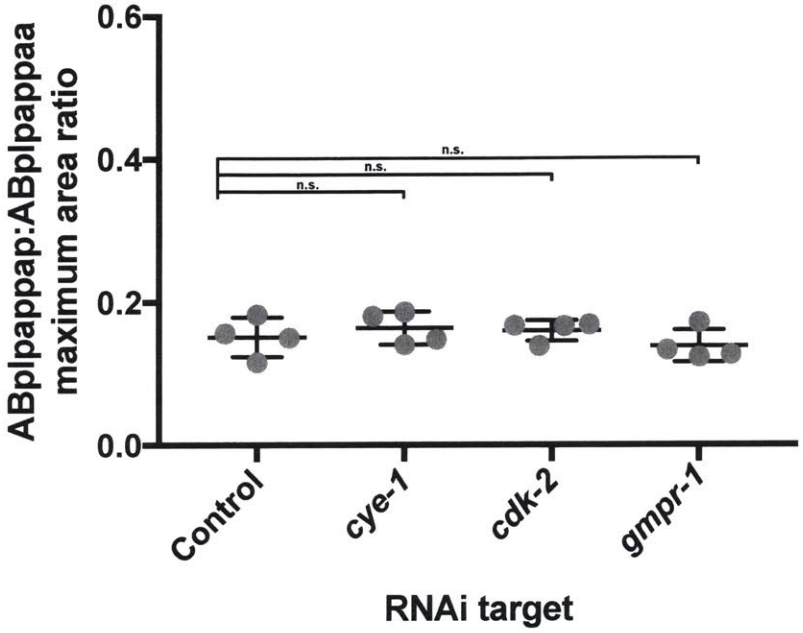
Supplemental Figure 6



Supplemental Figure 7 The unequal cell division of ABplpappap's mother is independent of *cye-1*, *cdk-2*, and *gmpr-1* gene function

Quantification of the ratio of ABplpappap maximum area to ABplpappaa maximum area in *ced-3(lf)* embryos with RNAi against control, *cye-1*, *cdk-2* or *gmpr-1* (5 embryos each) shows that these RNAi treatments did not affect the unequal cell division of ABplpappap's mother. n.s. – not significant. n=4 embryos each.

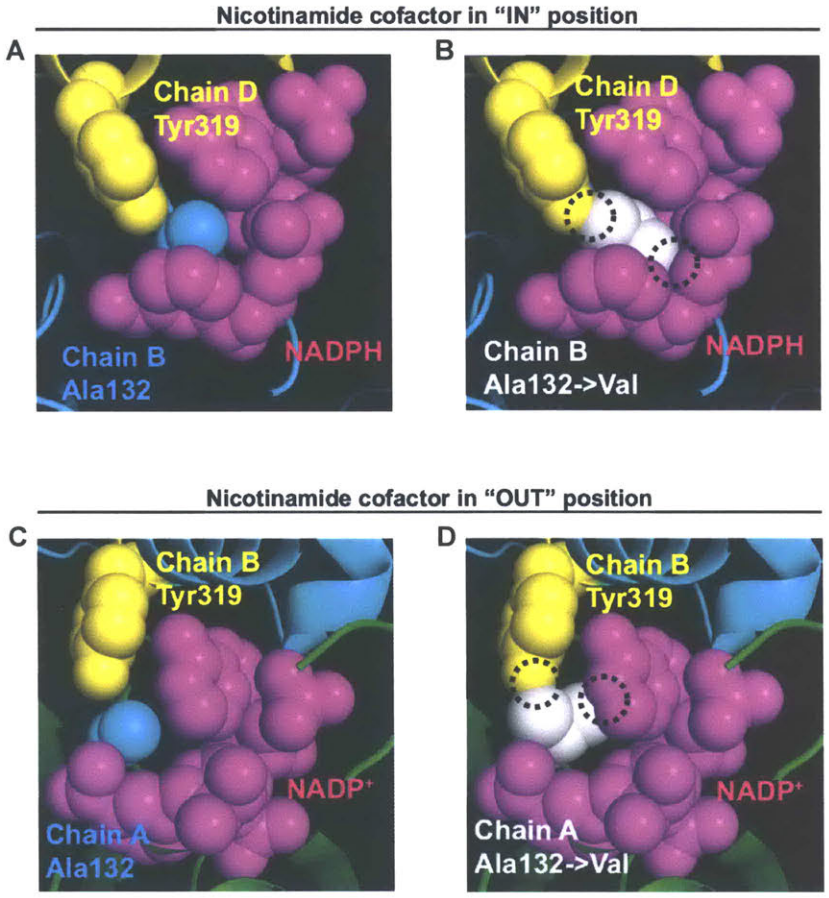
Supplemental Figure 7

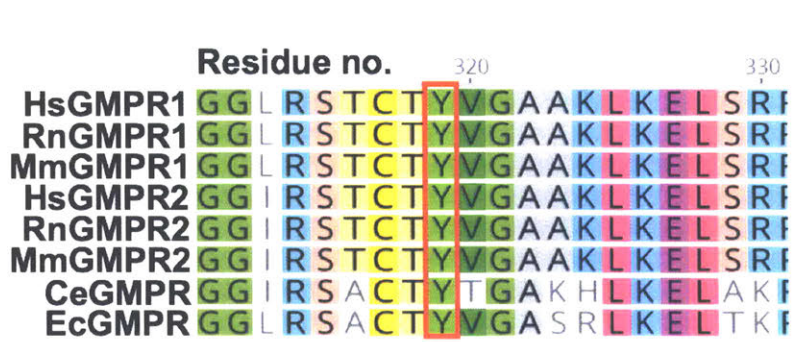


Supplemental Figure 8 The Ala132->Val mutation in GMPR-1 is predicted to disrupt GMPR-1 function

(A-D) Space-filling models of the conserved Ala132 (cyan) residue and Tyr319 (yellow) of an adjacent chain with the (A) "IN" and (C) "OUT" conformations of the NADPH/$NADP^+$ cofactor (magenta) as per the PDB model 2C6Q of the human GMPR2 enzyme (Patton *et al.*, 2011). The result of changing Ala132 to Val (grey) is shown for the (B) "IN" and (D) "OUT" conformations of the NADPH/$NADP^+$ cofactor (magenta). This change will likely introduce steric clashes (outlined in black dotted circles) with both the cofactor NADPH/$NADP^+$ and a Tyr319 amino acid residue of an adjacent chain. (E) Protein sequence alignment of shows a complete conservation of the Tyr319 residue in GMP reductase enzymes of different organisms. The Tyr319 is present on the interface of two adjacent GMP reductase protein chains and likely participates in maximal enzymatic function. Hs – *Homo sapiens*, Rn – *Rattus norvegicus*, Mm – *Mus musculus*, Ce – *Caenorhabditis elegans*, Ec – *Escherichia coli*.

