Chapter 2
Organogenesis of the *C. elegans* Vulva and Control of Cell Fusion

Nathan Weinstein and Benjamin Podbilewicz

Abstract The vulva of *Caenorhabditis elegans* is widely used as a paradigm for the study of organogenesis and is composed of seven toroids, formed by the migration of cells and the formation of homotypic contacts. Five of the toroids contain two or four nuclei and cell membrane fusion is one of the main driving forces during the morphogenesis of the vulva. The network of genes involved in the control of cell fusion during the formation of the vulva must determine which cells fuse and when. Especially during the formation of the vulval toroids, when those cells that fuse to form each ring, must not fuse with the neighbor cells, which form other separate rings. This is achieved through very fine control on the expression and function of several key genes.

Keywords Vulva morphogenesis · *Caenorhabditis elegans* · Cell fusion · Organogenesis · Signaling pathways · *eff-1* · *aff-1* · Wnt · Notch · RTK-Ras-ERK · Vulval toroids · Developmental genetics · Cell differentiation · Cell invasion · Anchor cell · Vulval precursors · Fate determination · Cell migration · Cell lineage · Cell polarization · Transcriptional control · Modeling · Uterine-vulval connection · Nematodes · Evolution · Evo-devo

N. Weinstein (✉)
ABACUS-Centro de Matemáticas Aplicadas y Cómputo de Alto Rendimiento, Departamento de Matemáticas, Centro de Investigación y de Estudios Avanzados CINVESTAV-IPN, Carretera México-Toluca Km 38.5, La Marquesa, Ocoyoacac, Estado de México 52740, Mexico
e-mail: nathan.weinstein4@gmail.com

B. Podbilewicz (✉)
Department of Biology, Technion—Israel Institute of Technology, Haifa 32000, Israel
e-mail: podbilew@tx.technion.ac.il

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2.1 Background

The *C. elegans* vulva is a sexual and egg-laying organ specific to the hermaphrodite that develops after the formation of the embryo. The vulva is composed of a pile of seven epithelial toroids that contain a total of 22 cell nuclei and connect the uterus with the exterior. The toroids are in a ventral to dorsal order before eversion: vulA, vulB1, vulB2, vulC, vulD, vulE and vulF (Fig. 2.1).

The functions of the vulva are egg laying and copulation; both functions require the vulva to open, forming a channel that connects the internal reproductive organs to the exterior. The uterine seam cell (utse) forms a barrier between the vulva and the uterus (hymen) that is probably broken during the first egg laying or the first copulation. The shape of the vulva and the fact that the vulE ring is attached to the seam cells causes it to remain closed until the vulval muscles contract to allow egg laying (Sharma-Kishore et al. 1999; Lints and Hall 2009).

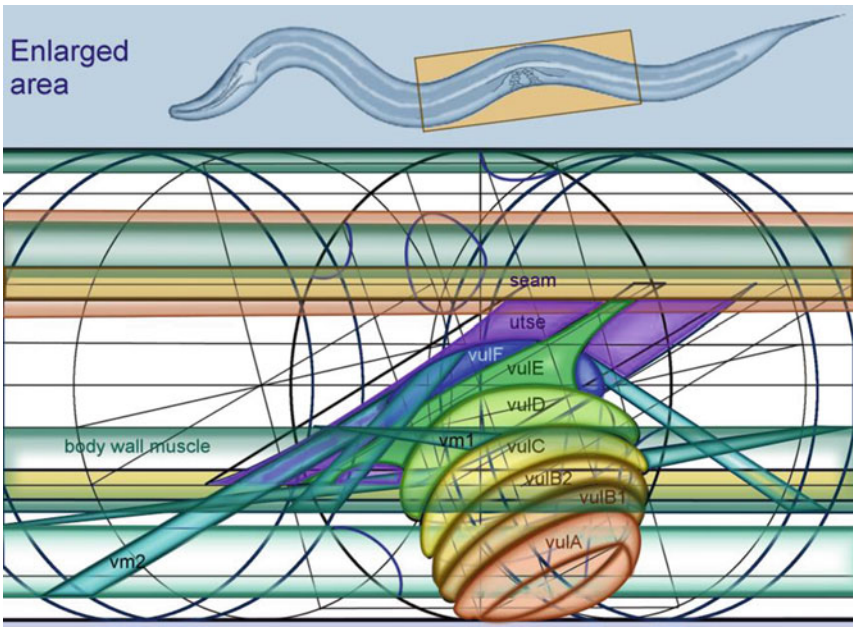

Fig. 2.1 The vulva of *Caenorhabditis elegans* at the late L4 stage before eversion. vulA cells are shown in *auburn*, vulB1 cells in *dark orange*, vulB2 cells in *light orange*, vulC cells in *yellow*, vulD in *olive green*, vulE in *forest green*, vulF in *blue*, muscle cells in *blue green* and *utse* in *purple*

2.1.1 *The Vulva of* C. elegans *as a Genetic Model Organ*

The vulva is a superb developmental genetic model for the study of organogenesis because the lineage of the cells that form the vulva, and the effects of numerous mutations on vulval development are easy to observe during the entire life of the worm due to the fact that the vulva is not an essential organ in *C. elegans*. Many mutations that cause vulval phenotypes are viable. Some mutations that cause an egg laying defective (Trent et al. 1983) (Egl) phenotype, or prevent the formation of a vulva (Horvitz and Sulston 1980; Ferguson and Horvitz 1985) (Vulvaless, Vul), do not block self-fertilization in the worm, resulting in a bag of worms (Bag) phenotype, where the eggs hatch inside the worm. Other mutations cause the formation of multiple vulvae (Horvitz and Sulston 1980; Ferguson and Horvitz 1985) (Multivulva, Muv); bivulval (Biv) worms form two vulvae because of defective cell polarization. Other mutations cause morphological defects, such as the formation of a protruded vulva (Eisenmann and Kim 2000) (Pvl) or defective vulval eversion (Seydoux et al. 1993) (Evl).

2.1.1.1 Historic Overview of Vulva Research

Vulva research emerged from general studies about the development of *C. elegans*; specifically, the determination of the lineages of the vulval precursor cells (VPCs) was described as part of a study on the post embryonic lineages (Sulston and Horvitz 1977). After the cell lineages where known, two questions were asked. First, can similar cells replace vulval cells? This question led to the discovery of the vulval competence group by laser-mediated cell ablations. The vulval competence group is composed of six VPCs that have the potential to acquire any vulval fate (Sulston and White 1980). Second, which mutations may change the cell linages? This question lead to the discovery of some of the genes that affect vulval development (Horvitz and Sulston 1980).

Our knowledge about the signaling pathways involved in the control of vulval formation and the way in which those pathways are interconnected is based on screens for genes that when mutated cause (Ferguson and Horvitz 1985; Eisenmann and Kim 2000; Seydoux et al. 1993) or suppress different vulval phenotypes (Han et al. 1993; Clark et al. 1992, 1993; Aroian and Sternberg 1991; Beitel et al. 1990) as well as on reverse genetic studies (Ririe et al. 2008; Myers and Greenwald 2007; Fernandes and Sternberg 2007; Wagmaister et al. 2006a, b; Sundaram 2005a; Inoue et al. 2005; Hill and Sternberg 1992). Additionally many diagrammatic and computational models of vulval development (Kam et al. 2003; Fisher et al. 2005, 2007; Giurumescu et al. 2006; Sun and Hong 2007; Kam et al. 2008; Bonzanni et al. 2009; Giurumescu et al. 2009; Li et al. 2009; Fertig et al. 2011; Hoyos et al. 2011; Pénigault and Félix 2011a; Corson and Siggia 2012; Félix 2012; Félix and Barkoulas 2012; Weinstein and Mendoza 2013) have allowed the proposal of several predictions about the interaction between the signaling pathways. Some of

those predictions have been proven experimentally; furthermore, each dynamic model has helped us understand better the process of vulval formation.

Vulval morphogenesis has been studied by observing the whole process using electron and light microscopes both in the wild type (Sharma-Kishore et al. 1999) and in some mutant backgrounds (Eisenmann and Kim 2000; Seydoux et al. 1993; Shemer et al. 2000; Sapir et al. 2007; Green et al. 2008; Pellegrino et al. 2011; Farooqui et al. 2012). Additionally, reverse genetic studies addressing the genes involved in the morphogenesis of the vulva (Alper and Podbilewicz 2008; Schindler and Sherwood 2013; Schmid and Hajnal 2015) have clarified the role of different signaling pathways that control cell migration, fusion and invasion during the morphogenesis of the vulva.

2.1.2 Overview of Vulva Development

There are three main stages during vulval development: (i) Formation and maintenance of the vulval competence group, (ii) Vulval cell differentiation and proliferation, and (iii) Morphogenesis of the vulva.

The worm is born with two rows of six P cells in the mid-ventral region; some of these P cells are the progenitors of all vulval cells (Sulston and Horvitz 1977; Altun and Hall 2009; Sternberg 2005; Greenwald 1997). During the first larval stage (L1), the P cells first migrate to the ventral midline and then divide. Six central posterior daughters of the P cells become the vulval precursor cells (VPCs, P3.p-P8.p) (Sulston and Horvitz 1977; Altun and Hall 2009; Sternberg 2005; Greenwald 1997). During the second larval stage (L2), the gonadal anchor cell (AC) differentiates and the competence of the VPCs is maintained (Lints and Hall 2009; Wang and Sternberg 1999; Eisenmann et al. 1998).

During the end of the second larval stage (L2) the VPCs acquire the primary, secondary, or tertiary fates (Fig. 2.2, 28 h post hatching) (Sternberg 2005; Sternberg and Horvitz 1989), then the VPCs that acquired the secondary fate become polarized (Green et al. 2008). Following this step, the VPCs divide longitudinally (Fig. 2.2, 30 h), and the daughters of the VPCs that acquired the tertiary fate fuse with a hypodermal syncytium (hyp7). The remaining VPC daughters undergo a second longitudinal division (Fig. 2.2, 32 h).

During the third molt, the granddaughters of the VPC that acquired the primary fate divide transversely (T), the granddaughters of the secondary fate VPCs nearest to the AC, do not divide (N) a third time, the next secondary fate granddaughters nearest to the AC divide transversely, and the rest of the secondary fate granddaughters divide longitudinally (L) a third time (Fig. 2.2, 33 h, L3/L4) (Sharma-Kishore et al. 1999; Schindler and Sherwood 2013).

Vulval morphogenesis begins during L3, when the AC breaks the basement membrane separating it from the primary fate VPC daughters (Sherwood et al. 2005). Then the AC sends a projection that invades between the most proximal VPC granddaughters. Later, after three divisions, the descendants of the VPCs

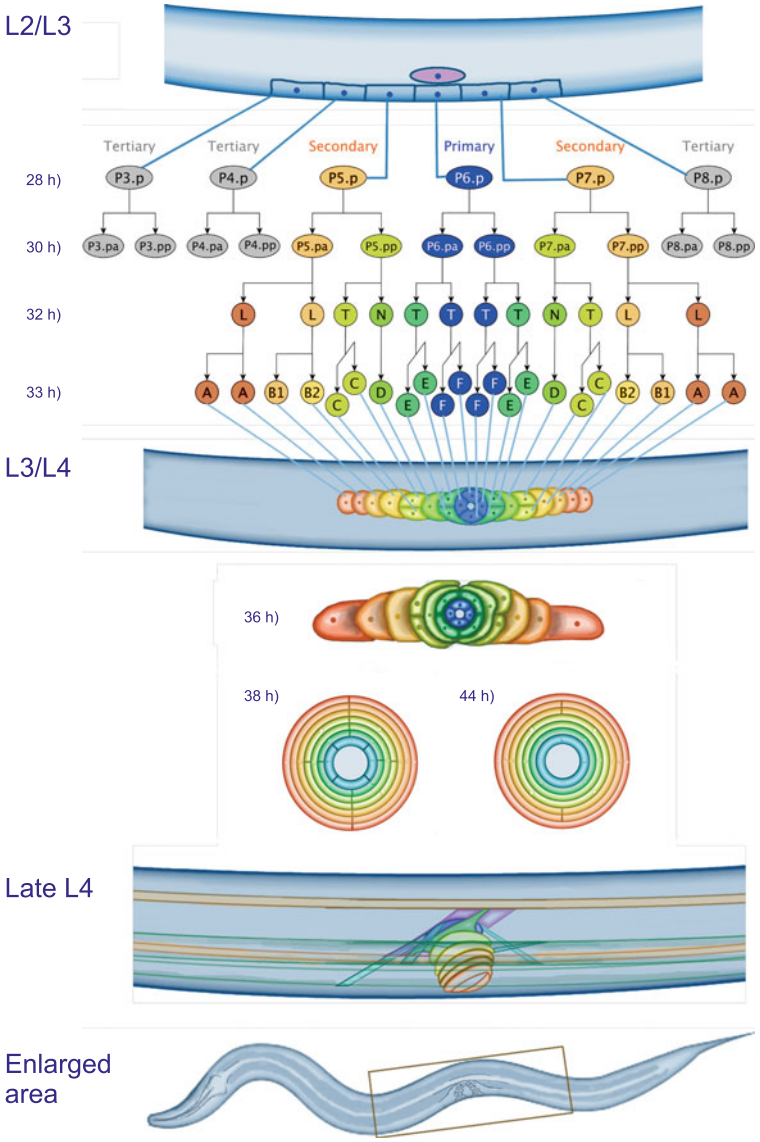

Fig. 2.2 Overview of vulval development. 28 h) The fate of the VPCs is determined (Primary fate in *blue*, secondary fate in *orange* and tertiary fate in *gray*). 30 h) The VPCs divide longitudinally and the daughters of tertiary fate VPCs fuse with hyp7. 32 h) The daughters of P5.p, P6.p, and P7.p divide longitudinally. 33 h) Some of the granddaughters of primary and secondary VPCs divide following the pattern LLTN TTTT NTLL where "T" represents a transverse division, "N" no division, and "L" a longitudinal division. L3/L4) The cells acquire adult vulval cell fates (vulA in *auburn*, vulB1 in *dark orange*, vulB2 in *light orange*, vulC in *yellow*, vulD in *olive green*, vulE in *forest green*, vulF in *blue*). 36 h) The VPCs migrate towards the center of the vulva. 38 h) Toroid formation. 44 h) Intratoroidal cell fusions. Late L4) Formation of the utse cell and muscle attachment. L2/L3, Late L4 and Enlarged area show lateral views. L3/L4 shows ventral views

migrate towards the center of the developing vulva (Fig. 2.2, 36 h). During the fourth larval stage (L4), the vulval toroids are formed (Fig. 2.2, 38 h), and some of the cells within the toroids fuse (Fig. 2.2, 44 h). Later the vulva invaginates allowing the formation of the vulval lumen. The vulval muscles attach to the vulva and are innervated. Next, the AC fuses with eight pi cells of the uterus during early L4, forming the utse cell (Fig. 2.2, Late L4). Finally, the vulva undergoes eversion resulting in a functional, adult vulva (Sharma-Kishore et al. 1999; Lints and Hall 2009; Schindler and Sherwood 2013; Gupta et al. 2012).

In the following sections we present the main signaling pathways involved in the molecular control of vulval development. Next, we will review; for each stage of vulval development what is known about the role of the different signaling pathways during that stage, some of the relevant existing models for that stage of development, and the predictions made based on those models.

Peter Abelard said *"Constant and frequent questioning is the first key to wisdom for through doubting we are led to inquire, and by inquiry we perceive the truth"* (Graves 1910); We will try to follow his advice and will include some of the questions that still need to be answered.

2.2 Three Signaling Pathways Involved in the Control of Vulval Development

The development of multicellular organisms requires directed cell polarization, differentiation and migration in order to generate different tissues and organs. One of the mechanisms involved in the regulation of these essential developmental processes are the signaling pathways. During vulval development, crosstalk between signaling pathways (*Notch, Wnt,* and *RTK-Ras-ERK)* coordinates the molecular mechanisms which direct cell differentiation (Sternberg 2005), migration (Pellegrino et al. 2011), fusion and shape (Alper and Podbilewicz 2008; Schindler and Sherwood 2011). These signaling pathways control the expression and activity of several target genes, including, actin, myosin, rho, *eff-1, aff-1, egl-17, lin-39, cki-1 and lin-12*. Here, we introduce the signaling pathways and in the next sections we will describe how they are involved in the control of each stage of vulval development.

2.2.1 Wnt Signaling

Wnt proteins are evolutionary conserved, secreted, lipid-modified glycoproteins that can function as morphogens that form concentration gradients to provide positional information to cells in developing tissues and also as short range signaling molecules (Clevers and Nusse 2012). Wnt proteins cause a wide variety of

responses including cell fate determination through the activation of specific target genes, and the control of cell polarity and migration by directly adjusting the cytoskeleton (Angers and Moon 2009).

Wnt proteins can activate different signaling mechanisms. The mechanism that has been studied in most detail is the canonical Wnt pathway, which controls the expression of specific target genes through the effector protein β-catenin and some members of the TCF/Lef1 family of HMG-box containing transcription factors (Sawa and Korswagen 2013) (Fig. 2.3). In the absence of Wnt signaling, β-catenins are targeted for degradation by a proteolysis promoting complex that consists of the scaffold protein Axin, the tumor suppressor gene product APC, and the kinases CK1 and GSK3β.

Canonical Wnt signaling in *C. elegans* (Fig. 2.3), begins with the FGF (Minor et al. 2013) retromer complex, AP-2 and MIG-14/Wntless mediated secretion of a Wnt ligand (Hardin and King 2008), such as: MOM-2, CWN-1, CWN-2, LIN-44 or EGL-20 (Gleason et al. 2006).

The Wnt ligand then binds to a Frizzled receptor; such, as MIG-1, LIN-17, MOM-5 or CFZ-2 (Gleason et al. 2006), located in the cell membrane of another cell, then the Wnt/Frizzled complex binds a Disheveled protein like DSH-1, DSH-2 or MIG-5 (Sawa and Korswagen 2013; Walston 2006), preventing the formation of APR-1/PRY-1/KIN-19/GSK-3β complexes which up regulate β-catenin degradation (Sawa and Korswagen 2013; Oosterveen et al. 2007; Korswagen et al. 2002;

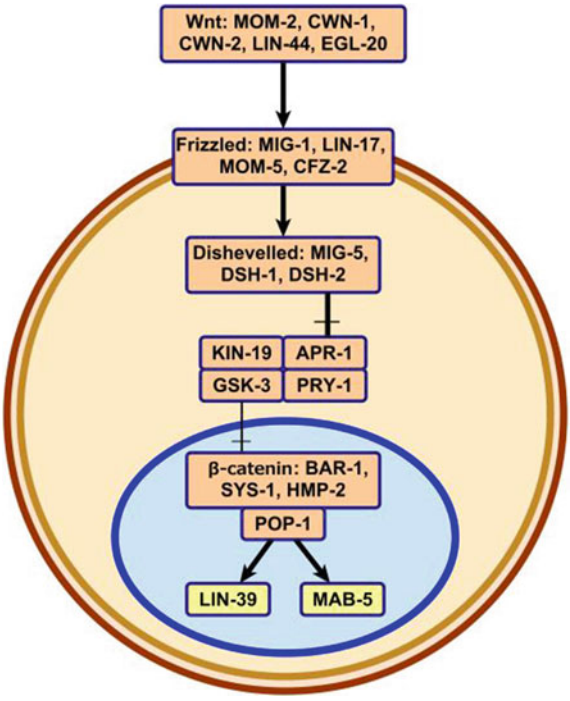

Fig. 2.3 Canonical Wnt signaling in *C. elegans*. *Pointed arrows* represent activating interactions and *blunt arrows* represent inhibitory interactions, *bold arrows* represent active interactions and *thin arrows* represent inactive interactions

Hoier et al. 2000). The β-catenins, HMP-2 (Costa et al. 1998), SYS-1 (Kidd et al. 2005; Liu et al. 2008) or BAR-1 (Eisenmann et al. 1998), bind to POP-1/Tcf, a HMG box-containing protein that is the sole *C. elegans* member of the TCF/LEF family of transcription factors (Sawa and Korswagen 2013), forming a protein complex that activates the expression of target genes such as the homeotic transcription factors *lin-39* (Eisenmann et al. 1998) and *mab-5* (Sawa and Korswagen 2013).