Supplemental Figure 8





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Chapter 4

Future Directions

Identifying the regulators of caspase-independent apoptosis in extruded cells

Cells extruded from caspase-deficient *C. elegans* embryos display many hallmarks of apoptosis, including chromatin condensation, DNA fragmentation and phosphatidylserine exposure (Denning *et al.*, 2012). It is still an open question as to what genes drive apoptosis in these cells in the absence of caspases. Interestingly, some cells that are not extruded also undergo apoptosis in caspase-deficient *C. elegans* animals (Denning *et al.*, 2013). These two forms of caspase-independent apoptotic deaths might share the mechanism of apoptotic death. To identify this mechanism, an EMS mutagenesis screen could be performed for mutant embryos in which ABplpappap, a cell extruded in caspase-deficient embryos, shows signs of differentiation after extrusion. Dan Denning (Denning *et al.*, 2012) and I have shown that blocking ABplpappap extrusion leads to the generation of an extra excretory cell, suggesting that when it survives, it (or its descendant) differentiates into an excretory cell. Therefore, an EMS mutagenesis screen for embryos in which an extruded floating cell expresses a fluorescent excretory-cell reporter might identify genes that drive apoptosis in a caspase-independent fashion. The *nIs433[$P_{pgp\text{-}12}$ ::4xNLS::GFP]* transgene might not be suitable for this screen, as this reporter becomes visible only after hatching. Instead, the *syIs107[lin-3::GFP]* might be a better reporter for such a screen, as it starts to get expressed in the excretory cell after ventral enclosure (when extrusion occurs) and is expressed through the L1 larval stage (Abdus-Saboor *et al.*, 2011).

Determining cell autonomous vs. cell non-autonomous function of genes required for cell extrusion by *C. elegans*

Whether a gene functions cell autonomously or cell non-autonomously can be determined by using genetic mosaic analysis (reviewed by Yochem and Herman, 2005). A mosaic animal has cells of different genotypes. Genetic mosaics of *C. elegans* can be generated when a mutant for a certain gene carrying an extra-chromosomal array or a free duplication with a wild-type copy of the same gene spontaneously loses the array or free duplication in some but not all cells. Such loss occurs at an appreciable rate because of the unstable nature of extra-chromosomal arrays or free duplications during mitosis. Expressing a wild-type version of a gene in a specific tissue of a mutant for that gene can also be used to create genetic mosaics. If the mutant phenotype in a tissue depends only on the genotype of the same tissue, then the gene functions cell autonomously. If not, the gene functions at least partly cell non-autonomously.

Because my work has mainly utilized RNAi as a genetic tool, I have not had the opportunity to use mosaic analysis to determine whether cell-cycle genes function cell autonomously or cell non-autonomously for cell extrusion. I have, however, been able to show using cell-cycle reporters that the cell cycle is affected in the same cell (ABplpappap) that would otherwise be extruded but is not extruded in embryos with RNAi against cell cycle genes *cye-1* or *cdk-2*. This finding indicates a cell-autonomous function of the genes *cye-1* and *cdk-2* in cell extrusion. Mosaic analysis using a *cye-1* or *cdk-2* mutant background would

confirm this finding, with the expectation being that the Tex phenotype arising from a block in ABplpappap extrusion would occur only if the rescuing extra-chromosomal array were lost in its lineage. A potential difficulty with such experiments is that many proteins involved in cell-cycle regulation are maternally provided. Therefore, corresponding mutants go through embryonic development without a problem and either arrest as larvae or grow to be sterile adults. In some cases, I have observed maternal rescue by the rescuing transgene, i.e., there was no Tex phenotype in mutant progeny without the transgene from a homozygous mutant mother with the transgene. This rescue occurred in cases of *pig-1(gm344)* and *gmpr-1(n6070)* mutants that produce the Tex phenotype. There are still other cases where the transgene expressing the wild-type version of a gene produces partial rescue or no rescue of the phenotype. This occurred for *shc-2* and *C45G7.4*, respectively (Daniel Denning, unpublished data). Given these observations, my suspicion is that the list of genes for which mosaic analysis by spontaneous array loss can be performed for the Tex phenotype might be rather small.

Another option would be to perturb processes like the cell cycle specifically in cells fated to be extruded. The problem with this option is that no known promoter can specifically express a protein of choice in cells fated to be extruded before this fate is sealed. Even the *egl-1* promoter that I have used in the integrated transgene *nIs861[Pegl-1::mCherry::PH::unc-54 3'UTR]* to mark the extruded cell ABplpappap expresses mCherry::PH in a large number of other

cells, making it largely unsuitable for mosaic analysis. Therefore, promoters that specifically express in cells fated to be extruded are needed.

Recent technological developments allow transcriptomic sequencing of low-input samples and even single cells (reviewed by Adiconis *et al.*, 2013; Bhargava *et al.*, 2014). Such technological developments would facilitate the transcriptomic analysis of extruded cells, which might allow identification of genes that are specifically expressed in such cells. The promoters of such genes could then be used to perform cell-specific perturbation experiments currently not possible.

Demonstrating sufficiency of cell-cycle arrest for cell extrusion by *C. elegans*

In this thesis, I have reported findings that indicate that an arrested S phase is required for cell extrusion by *C. elegans*. I have shown that the unequal generation of extruded cells that make them small in size, their entry into the cell cycle and a nucleotide imbalance (inferred from *gmpr-1(lf)* phenotype) that likely occurs in these cells are all necessary to induce an arrested cell-cycle state required for cell extrusion. However, I have not been able to show sufficiency, i.e., I have been not able to show that ectopically inducing an S-phase arrest in cells that normally don't undergo cell extrusion during embryonic development leads to their extrusion by *C. elegans* embryos. Such an experiment would first require identification of cell(s) that would not cause lethality if lost. So, for example, ABplpappaa (sister of the extruded cell ABplpappap) would not be a good target for such an experiment, as ABplpappaa's daughter, the excretory

cell, is required for survival (Sulston *et al.*, 1983). A second constraint comes from the requirement of a cell to be suitably positioned in space and time for it to undergo cell extrusion, i.e., a cell should exist around the time of ventral enclosure and must be located close to an exit point such as the ventral pocket or anterior sensory depression to be considered for the sufficiency experiment. If cells meeting these criteria are identified, the cell cycle in such cells would then need to be arrested in S phase and the cells tested for extrusion. For example, perhaps a molecular tool that causes an S-phase arrest specifically in such cells around the time extrusion occurs could be expressed by using a promoter that is appropriately spatially and temporally precise. Unfortunately, neither such molecular tools nor such promoters currently exist. Chemical agents such as hydroxyurea (HU), an inhibitor of the ribonucleotide reductase (RNR) enzyme, can be used to induce S-phase arrest (Elford, 1968; reviewed in Grallert and Boye, 2008). However, if an embryo were effectively treated with HU, no cell in the embryo would progress through S phase and the specificity of cell-cycle arrest required for extrusion would be lost. Such limitations make addressing the sufficiency question difficult to address using *C. elegans*. However, HU-induced increase in cell extrusion from mammalian epithelial monolayers (Chapter 3) suggests that S-phase arrest is most likely sufficient to drive cell extrusion from *C. elegans* embryos. Nonetheless, whether this is truly the case is an outstanding question that should be answered when the tools required to answer this question come into existence. Determining sufficiency might enable a deeper

analysis of the role of individual genes and the order in which they function in cell extrusion.

Exploring the role of cell-cycle regulators in other *C. elegans* cell deaths

I have showed that a unique cell-cycle state leads to cell extrusion. In the absence of caspases, cell extrusion eliminates cells that are otherwise fated to be killed by caspases (Denning *et al.*, 2012). It remains to be determined if the cell cycle pathway also promotes general apoptotic cell death in the presence of caspase activity. Although a large number of screens have been performed to identify regulators of cell death in *C. elegans* (reviewed by Conradt *et al.*, 2016), it would be difficult to identify essential genes, such as most cell-cycle regulators, from such screens. As RNAi has been instrumental in understanding the role of cell-cycle regulators in cell extrusion, RNAi could also be used to study the role of cell cycle genes, if any, in the regulation of cell death and would overcome the difficulty of dealing with mutants of essential genes.

Interestingly, at least two previous studies have implicated the roles of cell-cycle genes in specific developmental cell deaths in *C. elegans*. The first study found a role of the cell-cycle regulator genes *lin-35*, *efl-1* and *dpl-1*, which encode the *C. elegans* homologs of the mammalian proteins Rb, E2F and the E2F binding partner Dp1, in ventral nerve cord (VNC) cell deaths (Reddien *et al.*, 2007). Loss of function in these genes enhances survival of VNC cells fated to die by caspase-mediated apoptosis in backgrounds with weak caspase mutations (Reddien *et al.*, 2007). These genes are members of a group of genes called synMuv B that regulate vulval development by *C. elegans* (Ferguson and

Horvitz, 1989). Although this study might seem to provide a first link between cell cycle regulators and cell death, Reddien *et al.* (2007) argue that the role of *lin-35*, *efl-1* and *dpl-1* in VNC death is independent of their roles in cell cycle regulation, because other synMuv B class genes that regulate cell cycles (*lin-9*, *lin-15* and *lin-36*) do not regulate VNC deaths and a synMuv B class gene that does not regulate cell cycles (*lin-37*) does regulate VNC deaths (Reddien *et al.*, 2007).

The second study that implicated cell-cycle genes in abnormal cell death phenotypes was a candidate-based RNAi screen of a library of essential genes for the survival of Rn.aap cells in the male tail ray lineages. This screen was performed by Holly Johnsen, then a graduate student in the Horvitz laboratory (Holly Johnsen, MIT PhD Thesis 2016). From this screen, Holly identified the cell-cycle regulators *lin-23*, *cul-1* and *psf-3*, which encode the *C. elegans* homologs of the mammalian SCF E3 ligase components β-TrCP and Cul1 and the GINS complex protein PSF3, respectively. Holly excluded *lin-23* and *cul-1* from further analysis because RNAi against these genes appeared to produce more cells than those expected from the survival of cells fated to die. It is possible that cells divided after surviving in animals with these RNAi treatements. Holly examined a mutant homozygous for the putative null deletion allele of *psf-3*, *ok2828*, but did not find an abnormal cell-death phenotype in the ray lineage of these mutants. It should be noted, however, that the superficially normal phenotype of the *psf-3* deletion allele strain does not match the embryonic lethal phenotype caused by RNAi against this gene, as has been observed by many groups (Kamath *et al.*, 2003; Rual *et al.*, 2004; Sönnichsen *et al.*, 2005;

Zipperlen *et al.*, 2001). The Million Mutation project includes a library of 2007 mutagenized and sequenced *C. elegans* strains that contains an average of 8 non-synonymous mutations per gene (Thompson *et al.*, 2013). This library contains only one missense allele strain and no nonsense allele strains for *psf-3*, indicative of the essential nature of this gene. These observations suggest that *lin-23*, *cul-1*, *psf-3* and perhaps other cell-cycle genes might play a role in cell deaths that occur in the male tail ray lineages. The role of cell-cycle regulators in male tail ray lineage cell deaths should be examined more closely.