Canonical Wnt signaling is required for proper cell fusion control (Myers and Greenwald 2007; Pénigault and Félix 2011a; Eisenmann et al. 1998) and primary fate determination (Gleason et al. 2002, 2006; Wang and Sternberg 2000) during the formation of the *C. elegans* vulva.

A divergent canonical Wnt signaling pathway called the Wnt/β-catenin asymmetry pathway is one of the main mechanisms that control the polarization and differentiation of several somatic cells along the anterior-posterior axis (Sawa and Korswagen 2013; Yamamoto et al. 2011). Importantly, the Wnt/β-catenin asymmetry pathway is involved in the polarization of the vulval precursor cells P5.p and P7.p (Green et al. 2008).

The *C. elegans* Wnt/β-catenin asymmetry pathway (Fig. 2.4) is activated when a dividing cell is exposed to a gradient of Wnt ligands (Gleason et al. 2006). On the part of the cell that is exposed to a higher concentration of Wnt ligands (the right side in Fig. 2.4), the Wnt ligands bind to one of three Frizzled receptors on the membrane, LIN-17, LIN-18 or CAM-1 (Green et al. 2008; Gleason et al. 2006), and then a Dishevelled protein; specifically, MIG-5 DSH-1 or DSH-2 (Sawa and Korswagen 2013; Walston 2006), binds to the activated receptor. Meanwhile, the side of the cell that is exposed to a lower concentration of Wnts (left part of the cell in Fig. 2.4), accumulates WRM-1/LIT-1/APR-1 (Sawa and Korswagen 2013; Mizumoto and Sawa 2007) complexes in the membrane. Once the cell divides, the daughter cell exposed to a lower concentration of Wnt forms APR-1/PRY-1/KIN-19/GSK-3β complexes which activate β-catenin degradation (Sawa and Korswagen 2013; Oosterveen et al. 2007; Korswagen et al. 2002; Hoier et al. 2000). There are four β-catenins in *C. elegans* [WRM-1 (Takeshita and Sawa 2005), HMP-2 (Costa et al. 1998), SYS-1 (Kidd et al. 2005; Liu et al. 2008) and BAR-1 (Eisenmann et al. 1998)]. In the daughter cell exposed to a lower concentration of the Wnt ligand, the result is that POP-1 represses the transcription of certain target genes in the nucleus (left daughter cell in Fig. 2.4). In the daughter cell exposed to a higher concentration of Wnt, the formation of APR-1/PRY-1/KIN-19/GSK-3β complexes is inhibited, the concentration of SYS-1 rises and SYS-1/POP-1 complexes form and activate the transcription of certain target genes. Additionally, the SYS-1 unbound POP-1 binds to WRM-1/LIT-1 complexes that are transported outside of the nucleus, preventing the inhibition of the transcription of some target genes (Green et al. 2008; Sawa and Korswagen 2013; Takeshita and Sawa 2005; Phillips et al. 2007).

In summary, in the daughter cell that is exposed to a higher concentration of Wnt ligands, β-catenin degradation is inhibited and the concentration of POP-1 in the nucleus is reduced due to LIT-1 and WRM-1 action. Increasing the ratio of active

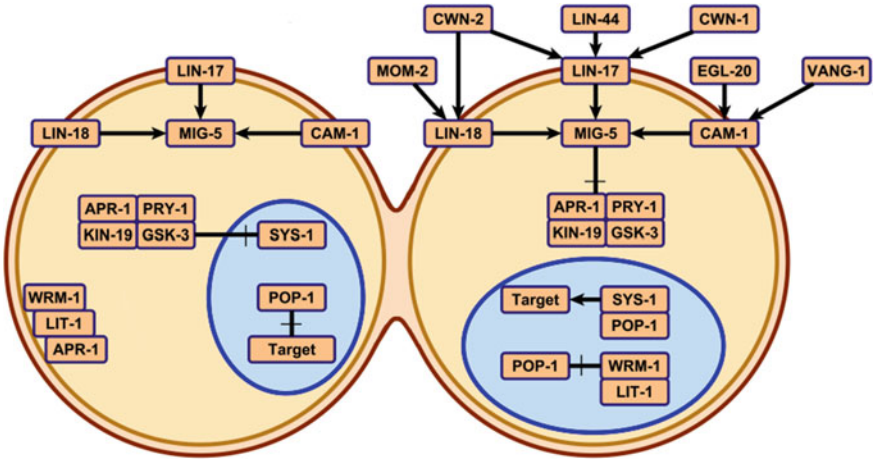

Fig. 2.4 The Wnt/β-catenin asymmetry pathway polarizes a cell that is about to divide. In this figure, the *right part* of the cell is exposed to a higher concentration of Wnt ligands. *Pointed arrows* represent activating interactions and *blunt arrows* represent inhibitory interactions, only active interactions are shown

β-catenin bound POP-1 to inhibitory free POP-1, that increased ratio allows the expression of certain target genes (Fig. 2.4, right). In the other daughter that is exposed to a lower concentration of Wnt ligands, the β-catenins are degraded and the expression of the target genes is inhibited (Fig. 2.4, left).

2.2.2 *Notch Signaling*

Notch is a fundamental signaling pathway that mediates cell differentiation during animal development (Greenwald and Kovall 2002; Andersson et al. 2011). Genetic analysis of Notch signaling in *C. elegans* has highlighted several characteristics of this essential pathway that are conserved in other animal species (Greenwald and Kovall 2002). The two *C. elegans* Notch proteins, LIN-12 and GLP-1 (Lambie and Kimble 1991), are required by several cell fate specification processes during development including vulval cell fate determination, and anchor cell differentiation. Additionally, the Notch pathway is required for proper germline development, regulation of tubular morphogenesis, and auto cell fusion in the digestive tract of *C. elegans* (Rasmussen et al. 2008).

Notch signaling is initiated by LAG-2 (Lambie and Kimble 1991; Zhang and Greenwald 2011a), DSL-1 (Chen and Greenwald 2004), APX-1 (Mello et al. 1994) or ARG-1 (Fitzgerald and Greenwald 1995), the four *C. elegans* DSL (Delta-Serrate-LAG-2) family ligands. The DSL ligand binds to LIN-12 or GLP-1 (Lambie and Kimble 1991), which are receptors orthologous to NOTCH; of these

two receptors, LIN-12 is more important during vulva development. After activation, LIN-12 is cleaved by the disintegrin-metalloproteases, ADAM family SUP-17 (Wen et al. 1997) or ADM-4 (Jarriault and Greenwald 2005) at the extracellular site 2. Following this processing, it undergoes another cleavage at the trans-membrane site 3 mediated by the γ-secretase protease complex conformed by SEL-12 or HOP-1 (Westlund et al. 1999), APH-1 (Goutte et al. 2002), APH-2 (Levitan et al. 2001), and PEN-2 (Francis et al. 2002). The resulting intracellular domain of LIN-12 is transported to the nucleus where it binds to LAG-1 (CSL) (Christensen et al. 1996) and SEL-8 (MASTERMIND) (Doyle et al. 2000), forming a complex (Greenwald and Kovall 2002) that activates the transcription of the target genes *ark-1, lip-1, dpy-23, lst-1, lst-2, lst-3, lst-4, mir-61*, and *lin-11* (Yoo et al. 2004; Marri and Gupta 2009), among others. Notch signaling includes at least two positive feedback circuits. First, LIN-12 activates the LAG-1/SEL-8 complex, which in turn activates *lin-12* and *lag-1* transcription (Christensen et al. 1996; Wilkinson et al. 1994; Choi et al. 2013; Park et al. 2013) and second, LIN-12 activates *mir-61* transcription, which causes VAV-1 down-regulation, and as a result promotes *lin-12* activity (Yoo and Greenwald 2005).

In summary, the Notch proteins are membrane receptors that bind DSL ligands. After the ligand binds a series of reactions cut, release and transport an intracellular fragment of Notch to the nucleus. The Notch fragment forms a protein complex that regulates the transcription of numerous target genes (Fig. 2.5).

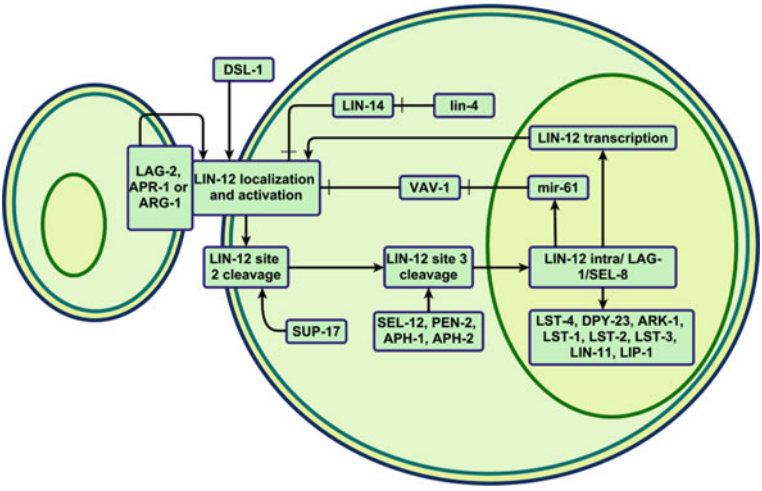

Fig. 2.5 Notch signaling in *C. elegans*. *Pointed arrows* represent activating interactions and *blunt arrows* represent inhibitory interactions

2.2.3 RTK-Ras-ERK

The small GTPase Ras has important functions in multiple signaling pathways, one of the most important and well conserved of these is the RTK-Ras-ERK pathway (Sundaram 2013). RTK-Ras-ERK signaling is conserved across many animal species and is used to control many different biological processes during development including cell proliferation (Xie et al. 2006; McKay and Morrison 2007). During *C. elegans* vulva development, RTK-Ras-ERK signaling is needed to allow the vulval cells to divide (Clayton et al. 2008), to prevent ectopic cell fusion (Pellegrino et al. 2011; Alper and Podbilewicz 2008), and to allow the specification of the primary vulval fate (Wang and Sternberg 2000).