Identifying and characterizing other genes that regulate cell extrusion

As described in Chapter 2, I identified a total of 27 genes from the genome-scale RNAi screen for the Tex phenotype. Of these, I have completely characterized the roles of only *cye-1*, *cdk-2*, *grp-1* and *cnt-2* in cell extrusion. Other cell-cycle genes identified from the genome-scale RNAi screen are likely required to produce the cell cycle state that drives cell extrusion. Still other genes identified in this screen, such as gene expression regulators and genes with vague or unknown function in cell extrusion, I have not yet characterized. Studying these genes and their roles in cell extrusion could reveal additional biological requirements for this process. It is possible that some of these genes are specifically expressed in cells fated to be extruded, in which case a powerful tool for further dissection of cell extrusion biology could be generated using their promoters.

Finally, I screened only those 55% of the genes in the *C. elegans* genome represented in the ORFeome RNAi library to find the 26 unique clones that

produce the Tex phenotype (Chapter 2, Table 2). That leaves 45% of *C. elegans* genes yet to be explored. Screening these genes might reveal additional genes, pathways and biological processes that regulate cell extrusion.

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Appendix

Genetic and other data for other genes

required for cell extrusion

C45G7.4

Loss-of-function mutations in *C45G7.4* were first identified by Daniel (Dan) Denning, a former postdoctoral fellow in the Horvitz Laboratory, from an EMS mutagenesis screens for the two-excretory cell (Tex) phenotype, which arises from survival of the cell ABplpappap. This cell is eliminated by caspase-mediated apoptosis in wild-type embryos and by cell extrusion in *ced-3(lf)* embryos. Dan identified mutations in *C45G7.4* to be causative in mutants from two separate screens. A screen for the Tex phenotype in the *ced-3(lf)* and *pig-1(lf)* genetic background yielded the *C45G7.4* alleles *n5431* (a splice-site mutation at the start of second exon) and *n5434* (R469*) (Figure 1A). RNAi against *C45G7.4* in *ced-3(lf)* animals generated the Tex phenotype (Figure 1D). Consistently, I identified *C45G7.4* from the genome-scale RNAi screen for the Tex phenotype in *ced-3(lf)* embryos (Chapter 2, Table 2).

C45G7.4 is a single RING finger domain protein that might function as an E3 ubiquitin ligase (Figure 1B). A large number of RING finger domain proteins have E3 ubiquitin ligase activity in vitro (reviewed by Deshaies and Joazeiro, 2009). Furthermore, RNAi against C45G7.4 leads to enhanced aggregation of transgenically expressed human α-synuclein in C. elegans (Hamamichi et al. 2008), suggesting a role in protein degradation. Hence, C45G7.4 might target one or more proteins for degradation to promote cell extrusion.

Both Dan and I had attempted to rescue the Tex phenotype produced by *C45G7.4(lf) ced-3(lf)* double mutants using vectors or amplified DNA that carried the wild-type *C45G7.4* genomic locus, but we were both unsuccessful in these

attempts. I attempted to identify the role of *C45G7.4* in cell extrusion by finding its interactors using yeast two-hybrid experiments using C45G7.4 as bait and found multiple interactors of C45G7.4 (Table 1). However, preliminary results of a candidate RNAi screen indicated that probably none of the identified interactors played a role in cell extrusion. I tried a second approach using a recently published technique called ubiquitin-activated interactor trap (UBAIT), which covalently links a RING or HECT-type E3 ubiquitin ligase to its interactor (schematic in Figure 1C; O'Connor *et al.*, 2015). Briefly, I expressed a fused version of a FLAG-tagged C45G7.4 linked to a ubiquitin moiety with a flexible linker in a *ced-3(lf)* background. Interestingly, transgenic expression of this fusion protein generated a Tex phenotype in *ced-3(lf)* mutants, suggesting a possible dominant-negative effect of this fusion protein. I performed an immunoprecipitation experiment on lysates of this strain using an anti-FLAG antibody and sent the immunoprecipitated protein for identification by mass spectrometry (MS). Although peptides from certain proteins were identified (Table 2), peptides corresponding to C45G7.4 were conspicuously absent. Additionally, preliminary testing of the proteins identified from MS by RNAi suggested that these proteins did not function in cell extrusion.

To determine how C45G7.4 might be expressed during embryogenesis in N2 embryos, I decided to perform single molecule Fluorescent In Situ Hybridization (smFISH) for *C45G7.4* mRNA molecules. I used a set of 46 smFISH probes targeting *C45G7.4* mRNA molecules (Table 3) conjugated to the fluorophore Alexa Fluor 647 (Life Technologies) at a final dilution of 1:100, as

previously described (Ji and van Oudenaarden, 2012). I found that *C45G7.4* mRNA was expressed during early embryogenesis and had a highly localized expression pattern, i.e., very specific cells expressed this mRNA (Figure 2). However, as the fixation procedure that is required for smFISH experiments makes it hard to identify individual cells by Nomarski optics, I could not establish which cells specifically expressed *C45G7.4*.

After finding genes involved in cell-cycle progression and unequal cell division from my genome-scale RNAi screen, I started using molecular tools to dissect the roles of these genes in cell-cycle progression by ABplpappap and the unequal cell division that generates ABplpappap, which is normally extruded in *ced-3(lf)* embryos. These tools included DHB- and PCNA-based cell-cycle phase reporters and mCherry-PH (Pleckstrin Homology domain of PLCδ) to label the membrane of all cells (see Chapter 3). I found using these tools that RNAi against *C45G7.4* in *ced-3(lf)* embryos perturbs the asymmetric cell division that generates ABplpappap and allows ABplpappap to proceed through the cell cycle, similar to the effect produced by *pig-1(RNAi)* (Figure 3A, 3B, 3C; Chapter 3, Figure 5). This finding indicates that *C45G7.4* is required for the unequal cell division that generates ABplpappap, along with the genes *pig-1*, *grp-1* and *cnt-2* (Chapter 3, Figure 5).