In order for the RTK/Ras/ERK signaling pathway (Sundaram 2013) to be activated in *C. elegans* (Fig. 2.6), first, a near neighbour cell must express and secrete the epidermal growth factor LIN-3/EGF (Hill and Sternberg 1992). In the wild type, the AC secretes LIN-3/EGF. The expression of LIN-3/EGF in the AC requires the function of the transcription factor HLH-2/E/Daughterless and an unidentified nuclear hormone receptor (NHR) (Hwang and Sternberg 2004). The expression of LIN-3 in vulF cells requires the function of *nhr-67* and *egl-38* (Fernandes and Sternberg 2007). LIN-3 is initially synthesized as a transmembrane protein, and LIN-3 needs to be cleaved proteolytically to generate a diffusible ligand (Sundaram 2013; Dutt et al. 2004). Additionally, the Synthetic Multivulva (SynMuv) genes, that include several chromatin modification pathways, regulate the expression of *lin-3* and prevent its ectopic expression in many tissues, including the hyp7 syncytium (Saffer et al. 2011).

Once LIN-3 is present in the extracellular microenvironment of a cell, LIN-3 may bind to the receptor LET-23/EGFR (Aroian and Sternberg 1991) and activate the RTK/Ras/ERK signaling pathway. The basolateral localization of LET-23 requires the function of ERM-1 (Haag et al. 2014) and a complex formed by three PDZ-domain proteins (LIN-2, LIN-7, and LIN-10) to localize LET-23/EGFR (Kaech et al. 1998). The LIN-2/7/10 complex also recruits EPS-8 to inhibit RAB-5 mediated LET-23 endocytosis (Stetak et al. 2006). ARK-1 (Hopper et al. 2000), SLI-1 (Jongeward et al. 1995), UNC-101 (Lee et al. 1994), DPY-23 (Yoo et al. 2004), LST-4 (Yoo et al. 2004), RAB-7 (Skorobogata and Rocheleau 2012), several members of the ESCRT complex (Skorobogata and Rocheleau 2012) and an AGEF-1/Arf GTPase/AP-1 ensemble (Skorobogata et al. 2014), all negatively regulate signaling, most likely by promoting LET-23 endocytosis and lysosomal degradation. DEP-1 inhibits LET-23 function, most likely through direct dephosphorylation of key tyrosine residues (Berset et al. 2005).

When LIN-3 binds to LET-23, the receptor dimerizes and phosphorylates its C-terminal region exposing phospho-tyrosine residues that serve as docking sites for the cytosolic phospho-tyrosine binding adaptor protein SEM-5 (Clark et al. 1992; Hopper et al. 2000; Worby and Margolis 2000). Activated SEM-5 then recruits SOS-1 (Worby and Margolis 2000; Chang et al. 2000), a Guanine Nucleotide Exchange Factor (GEF), which activates LET-60/Ras (Han et al. 1990)

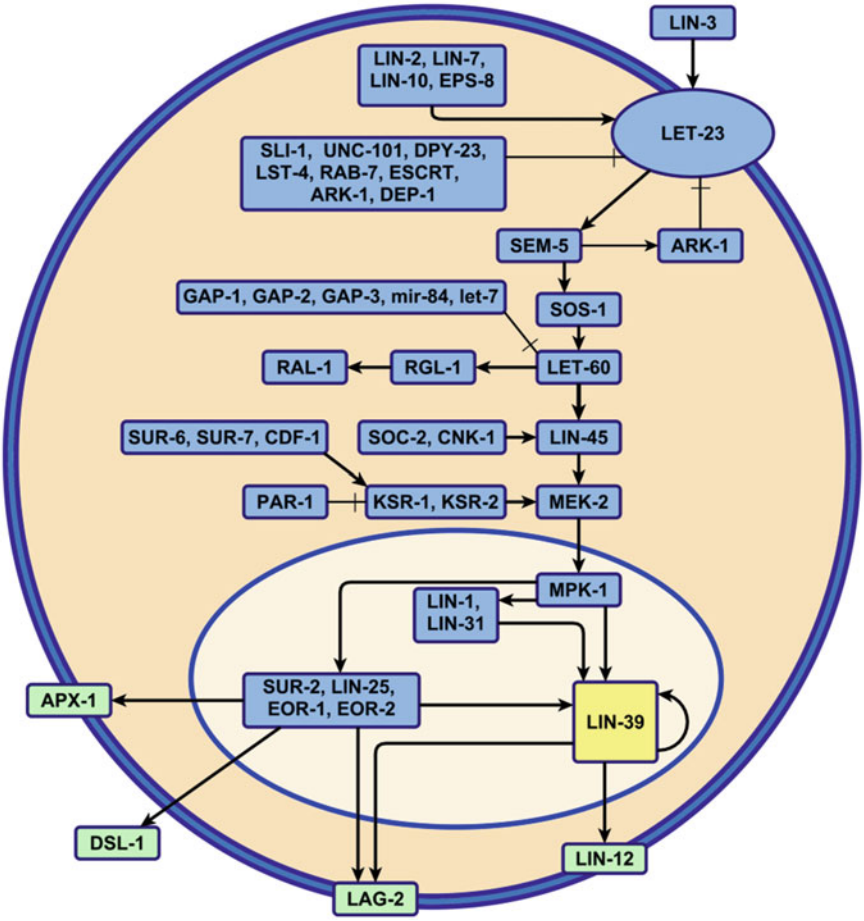

Fig. 2.6 RTK/Ras/ERK signaling in the vulva of *C. elegans. Pointed arrows* represent activating interactions and *blunt arrows* represent inhibitory interactions, *bold arrows* represent active interactions and *thin arrows* represent inactive interactions

by stimulating conversion of LET-60-GDP to LET-60-GTP (Chang et al. 2000). The GTPase Activating Proteins [GAP-1, GAP-2 and GAP-3 (Stetak et al. 2008; Hajnal et al. 1997; Hayashizaki et al. 1998)] stimulate conversion of LET-60-GTP to LET-60-GDP, inhibiting LET-60 function. Furthermore, *let-60* is negatively regulated by two microRNAs: *mir-84* and *let-7* (Johnson et al. 2005).

If the extracellular concentration of LIN-3 is not very high, LET-60-GTP may activate RGL-1, which in turn activates RAL-1, and that promotes secondary VPC fate determination (Zand et al. 2011). Alternatively, if the concentration of LIN-3 is sufficiently high, GTP-bound LET-60 may initiate LIN-45/Raf activation (Han et al. 1993; Hsu et al. 2002). Additionally, LIN-45 is activated by SOC-2 (Yoder 2004) mediated dephosphorylation at certain sites and CNK-1 (Rocheleau et al. 2005)

mediated phosphorylation at other sites. LIN-45 then binds to the scaffold proteins [KSR-1 and KSR-2 (Ohmachi et al. 2002)], that are also activated by SUR-6. KSR-1 and KSR-2 are likely inhibited by PAR-1 and activated by high levels of zinc and the zinc transporter proteins CDF-1 and SUR-7 (Yoder 2004).

The LIN-45/KSR-1/KSR-2 complex phosphorylates and activates MEK-2 (Rocheleau et al. 2005; Wu et al. 1995), which in turn phosphorylates and activates MPK-1 (Lackner and Kim 1998). MPK-1 then moves to the nucleus, where it phosphorylates and activates several target proteins [LIN-1 (Jacobs et al. 1998), LIN-31 (Tan et al. 1998), EOR-1, EOR-2 (Rocheleau et al. 2002; Howell et al. 2010), LIN-39 (Wagmaister et al. 2006a; Eisenmann et al. 1998; Clandinin et al. 1997; Maloof and Kenyon 1998)] and two subunits of the Mediator complex [SUR-2 and LIN-25 (Sundaram 2013; Singh and Han 1995; Tuck and Greenwald 1995; Nilsson et al. 1998)]. Unphosphorylated LIN-1 and LIN-31 inhibit the expression of *lin-39*. Conversely, phosphorylated LIN-1 and LIN-31 are required for the upregulated expression of *lin-39* in P6.p (Wagmaister et al. 2006a, b; Tiensuu 2005; Leight et al. 2015). Phosphorylated LIN-39 activates its own expression (Wagmaister et al. 2006a; Maloof and Kenyon 1998), and the transcription of *lin-12* and *lag-2* (Takács-Vellai et al. 2007). Furthermore, the Mediator complex activates the expression of *apx-1, dsl-1* and *lag-2* (Zhang and Greenwald 2011a; Chen and Greenwald 2004). Additionally, unphosphorylated LIN-1 inhibits the expression of *lag-2* (Zhang and Greenwald 2011b) and the phosphorylation of LIN-1 in P6.p may be necessary to overcome this inhibition.

2.3 Formation and Maintenance of the Vulval Competence Group

When the worm hatches, about 12 h after the egg is laid, it has two rows that contain six ventral P cells each. These epidermal cells are called P1/2, P3/4, P5/6, P7/8, P9/10, P11/12 in anterior to posterior order (Fig. 2.7, 0 h). During the first larval stage (L1) the P cells migrate towards the ventral midline so that 10 h later there is only one row of cells, P1-P12 (Altun and Hall 2009), the migration of P cells towards the ventral midline requires the function of *ref-2*(+) (Alper and Kenyon 2002), *rho-1*(+), *unc-73*(+), *let-502*(+) (Spencer et al. 2001) and *ect-2*(+) (Morita et al. 2005). Following the formation of the single row, when the P cells undergo a longitudinal division, the anterior daughter cells acquire a neuronal fate and detach from the hypodermis, while the posterior daughters acquire a hypodermal fate (Sulston and Horvitz 1977; Chisholm and Hsiao 2012) (Fig. 2.7, 10 h). In parallel, during the second larval stage (L2) Notch signaling specifies which gonadal cell; Z1.ppp or Z4.aaa, becomes the anchor cell (Park et al. 2013) (Fig. 2.7, 12 h).

During both L1 and L2, canonical Wnt and RTK/Ras/MAPK signaling maintain the competence of the vulval precursor cells (VPCs) by inhibiting cell fusion

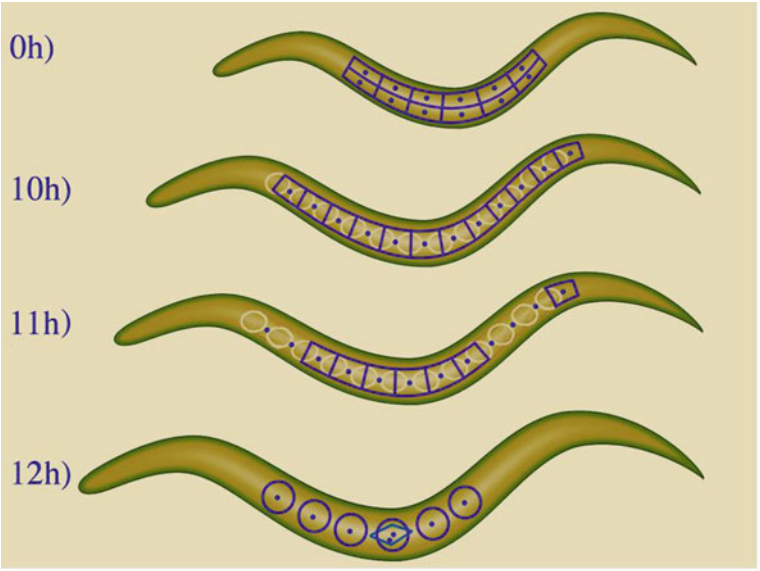

Fig. 2.7 Formation and maintenance of the vulval competence group. In all figures of worms anterior is left unless otherwise specified. Here we show ventral views. Larval phase L1: 0 h) Newly hatched worm with two rows of P cells shown in *purple*, 10 h) Worm with only one row of P cells which divided longitudinally, their anterior daughters are shown in beige and produce a neuronal linage, while the posterior daughters produce a hypodermal linage and are shown in *purple*, 11 h) P1.p, P2.p, P9.p, P10.p and P11.p fuse with hyp7 (only their nuclei, as *purple dots*, are shown), P12.p later becomes hyp12, 12 h) Larval phase L2: The VPCs (Shown in purple, P3.p, P4.p, P5.p, P6.p, P7.p, P8.p) remain unfused and the anchor cell has formed (Shown in *blue*) is positioned dorsal to the P6.p cell

(Eisenmann et al. 1998; Shemer and Podbilewicz 2002), and cell cycle quiescence is maintained by the Cyclin-dependent Kinase Inhibitor CKI-1 (Buck et al. 2009).

2.3.1 *How Are the P Cells Polarized to Form Epidermal and Neuronal Linages?*

The Wnt/β-catenin asymmetry pathway signaling is one of the main mechanisms by which cells are polarized during the development of *C. elegans* (Green et al. 2008; Sawa and Korswagen 2013; Yamamoto et al. 2011) (Fig. 2.4) and it is the main mechanism involved in P cell polarization (Tan 2013).

Multiple genes are likely targets of Wnt/β-catenin asymmetry pathway in P cells and are involved in hypodermal differentiation [e.g. *elt-1, lin-26, elt-3, nhr-25, grh-1* and *nhr-23* (Chisholm and Hsiao 2012)]. *lin-22* and *cbp-1* inhibit the neuronal

fate in some epidermal cells, and the genes *lin-32, hlh-2 and hlh-14* are necessary for neuronal fate specification (Hobert 2010).

2.3.2 Notch Signaling and the Formation of the Anchor Cell

The anchor cell (AC) is the source of the signal (LIN-3/EGF) that induces the VPCs to differentiate and is essential for vulva development. Accordingly, AC ablation prevents the formation of the vulva causing the VPCs to fuse with hyp7 during L1 (Kimble 1981; Sternberg and Horvitz 1986).

Two gonadal cells; namely Z1.ppp and Z4.aaa have the potential to become the AC. The process by which one cell becomes the AC and the other one becomes a VU (ventral uterine) cell during early L2 depends on the order of formation of the cells and the outcome of the competition between the two cells for the expression of LAG-2.

Initially both Z1.ppp and Z4.aaa express LIN-12/Notch and LAG-2/Delta. Any initial difference in *lin-12* activity is amplified because the protein LIN-12 activates the LAG-1/SEL-8 complex, which in turn activates *lin-12* transcription forming a positive feedback loop. The amplified difference in *lin-12* activity, causes one cell to accumulate more LIN-12 in its membrane and stop transcribing *lag-2* and that cell then differentiates into a VU cell. The other cell expresses *lag-2* at a higher level, begins expressing *lin-3,* stops expressing *lin-12*, and becomes the AC (Park et al. 2013).

Another set of two genes with important functions during the formation of the AC are *nhr-67* and *hlh-2 (*which encode a nuclear hormone receptor and helix-loop-helix transcription factor respectively): *nhr-67* is required for the expression of both *lag-2* in the AC and *lin-12* in all three VU cells and their descendants. When *nhr-67* function is compromised, the presumptive VU cell adopts an AC identity (Verghese et al. 2011), *hlh-2* function is required by the AC to express *lag-2* and *lin-3* (Park et al. 2013), and *hda-1*, which encodes a component of NuRd (the nucleosome remodeling and deacetylation complex), is also required for AC differentiation, and functions upstream of *egl-43* and *nhr-67* (Ranawade et al. 2013).

2.3.3 Wnt and RTK/Ras/MAPK Signaling Maintain the Competence of the VPCs

The VPCs are formed during L1 and they must not fuse or differentiate until the end of L2. Canonical Wnt (Sawa and Korswagen 2013) and Ras (Sundaram 2013) signaling maintain the competence of VPCs, mainly by activating the expression of

lin-39 (Eisenmann et al. 1998). The activity of the Hox gene *lin-39* is necessary for the formation and competence of the VPCs and the expression of the Hox genes *mab-5* and *ceh-13* acts as a boundary for the vulval competence group (Tihanyi et al. 2010; Pénigault and Félix 2011b).

During L1, *ref-2*(+) activity is needed to generate Pn.p cells and both *lin-39*(+) and *ref-2*(+) activity is required to repress EFF-1 (Epithelial Fusion Failure-1) and keep Pn.p cells unfused. LIN-39 together with its cofactors CEH-20 and UNC-62, activates the expression of *ref-2*, The posterior VPCs P7.p and P8.p express MAB-5, another Hox gene that activates the expression of *ref-2* (Alper and Podbilewicz 2008; Alper and Kenyon 2002; Shemer and Podbilewicz 2002; Shemer et al. 2004). The migration of P cells toward the ventral cord does not happen in *ref-2* loss of function mutants, in which the P cells may fuse with hyp7, undergo cell death, or divide and then die. Additionally, weak *ref-2(RNAi)* causes P3.p-P6.p to fuse with hyp7 (Alper and Kenyon 2002). The activity of *ref-1*(+) is required by P9.p, P10.p, and P11.p to fuse with hyp7 (Alper and Kenyon 2001). Furthermore, in *lin-39(lf)* single mutants, *eff-1* expression is allowed, causing all Pn.p cells to fuse and contribute their nuclei to the surrounding hypodermis. In *eff-1(lf)* single mutants, none of the cells are able to fuse with the hypodermis (Alper and Podbilewicz 2008). Two GATA-type zinc finger transcription factors, ELT-5/EGL-18 and ELT-6, prevent ectopic Pn.p cell fusion during L2; ELT-5 and ELT-6 are expressed in the VPCs during L2 and loss of both *elt-5* and *elt-6* function results in inappropriate fusion of the vulval precursor cells with hyp7. LIN-39 and CEH-20 are transcriptional regulators of one isoform of *elt-5/egl-18* (Alper and Podbilewicz 2008; Koh et al. 2002). In summary, Wnt and Ras signaling control the activity of numerous transcription factors that maintain VPCs competence and prevent their fusion by repressing the expression of the effector fusion protein EFF-1 during the L1 larval stage.

2.3.4 *The Molecular Mechanism Involved in the Maintenance of Cell Cycle Quiescence During Late L1 and L2*

Developmental timing control is one of the fundamental issues during the formation and growth of biological organisms. Even subtle changes in genes involved in the control of developmental timing can cause lethal defects or produce a phenotype that confers an evolutionary advantage to an organism. In the nematode *C. elegans* the heterochronic genes encode some of the most important components of the molecular mechanism involved in the control of development timing (Moss 2007). The following heterochronic genes are some of the main regulators of vulval developmental timing: *lin-4* encodes a microRNA, *lin-14* (Ruvkun and Giusto 1989) encodes a transcription factor that promotes L1 cell fates, *hbl-1* encodes a transcription factor related to *Drosophila's* hunchback (Fay et al. 1999), and *lin-28* (Moss et al. 1997) encodes a cytoplasmic protein with a cold shock domain and

zinc finger motifs. Both LIN-28 and HBL-1 promote certain aspects of L2 cell fates (Vadla et al. 2012) and loss of *lin-14* or *lin-28* function causes a precocious transition from G1 to S, and early VPC divisions (Schindler and Sherwood 2013).