How might *pig-1(lf) C45G7.4(lf)* double mutant produce a Tex phenotype? I believe that a certain size threshold exists above which ABplpappap can survive the cell-killing action of the CED-3 caspase. Loss of two pathways for unequal cell division, which likely function in parallel for the division that generates

ABplpappap, might allow ABplpappap to cross this size threshold. This threshold is not cleared by ABplpappap in *pig-1(lf)* single mutants as ABplpappap dies by caspase-mediated apoptosis in these mutants (Denning *et al.*, 2012). Crossing such a size threshold might also allow ABplpappap to complete the cell cycle and not be trapped in an S-phase arrest, thus preventing its extrusion. However, further experimentation and data are needed to provide corroboration for this hypothesis.

Actin-myosin regulators

From the genome-scale RNAi screen, I identified that proper function of *rho-1* and *mlc-5* is required to prevent the Tex phenotype in *ced-3(lf)* animals. These genes encode *C. elegans* homologs of the mammalian RhoA GTPase, a regulator of actin-myosin dynamics, and a myosin light chain molecule, respectively, suggesting that the functioning of the actin-myosin pathway might be required for cell extrusion. A similar requirement of the actin-myosin pathway in cell extrusion has been demonstrated for cell extrusions by vertebrates and *Drosophila* (see Chapter 1). From a candidate screen for other regulators of actin-myosin dynamics, I also identified *let-502* and *mlc-4*, which encode *C. elegans* homologs of the mammalian Rho Kinase and regulatory myosin light chain molecules (Figure 4A). RNAi against *let-502* produced a relatively high penetrance of the Tex phenotype and relatively low lethality and morphological defects. This finding allowed me to study the role of this pathway for ABplpappap cell extrusion.

From an examination of ten *ced-3(lf)* embryos treated with RNAi against *let-502* and expressing HIS-72::GFP and mCherry::PH in *egl-1*-expressing cells (including ABplpappap), I found that ABplpappap was not extruded in four of the embryos. This suggested that the actin-myosin pathway is required for ABplpappap cell extrusion (Figure 4B, 4C).

It is not clear, however, where this pathway acts for cell extrusion. Previously described roles of the RHO-1 – MLC-4 pathway suggest that it could be involved in the regulation of the unequal cell division of ABplpappap's mother, as in the case of its role in B cell asymmetric cell divisions (Wu and Herman, 2006), or it could be involved in generating the forces that cause the extrusion of cells during epidermal cell migrations (Fotopoulos *et al.*, 2013; Wernike *et al.*, 2015). Future work will further clarify the role and site of action of the actin-myosin regulation pathway in cell extrusion by *ced-3(lf)* embryos.

Figure 1 *C45G7.4* functions to prevent the Tex phenotype and UBAIT could be used as an approach to identify its targets

a) The *C45G7.4* locus is shown with the locations of the *n5431* and *n5434* mutations identified from EMS mutagenesis screens for the Tex phenotype in *ced-3(lf)* and *pig-1(lf)* mutant backgrounds, respectively.

b) A diagram of the C45G7.4 protein shows the location of the RING domain in this protein. No other recognizable domains are present in this protein.

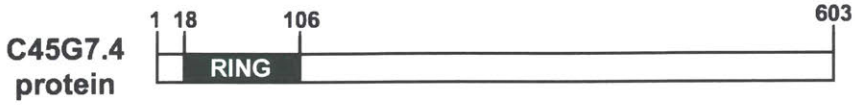
c) A schematic shows the workflow of a UBAIT experiment that might covalently trap the interactors and targets of C45G7.4

d) Table shows the Tex penetrance produced by RNAi treatments of and mutations in the gene *C45G7.4* in a *ced-3(lf)* background.

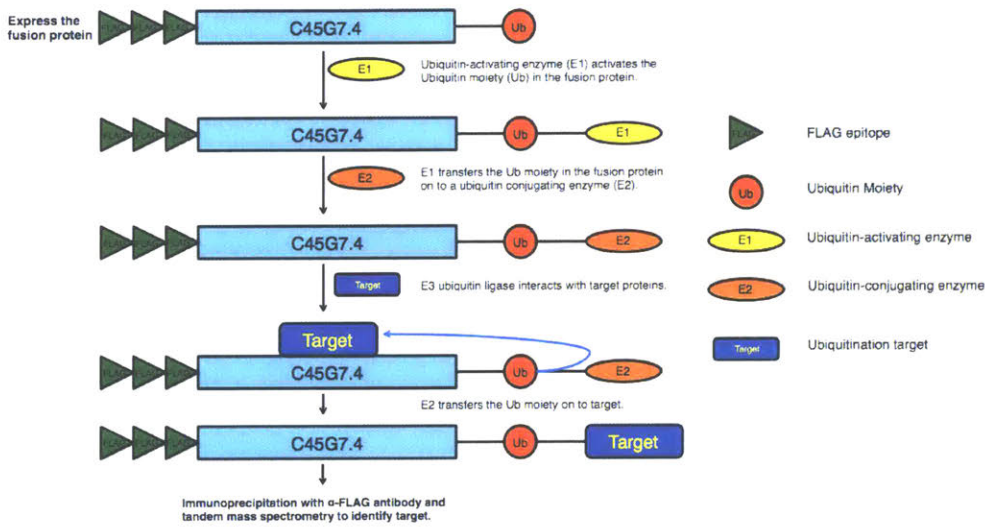
Figure 1

A

B



C



D

Knockdown or knockout of *C45G7.4* produces the Tex phenotype in *ced-3(lf)* mutants

genotype*	% animals w/ >1 excretory cell (n)
ced-3(n3692)	1 (98)
C45G7.4(n5431)	0 (77)
C45G7.4(n5431) ced-3(n3692)	58 (173)
ced-3(n3692); vector(RNAi)	2 (104)
ced-3; C45G7.4(RNAi ORFeome)	50 (102)
ced-3; C45G7.4(RNAi Ahringer)	44 (80)

*contained *nIs433* or *nIs434*[$P_{pgp\text{-}12}$:: *4xNLS-gfp*]

Figure 2 *C45G7.4* mRNA expression occurs during early embryogenesis and is highly localized

Figure panels show *C45G7.4* mRNA is expressed during early embryogenesis and occurs in very specific cells during embryonic development.