CKI-1, a *C. elegans* p21/p27 cyclin-dependent kinase inhibitor that inhibits cell cycle progression (Hong et al. 1998), is first expressed in the late L1 stage and is absent when the VPCs divide, and loss of *cki-1* function results in precocious VPC divisions. During L1 and L2, *cki-1* expression is regulated by *lin-14* (Hong et al. 1998), *lin-25, sur-2, mdt-13, mdt-23*, *lin-1* and *lin-31* (Clayton et al. 2008). The proteins SUR-2, LIN-25, LIN-1 (Jacobs et al. 1998) and LIN-31 (Tan et al. 1998) act as effectors of RTK/Ras/MAPK signaling (Sundaram 2013), which is activated during L3 before the VPCs divide, suggesting that Ras signaling is necessary for the activation of the cell cycle in VPCs (Schindler and Sherwood 2013; Clayton et al. 2008). CKI-2 is another *C. elegans* cyclin dependent kinase inhibitor, and the presence of active CKI-2 is enough to cause cell cycle quiescence, the redundancy between CKI-1 and CKI-2 may explain why CKI-1 RNAi only causes one additional round of cell division in VPCs (Buck et al. 2009).

In summary, the worm is born with two rows of P cells that have six cells each, during the first juvenile stage L1 the P cells undergo a *rho-1* mediated migration towards the ventral midline forming one row of P cells (intercalation by convergent extension), then the P cells divide longitudinally, their anterior daughters acquire a neuronal fate and their posterior daughters acquire a hypodermal fate. Some of the Pn.p cells fuse with hyp7. Wnt and RTK/Ras/MAPK signaling maintains the differentiation potential of the VPCs P3.p, P4.p, P5.p, P6.p, P7.p and P8.p in part by inhibiting their fusion with hyp7. One of the main molecular mechanisms involved in the maintenance of cell cycle quiescence during L1 and L2 is the activation of *cki-1* by *lin-14, lin-1* and *lin-31*. After the L2/L3 molt the miRNA *lin-4* inhibits LIN-14 expression and RTK/Ras/MAPK signaling negatively regulates *cki-1* transcription, allowing the progression of the cell cycle in the VPCs.

2.4 Vulval Cell Proliferation and Differentiation

During late L2, after the VPCs form, and Wnt and Ras signaling preserves their competence; the AC differentiates, the heterochronic miRNA *lin-4* is activated and *cki-1* activity is inhibited. At this developmental stage, the vulval precursor cells are ready to respond to the extracellular signals that guide them to differentiate, into a primary, secondary or tertiary fate (Fig. 2.2). The primary fate is characterized by the expression of *egl-17* (Burdine et al. 1998) and the transversal division of its granddaughters. The secondary fate is characterized by the expression of *lin-11* (Gupta and Sternberg 2002) and *lip-1* (Berset et al. 2001) and the diverse planes of division of its granddaughters; the most proximal do not divide, the next most proximal divide transversally and the rest divide longitudinally. The tertiary fate is characterized by one longitudinal division, where the two resulting daughter cells fuse with hyp7 (Sharma-Kishore et al. 1999; Sternberg 2005).

After the VPCs acquire their fate, all of them divide longitudinally once. Primary and secondary fate VPC daughters divide longitudinally again. Later all primary fate granddaughters and two secondary fate granddaughters divide transversally and four secondary fate granddaughters divide longitudinally (Sharma-Kishore et al. 1999) (Fig. 2.2). The resulting 22 cells are induced by the anchor cell, the anal depressor muscle, epithelial cells near the tail (Green et al. 2008; Gleason et al. 2006; Pénigault and Félix 2011b) and each other to differentiate into one of the seven adult vulval cell types (vulA-vulF) (Ririe et al. 2008; Schindler and Sherwood 2013; Gupta et al. 2012).

2.4.1 Current Understanding of VPC Fate Determination

All VPCs have a similar differentiation potential before induction; specifically, a VPC may acquire the primary fate and express *egl-17*, acquire the secondary fate and express *lin-11* and *lip-1*, or acquire the tertiary fate, which does not express any specific markers known to date. Cell ablation experiments have shown that if one VPC is experimentally removed, the nearest neighbor acquires the fate that would correspond to the ablated cell had it not been removed (Sulston and White 1980; Sternberg and Horvitz 1986). Furthermore, if all VPCs except P3.p are ablated, P3.p may acquire the primary or the secondary fate, depending on how far it is located from the AC (Sternberg and Horvitz 1986).

After the AC cell forms during early L2, it begins secreting LIN-3/EGF (Hill and Sternberg 1992; Hwang and Sternberg 2004), MOM-2 and LIN-44 (Green et al. 2008). Soon after, the concentration of those ligands around the nearest VPC (P6.p) rises, resulting in the activation of RTK/Ras/ERK signaling. After the L2/L3 molt, about 25 h post-hatching; P6.p expresses *lag-2, apx-1, dsl-1* (Chen and Greenwald 2004), *lin-39* (Wagmaister et al. 2006b) and the primary fate marker *egl-17* (Fisher et al. 2007; Cui and Han 2003) (Fig. 2.8, top). Canonical Wnt signaling is also required for the determination of the primary fate (Eisenmann et al. 1998).

During the larval stage L2, LIN-14 activity inhibits LIN-12, but in the successive L3 stage, the miRNA *lin-4* is expressed and binds to the mRNA of *lin-14,* targeting it for degradation and that allows Notch signaling to be activated (Li and Greenwald 2010). The secondary fate is redundantly induced by Notch and Ras signaling; three hours after acquiring the primary fate, neighboring VPCs may induce the determination of the secondary fate by expressing one of three DSL ligands; explicitly, APX-1 and LAG-2 stay in the membrane of the VPC that expresses them, which means that the neighbor VPC must be in physical contact with the cell in order to induce it. However, DSL-1 may act at a distance because it is secreted (Hoyos et al. 2011). After the DSL ligands bind they activate Notch signaling which directly activates the transcription of several genes that are called lateral signal targets, such as the secondary fate marker *lip-1* (Sundaram 2005b) and also directly or indirectly activates the expression of the secondary fate marker *lin-11* (Marri and Gupta 2009; Gupta and Sternberg 2002) (Fig. 2.8, bottom).

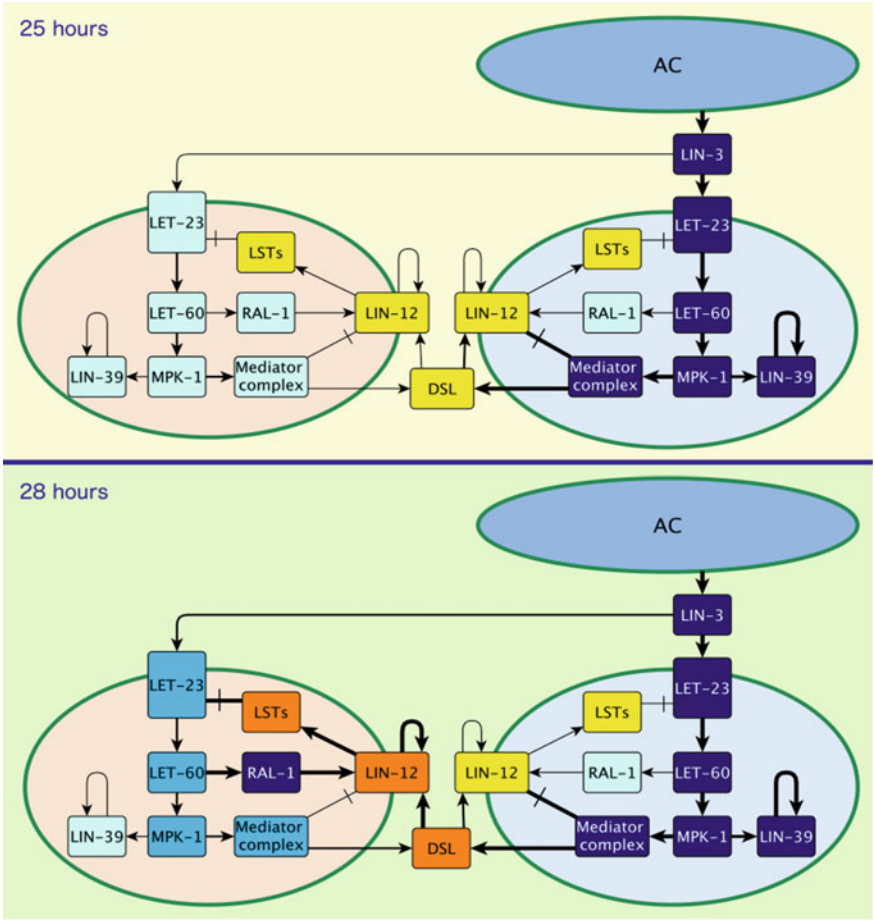

Fig. 2.8 The mechanism involved in VPC fate determination: *Pointed arrows* represent activating interactions and *blunt arrows* represent inhibitory interactions. The width of an *arrow* represents its activity level. The components of RTK/Ras/ERK are shown in *blue*, the darker the *blue* is; the more active the component is. The components of the Notch signaling pathway are shown in orange if they are active, if they are not active, they are shown in yellow. Twenty-five hours after the birth of the worm the VPC P6.p (*light blue*) responds to the inductive signal and begins expressing primary fate markers and DSL ligands, Notch signaling is inhibited and the primary fate is stabilized by the self-activation of LIN-39. Three hours later P5.p (*light orange*) and P7.p (not shown) respond to mild inductive signaling and the lateral signal from P6.p, acquire the secondary fate, express the lateral signal targets and inhibit RTK/Ras/ERK signaling. The secondary fate is stabilized by LIN-12 self-activation

Two possible molecular mechanisms may explain how an isolated VPC, in an extracellular microenvironment with a moderate concentration of LIN-3 can acquire the secondary fate: the moderate concentration of LIN-3 may activate Ras signaling but instead of activating LIN-45/Raf, LET-60/Ras activates RGL-1 (Omitted in

Fig. 2.8 for simplicity) which in turn activates RAL-1. RAL-1 directly or indirectly activates the expression of the lateral signal targets (Zand et al. 2011). Another option is that the isolated VPC begins to secrete DSL-1, forming an autocrine loop that activates Notch signaling and the expression of the lateral signal targets (Hoyos et al. 2011).

Several positive feedback circuits stabilize the vulval fates; specifically, in the primary fate, phosphorylated LIN-39 activates its own transcription (Wagmaister et al. 2006a; Maloof and Kenyon 1998). In secondary fate cells the LIN-12/LAG-1/SEL-8 complex activates *lin-12* and *lag-1* transcription (Christensen et al. 1996; Wilkinson et al. 1994; Choi et al. 2013; Park et al. 2013). Then LIN-12 activates *mir-61* transcription, which causes VAV-1 down-regulation, and as a result promotes *lin-12* activity (Yoo and Greenwald 2005). RTK/Ras/ERK and Notch signaling inhibit each other in all VPCs (Yoo et al. 2004; Sundaram 2005b); RTK/Ras/ERK inhibits Notch when the Mediator complex which is one of the main effectors of RTK/Ras/ERK, promotes the endocytosis of LIN-12 (Shaye and Greenwald 2005). Notch inhibits RTK/Ras/ERK because several of the lateral signal targets inhibit different components of RTK/Ras/ERK; in particular LIP-1 negatively regulates the activity of MPK-1 (Sundaram 2005a; Berset et al. 2001) and ARK-1 inhibits LET-23 (Hopper et al. 2000). It is not known precisely which of the molecules that compose RTK/Ras/ERK are targeted for inhibition by *lst-1, lst-2, lst-3, lst-4* and *dpy-23* (Yoo et al. 2004).

The models of the molecular network that controls VPC fate specification together with the data obtained by cell ablation experiments as well as forward and reverse genetics studies, make VPC fate determination one of the best-known processes of cell differentiation. Both the sequential control mechanism (Simske and Kim 1995) and the gradient-based mechanism (Katz et al. 1995) for the control of VPC fate specification exist and are sufficient for the correct differentiation of VPCs (Fig. 2.8).

2.4.2 VPC Polarization and Longitudinal Divisions

The network of molecules involved in the control of the cell cycle, is interconnected with the network of molecules involved in the control of VPC fate determination, and both processes are synchronized (Euling and Ambros 1996; Nusser-Stein 2012).

During L3, *lin-4* undermines cell cycle quiescence by inhibiting the translation of LIN-14, because LIN-14 positively regulates the transcription of *cki-1* (Kirienko et al. 2010). Moreover, unphosphorylated LIN-1 and LIN-31 also positively regulate *cki-1*, and both LIN-1 and LIN-31 are phosphorylated by Ras signaling further weakening cell cycle quiescence (Clayton et al. 2008). As a result, a short time after the fates of the VPCs are determined, the VPCs divide longitudinally once.

The gradients of four Wnt ligands determine the polarity of the VPCs: First EGL-20 is secreted by cells located in the tail exposing the posterior end of the

VPCs to a higher concentration of EGL-20. Thus establishing what is described as the ground polarity of the VPCs via the Ror receptor tyrosine kinase CAM-1 (Forrester et al. 1999) and the Planar Cell Polarity component Van Gogh/VANG-1 (Green et al. 2008). The AC secretes LIN-44 and MOM-2 forming a gradient of both ligands (Green et al. 2008). The sex myoblasts (SM) that require EGL-17/FGF from P6.p to migrate toward the correct location that is dorsal from the AC, secrete CWN-1 (Minor et al. 2013). CWN-1, LIN-44 and MOM-2 bind to LIN-17 and LIN-18, activating the Wnt/β-catenin asymmetry pathway and reversing the polarity of P7.p, which now has an anterior facing polarity referred to as "refined polarity" and strengthening the posterior polarity of P5.p (Green et al. 2008) (Fig. 2.4).

After the secondary fate VPCs are polarized and following the L2/L3 molt, the VPCs undergo a longitudinal division, the two daughters of P6.p both express *egl-17* (Burdine et al. 1998). Wnt signaling from the AC and lateral signaling from the primary fate neighbors, up regulate the expression of *lin-11* and *lip-1*. As a result, P5.pp and P7.pa, the proximal daughters of secondary fate cells express *lin-11* (Gupta and Sternberg 2002) and *lip-1* (Berset et al. 2001) at a higher level than the distal daughters P5.pa and P7.pp (Fig. 2.4).

Subsequently, the daughters of the tertiary fate VPCs; namely, P3.p, P4.p and P8.p fuse with hyp7. P6.pa and P6.pp do not fuse with hyp7 because Ras and Wnt signaling via ELT-6, EGL-18 and LIN-39 inhibits the transcription of EFF-1 (Alper and Podbilewicz 2008), additionally, LIN-39 activates the transcription of *egl-18* and *elt-6*, and ELT-6 positively regulates the transcription of *lin-39* forming a positive feedback loop (Liu 2014). The mechanism that precludes the daughters of second fate cells from fusing with hyp7 is less clear, but two processes are likely to be involved, first moderate Ras and Wnt signaling from the AC may suffice to inhibit EFF-1; second, in some *lin-12* gain of function mutants, the anchor cell does not form and all the VPCs acquire the secondary fate, and those secondary fate cells do not fuse with hyp7. The molecular mechanism mediating *eff-1* inhibition may involve Notch signaling due to the fact that some regulatory regions of *eff-1* contain candidate LAG-1/CSL binding sites. Additionally, Notch signaling inhibits *eff-1* during the formation of the digestive tract of *C. elegans* (Rasmussen et al. 2008).

Furthermore, the three CDK/Cyclin complexes that are the main regulators of cell cycle progression regulate Notch signaling as well. Specifically, the function of the CDK-4/CYD-1 complex that is needed for G1 progression inhibits the endocytosis of LIN-12 (NOTCH), stabilizing its localization on the plasma membrane; the CDK-2/CYE-1 complex functions to allow the G1/S transition and inhibit the proteolysis of LIN-12-intra, a fragment of LIN-12 that functions as a transcription factor in the nucleus. The activity of the CDK-1/CYB-3 complex is required for the G2/M transition and also activates the export from the nucleus and the degradation of LIN-12-intra (Nusser-Stein 2012).

In summary, the heterochronic gene *lin-14* and the transcription factors LIN-1 and LIN-31 keep the cell cycle quiescent during L2. During L3 *lin-4* microRNA inhibits *lin-14* function and RTK/Ras/ERK signaling phosphorylates LIN-1 and LIN-31, allowing the VPCs to divide longitudinally. RTK/Ras/ERK and Notch

signaling inhibit the fusion of primary and secondary fate VPC daughters respectively, tertiary fate daughters fuse with hyp7, and the daughters of the VPCs that do not fuse with hyp7 divide longitudinally.

2.4.3 *The Third Division of the VPCs and Differentiation of Adult Vulval Cells*

After the second longitudinal division, the patterns of gene expression of the granddaughters of the VPCs are almost the same as those of their parent cells and different from those of their daughters (Table 2.1). Yet at this stage the granddaughters of the VPCs are assigned a fate; from proximal to distal from the center of the developing vulva, P6.pap and P6.ppa, are assigned the vulF fate, P6.paa and P6.ppp are assigned the vulE fate, P5.ppp and P7.paa are assigned the vulD fate, P5.ppa and P7.pap are assigned the vulC fate, P5.pap and P7.ppa are assigned the vulB fate and P5.paa and P7.ppp are assigned the vulA fate (Sharma-Kishore et al. 1999; Schindler and Sherwood 2013; Gupta et al. 2012) (Fig. 2.2).

The third division of some of the granddaughters of the VPCs (Fig. 2.9) occurs during the L3/L4 molt; specifically, first the vulE precursor cells divide transversally, then, the anterior vulA and vulB precursors divide longitudinally, next, the posterior precursors of vulA and vulB divide longitudinally, and last, the precursors of vulC and vulF divide transversally forming the characteristic pattern of cell division "LLTN TTTT NTLL" where T represents a transversal division, N stands for no division and L represents a longitudinal division (Sharma-Kishore et al. 1999).

The third division of the granddaughters of secondary fate VPCs requires *cog-1* and *bed-3* activity (Inoue and Sternberg 2010), and *bed-3* positively regulates *lin-39* transcription (Liu 2014). The mechanism that controls the direction of the third division is poorly understood; specifically, loss of function of both Rac-like GTPases *ced-10* and *mig-2* or the guanine nucleotide exchange factor (GEF) *unc-73*/Trio or *lin-40*/MTA, a component of the NuRD complex, causes vulC and vulE cells to divide longitudinally or obliquely instead of transversely. Additionally, some *lin-11* mutations also cause vulC and vulD cells to divide longitudinally (Kolotuev and Podbilewicz 2008), furthermore, Wnt signaling regulates both the expression of *lin-11* (Marri and Gupta 2009) and spindle rotation of the EMS and ABar blastomeres (Hardin and King 2008), this together with the fact that several Wnt ligands are expressed by the AC and vulval cells suggests that Wnt signaling may also function in the determination of the direction of the division of VPC granddaughters, but this has yet to be proven experimentally.

The transcription factors *cog-1, egl-38, lin-11, lin-29, nhr-67* and *zmp-1* regulate each other forming part of the gene regulatory network that is involved in the determination of adult vulval fates (Ririe et al. 2008; Fernandes and Sternberg 2007; Schindler and Sherwood 2013; Gupta et al. 2012). Unfortunately our

Table 2.1 The patterns of expression of the important transcription factors, or cell fate markers: *lin-39* (Wagmaister et al. 2006b), *nhr-67* (Fernandes and Sternberg 2007), *cog-1* (Palmer et al. 2002), *zmp-1* (Wang and Sternberg 2000; Kirouac 2003), *egl-38* (Rajakumar and Chamberlin 2007), *lin-11* (Gupta and Sternberg 2002), *lip-1* (Berset et al. 2001) *and egl-17* (Burdine et al. 1998) in vulval cells during L3, L4 and adulthood (Gupta et al. 2012)

	vulA	vulB1	vulB2	vulC	vulD	vulE	vulF
LIN-11	L3, early/mid L4	L3, early/mid L4	L3, early/mid L4	L3, early/mid L4	L3, early/mid L4		
LIP-1	Late L3	Late L3	Late L3	Late L3	Late L3		
LIN-39	L4					Late L3	Late L3
COG-1	Early L4	Early L4	Early L4	Early/mid L4	Early/mid L4	Late L3, early/mid L4	Late L3, early L4
EGL-17				Late L4, adult	Late L4, adult	Late L3	Late L3
ZMP-1	Adult				Late L4, adult	Late L4, adult	
EGL-38							Mid/late L4
NHR-67	Late L4, adult	Late L4, adult	Adult	Adult	Adult		

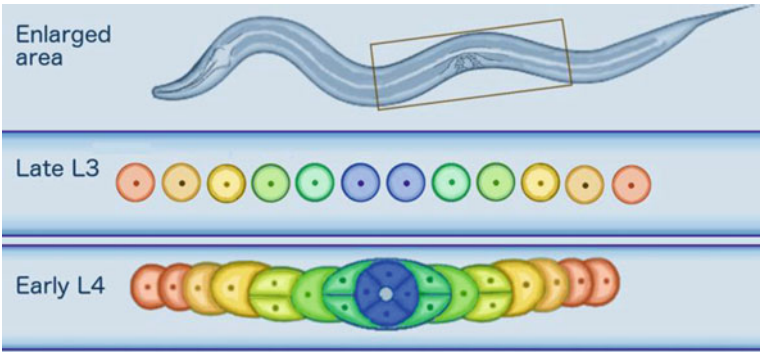

Fig. 2.9 Third division of the vulval cells: late L3 is a side view following the second division and early L4 is a ventral view after the third division. Color code is as in Fig. 2.2

knowledge about this network is not sufficient to build a model with a dynamic behavior that produces seven different stable patterns of gene expression that correspond to all the vulval cell types (Fig. 2.10).