Figure 2

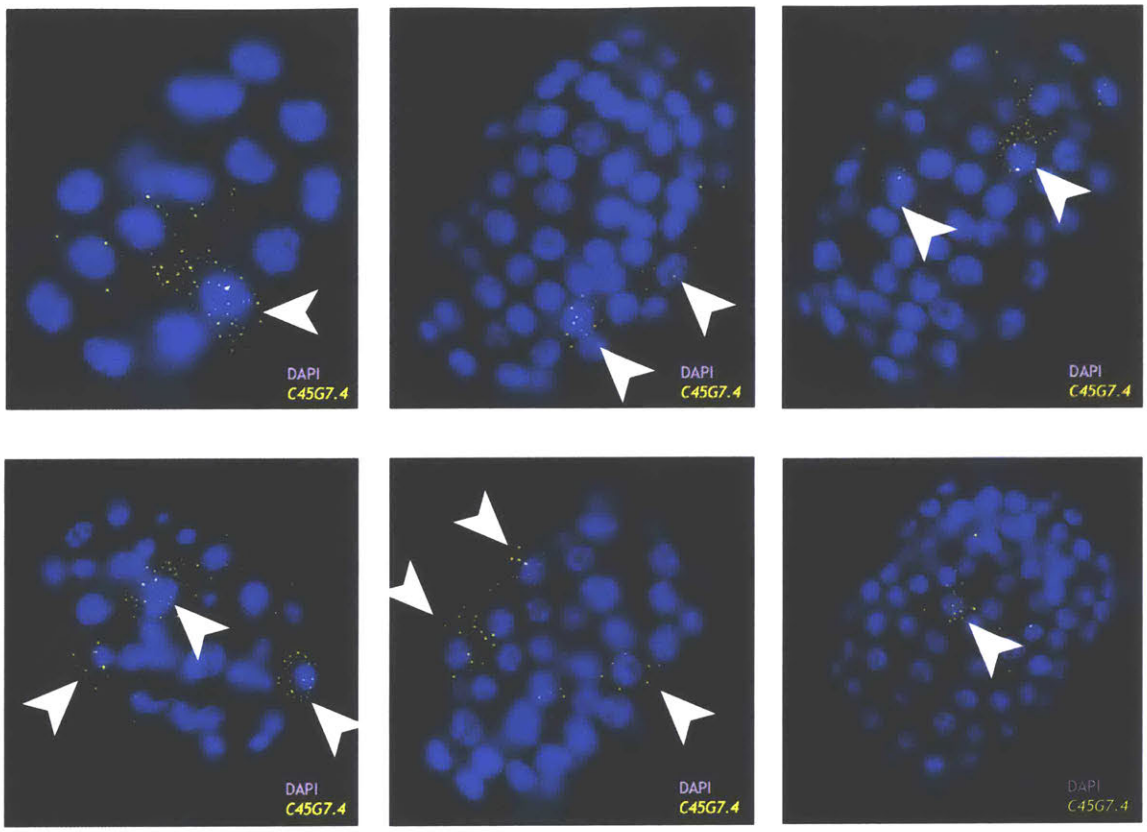

DAPI
C45G7.4
DAPI
C45G7.4
DAPI
C45G7.4
DAPI
C45G7.4
DAPI
C45G7.4
DAPI
C45G7.4

Figure 3 *C45G7.4* functions in the unequal cell division of ABplpappap's mother and prevents unrestricted cell-cycle progression by ABplpappap

a) Representative micrographs show a relatively larger ABplpappap is generated in embryos treated with RNAi against *C45G7.4* as compared to control. Scale bar, 10 µm.

b) Quantification of the relative sizes of ABplpappap and its sister show that *C45G7.4* function is required for the unequal cell division that generates ABplpappap. However, the effect of knockdown of *C45G7.4* is likely smaller than knockdown of *grp-1* by RNAi on this unequal division, suggesting either ineffective knockdown or a smaller role of *C45G7.4* in this process. $p<0.01$ (two-tailed T-test performed on log-transformed values of the ratios). n=4 embryos each. Note that values of the ratios for control and *grp-1* have been reused from Figure 5 of Chapter 3.

c) Time-lapse micrographs obtained using confocal microscopy of *ced-3(lf)* embryos expressing GFP::PCN-1 treated with RNAi against *C45G7.4* shows unperturbed progression through the cell cycle by ABplpappap ending with its division. The inset in each figure shows a magnified view of ABplpappap, the cell marked by an arrowhead. GFP::PCN-1 initially displays a localization pattern indicating S-phase at t_0, which then gradually changes to that indicating a phase between S and M (presumably G2), and then eventually changes to reflect mitosis of ABplpappap. Scale bar, 10 µm.

Figure 3

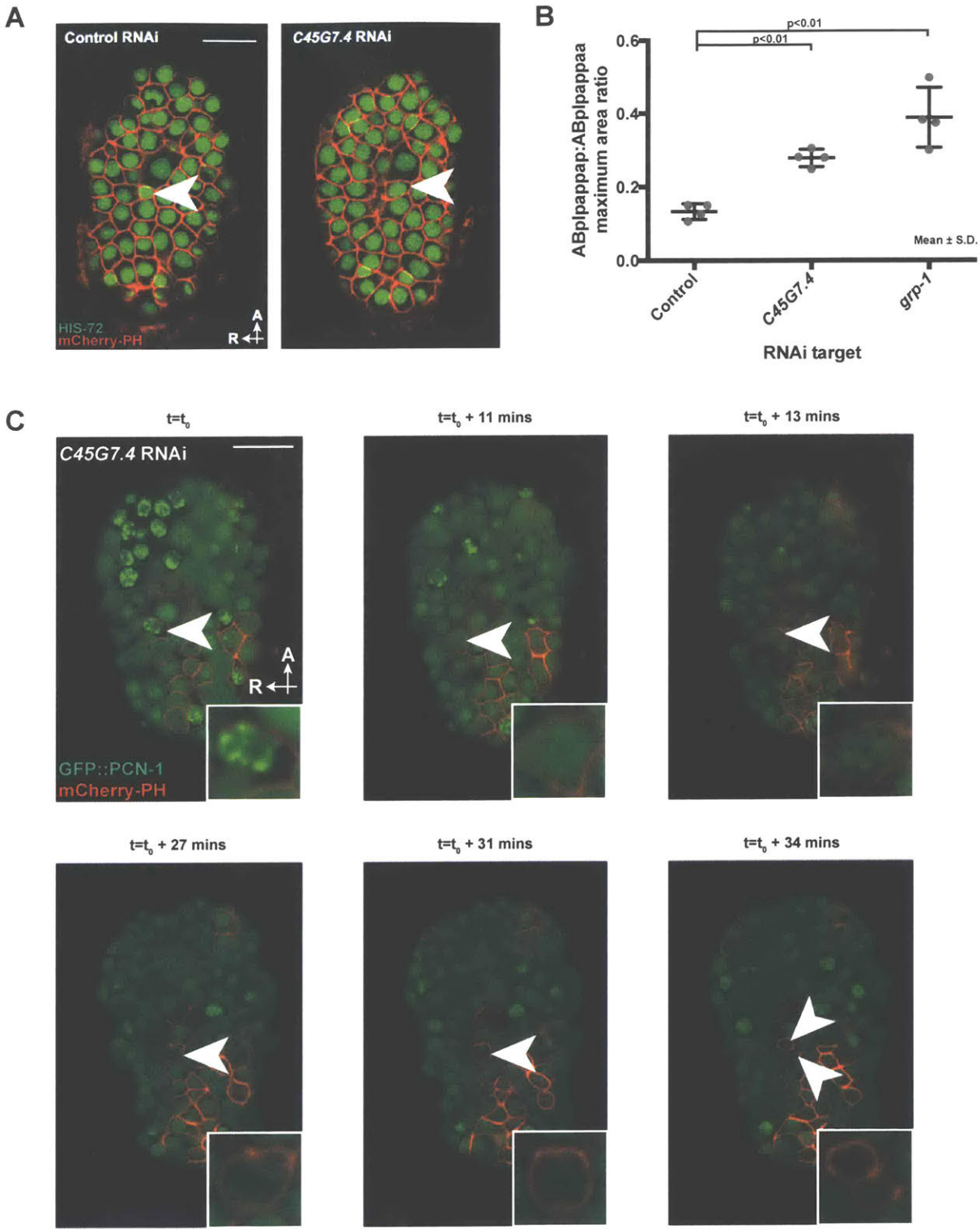

A
Control RNAi
C45G7.4 RNAi
HIS-72
mCherry-PH
A
R
B
p<0.01
p<0.01
ABplpappap:ABplpappaa maximum area ratio
0.6
0.4
0.2
0.0
Mean ± S.D.
Control
C45G7.4
grp-1
RNAi target
C
t=t0
C45G7.4 RNAi
A
R
GFP::PCN-1
mCherry-PH
t=t0 + 11 mins
t=t0 + 13 mins
t=t0 + 27 mins
t=t0 + 31 mins
t=t0 + 34 mins

Figure 4 The Rho – Rho Kinase – MLC pathway is required for ABplpappap cell extrusion

a) Table with Tex penetrance produced by RNAi against the genes *rho-1*, *let-502* and *mlc-4* in a *ced-3(lf)* genetic background show that these genes that encode actin-myosin regulators are required to prevent the Tex phenotype.

b) Time-lapse micrographs obtained using confocal microscopy of *ced-3(lf)* embryos expressing HIS-72::GFP and mCherry::PH in *egl-1*-expressing cells treated with RNAi against *let-502* shows that ABplpappap can evade extrusion as a result of *let-502(RNAi)* treatment. Scale bar, 10 μm.

c) A lateral view of the embryo in (b) shows that ABplpappap is not extruded, but is rather incorporated into the body of the developing embryo.

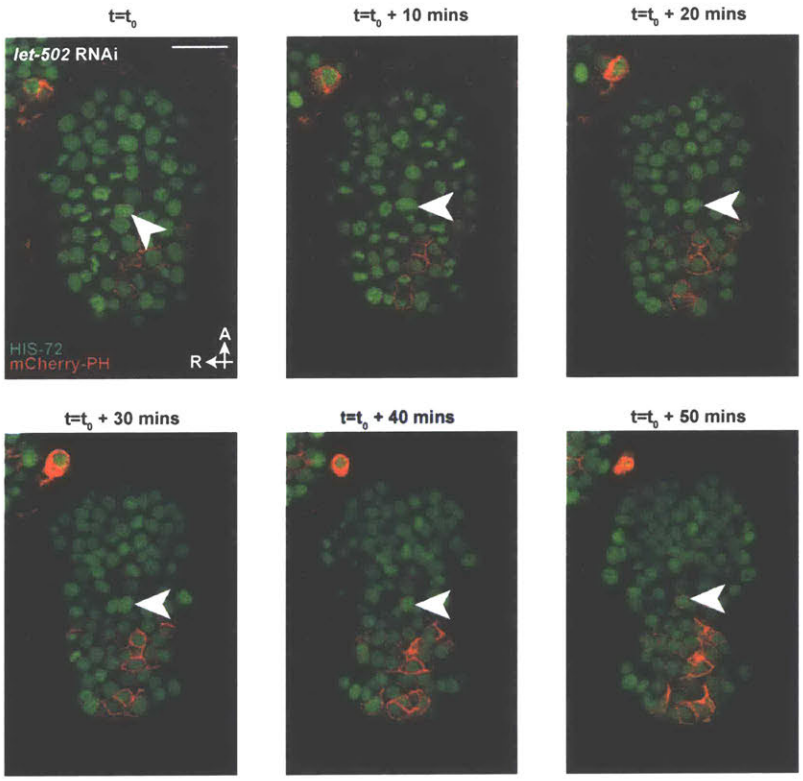
Figure 4

A

genotype*	% animals w/ >1 excretory cell (n)
ced-3(lf); control RNAi	2 (159)
ced-3(lf); rho-1(RNAi)	66 (40)
ced-3(lf); let-502(RNAi)	38 (104)
ced-3(lf); mlc-4(RNAi)	22 (95)

*contains *nls433[P_{pgp-12}::NLS-gfp]*

B



C

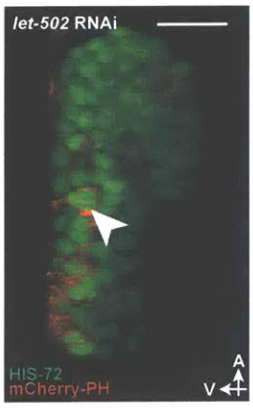


Table 1 List of potential interactors of C45G7.4 identified by yeast two-hybrid experiment.

Table 1

No.	Y2H interactor
1	*acs-17*
2	*cct-7*
3	*test-1*
4	*ubq-2*
5	*F54A3.5*
6	*cab-1*
7	*mlc-2*
8	*act-3*
9	*act-4*
10	*far-1*
11	*let-767*
12	*Y69A2AR.18*
13	*clp-7*
14	*R09B3.3*
15	*eef-1B.1*
16	*T27E7.1*
17	*far-2*
18	*cyn-7*
19	*asp-1*
20	*obr-3*
21	*immp-1*
22	*fkb-5*
23	*ndk-1*
24	*F46G10.1*
25	*hsp-1*

Table 2 List of potential interactors and/or targets of C45G7.4 identified by UBAIT.

Table 2

No.	Protein(s) containing peptide ID'd by MS	Total no. of peptides ID'd	No. of unique peptides ID'd
1	FTT-2/PAR-5	3	3
2	LEC-7	12	6
3	RPS-14	4	2
4	RPS-2	6	2
5	RPS-27	5	2
6	RPS-3	3	2
7	RPS-4	8	5
8	RPS-0	5	3
9	RPL-13	3	2
10	RPL-19	2	2
11	RPL-22	5	2
12	RPL-23	8	3
13	RPL-3	3	1
14	RPL-31	3	1
15	RPL-6	3	3
16	RPL-7A	4	2
17	RPL-8	3	2
18	ACT-1/ACT-2/ACT-3/ACT-4	122	15
19	H28O16.1	3	2
20	CAV-1	7	4
21	EFT-3	18	6
22	EEF-2	5	3
23	INF-1	3	2
24	RAN-1	3	1
25	HSP-1	19	8
26	DAF-21	8	4
27	HTZ-1/HIS-3	3	1
28	HIS-1	6	3
29	UNC-104	4	1
30	MLC-4	25	7
31	MLC-3	5	4
32	MLC-5	21	6
33	MYO-3	7	4
34	MYO-4	24	15
35	F01G4.6	8	4
36	UBQ-1/UBQ-2	5	4
37	SIP-1	5	2
38	MEC-7/TBB-2/TBB-4	4	2
39	VIT-2	9	5
40	VIT-2/VIT-1	39	20
41	VIT-3/VIT-4	4	2
42	VIT-3/VIT-4/VIT-5	43	25
43	VIT-4	4	2
44	VIT-5	6	3
45	VIT-6	48	25

Table 3 List of probe sequences used to perform smFISH experiments for *C45G7.4* mRNA.

Table 3

No.	Probe sequence
1	CTGTGATGTCGTTCATTCTG
2	GGAGTCAAGGATCACTGAGG
3	AACACCTCGAAGCAGACTTC
4	TTGGTGGACGATTCTCATGA
5	CAGAAGGTAGTACTCCTGAT
6	GCATGTTGGGCATTTAATGG
7	ACGAGCCGTATTTGTTGTAA
8	GATCATAATTGGTTGGTGCC
9	CGGAATAGGTTCTCGAGGAT
10	TGTGACGTTTTGCTCTTTTC
11	TGGCATTTTTGTGGCAACAG
12	CAATCGGCGCATCGAAAGTG
13	TTTTTCTGCGAGATTTTCCG
14	GTACTGGTACAATCCACACA
15	CATTCCAGGCAAACGATTGT
16	TGAGTTGGTTGTGGTGAAGG
17	TGAGCAGATTGTGTTTCTCG
18	CGACGCATGACGTCATCAAT
19	AACTGGTGGGAAGAGGATCG
20	GATTGCTCGTCTCGAATAGT
21	TCTTTTCCAACTGAGCTGAG
22	AAGGTTAGGCTAGTTGGTTT
23	CCTGGTAAGTGGTATCATTC
24	ATAGGGTGGATTTGTTCAGG
25	GACACTCTGGATTGACGGAT
26	GGAATAAGGAGTCGTCGGTT
27	GTCAGGGAACTTATCGACTC
28	ACATTGGGTGTTGATGCATA
29	CCTTGGAGGATACTGTAGAG
30	GACATGATCTTCTGCTTTCG
31	GATCCATGTCAAGCATGGAA
32	CAAAGATCGGCACCATCGAA
33	GGTTGAGTTGGTAGAAGCAG
34	CCCGACGAACTTGTTTTTAG
35	ATGTCACATCTTCGGTACTT
36	ATAACTGCACTGAACCTGCT
37	GGATGATGGGATGGGAAGTG
38	CAAGTACGCCAAATCATCCA
39	TCCGCGACATCACTATACAA
40	AGCTTCGATTAGGCAGATTG
41	CAATTTCAGTTAGGCCACTG
42	AGACGTTCTTCGGATCTTTG
43	GCATTCTGTCAAATGCTCAT
44	TTTTGACGTTTTTCGGAGCT
45	GCAATAAAGCATCCGCCAAA
46	GAGAATCATTAGCCTTCTCT

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