A summary of vulval cell proliferation and differentiation: After the L2/L3 molt, the vulval precursor cells respond to the extracellular signals that guide them to differentiate, into a primary, secondary or tertiary fate. Then, all VPCs divide longitudinally once, later the tertiary fate cells fuse with hyp7 and the remaining VPC daughters divide longitudinally again. After that, vulD cells do not divide, all vulC, vulE and vulF precursor cells divide transversally and all vulA, vulB1, and vulB2 precursor cells divide longitudinally (Sharma-Kishore et al. 1999) (Figs. 2.7 and 2.9). The resulting 22 cells induce each other and respond to signals from the surrounding tissue to differentiate into vulA, vulB1, vulB2, vulC, vulD, vulE, and vulF cells (Ririe et al. 2008; Schindler and Sherwood 2013; Gupta et al. 2012).

2.4.4 VPC Fate Determination Research and Modeling

VPC fate determination is one of the best examples of cell-to-cell induction (Gilbert 2013), and has been studied more than any other stage of vulval development. Before the details about the signaling pathways involved where known, diagrammatic models of the process contributed a lot to our understanding of VPC fate determination (Sternberg and Horvitz 1986, 1989).

Sternberg and Horvitz (Sternberg and Horvitz 1986) proposed two important diagrammatic models for VPC fate determination; (a) the gradient model, where a gradient of inductive signal from the anchor cell induces the closest VPC to the anchor cell (AC), to acquire the primary vulval fate, and the next nearest cells are induced to acquire the secondary vulval fate, and (b) the sequential model according to which, fate determination happens in two stages, first the AC induces

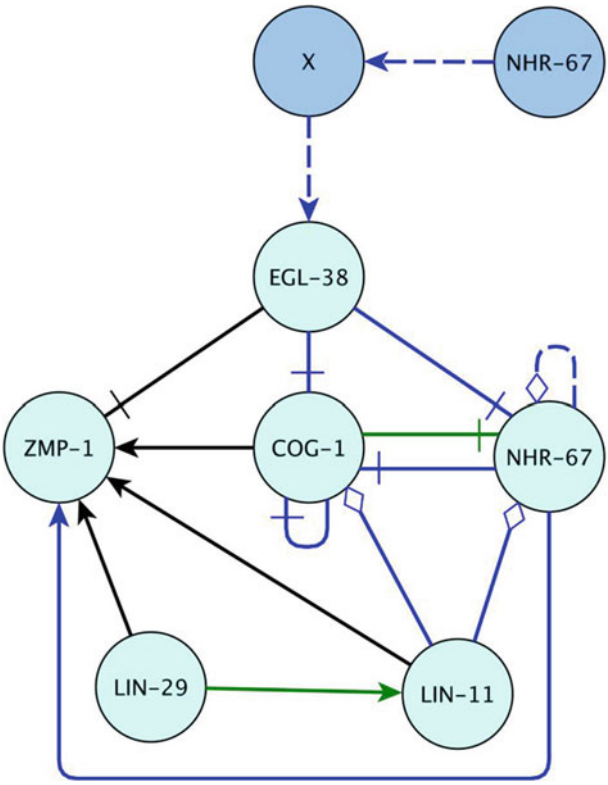

Fig. 2.10 Model of the gene regulatory network involved in vulval cell differentiation: regular *arrowheads* represent activation, *blunt arrows* represent inhibition and *white*, *rhomboid arrowheads* represent both inhibition and activation in different cell types, *discontinuous arrows* represent predictions. The *black*, *blue* and *green* interactions in the figure are supported by evidence from Ririe et al. (2008), Fernandes and Sternberg (2007), Inoue et al. (2005) respectively

the nearest VPC to acquire the primary fate, and second, the cell that acquired the first fate induces its neighbors to acquire the secondary vulval fate by means of a lateral signal.

Another diagrammatic model of vulval fate determination was the result of an effort to integrate the effects of single mutations reported by other studies (Ferguson and Horvitz 1985; Greenwald et al. 1983) and some double mutants, which the authors observed experimentally (Sternberg and Horvitz 1989). This model combined a graded inductive signal from the AC with a lateral signal and its receptor, LIN-12, and predicted the existence of mutual inhibition between the primary, secondary and tertiary fates.

After the two modeling efforts mentioned above, the need to find the molecules involved in the different signaling pathways involved in VPC fate determination, and the epistatic order of those molecules within the pathways was very clear. The

research effort that followed lead to a much better understanding of RTK-Ras-ERK (Sundaram 2013), FGF (Sundaram 2013), Notch (Greenwald and Kovall 2002), and Wnt (Sawa and Korswagen 2013) signaling pathways, and the crosstalk between them (Myers and Greenwald 2007; Sundaram 2005a; Minor et al. 2013; Takács-Vellai et al. 2007). These important signaling pathways, and the crosstalk between them form a molecular regulatory network. This system contains many positive and negative feedback circuits, and dynamic models are required to understand its complex behavior.

After more information about the signaling pathways was available, some modeling efforts focused on the importance of the sequential control during VPC fate determination (Kam et al. 2003; Fisher et al. 2005, 2007; Kam et al. 2008; Nusser-Stein 2012), in one of the studies, where the authors designed a dynamic model based on the diagrammatic model proposed by Sternberg and Horvitz in 1989 (Sternberg and Horvitz 1989), the dynamic behavior of the model emphasized the importance of time control during VPC fate determination. The model even required the existence of a mechanism that prevents the neighbors of a differentiating VPC from differentiating at the same time (bounded asynchrony) (Fisher et al. 2005). In 2007 Fisher et al, extended their model by including several crosstalk mechanisms between the RTK-Ras-ERK and Notch signaling pathways, which resulted in a model that is more robust to variations in synchronicity between VPCs. The researchers demonstrated experimentally the existence of a three-hour time delay between the determination of the primary fate and the determination of the secondary fate. In another effort (Nusser-Stein 2012), the authors demonstrated that the cell cycle and Notch signaling are coordinated by three molecular interactions. Finally, Fisher et al. used the cell cycle as a scheduler (the part of the model that controls timing of events) for another dynamic model of VPC fate determination.

Other modeling efforts have focused on the importance of the gradients of concentration of the inductive and lateral signals. These models used a set of differential equations to explore the way in which the mutual inhibition between the two signaling pathways affects the dynamic behavior of the system (Giurumescu et al. 2006, 2009; Hoyos et al. 2011; Corson and Siggia 2012). Giurumescu et al. (2006), proposed that coupling inductive and lateral signals amplifies cellular perception of the inductive signal gradient and polarizes lateral signaling, both of which enhance fate segregation beyond that achievable by an uncoupled system. In their following modeling effort (Giurumescu et al. 2009), the same authors built a multicellular version of their model that included the six VPCs, and used the parameters of the model to build a phenotype phase diagram, where each point represents a different fate pattern, such as the wild type, which is (3, 3, 2, 1, 2, 3). The authors used the model to predict several possible fate patterns, for example, they predict the most likely phenotypes for different levels of inductive signal, and additionally their model offers an interesting evolutionary perspective, because changing the parameters of the model it is possible to simulate the process of fate determination in the related nematodes *C. briggsae* and *C. remanei*.

Hoyos et al.'s model (2011) was the first to propose that the network of molecules involved in VPC fate determination may use a morphogen-based

and/or a sequential mechanism of induction. It was also the first model to include a mechanism, which allows an isolated VPC to acquire a secondary fate; the isolated VPC, in an extracellular micro-environment with a moderate concentration of inductive signal, begins secreting DSL-1, which is, one of the main components of the lateral signal. Next, the isolated VPC responds to the lateral signal by acquiring the secondary fate. The authors then proved experimentally that *dsl-1(lf)* reduced the likelihood that an isolated VPC may acquire the secondary fate. Changing the parameters of this model it is also possible to simulate the process of fate determination in the related nematodes *C. brenneri, C. briggsae* and *C. remanei,* offering an interesting evolutionary perspective. The authors proposed that the differences between the species are due to changes in the dynamics of gene expression, as opposed to changes in the topology of the network.

The large amount of experimental information with regards to the network of molecules involved in VPC fate determination has made the system very appealing to test novel modeling techniques for biological molecular networks (Sun and Hong 2007; Kam et al. 2008; Bonzanni et al. 2009; Li et al. 2009; Fertig et al. 2011; Corson and Siggia 2012). One of these models (Corson and Siggia 2012) is particularly interesting because it allows the simulation and visualization of the commitment of the VPCs to their fates. Specifically, the authors designed and implemented a methodology to build an epigenetic landscape of the process of VPC fate determination. Epigenetic landscapes (Ferrel 2012) attach a certain potential for differentiation to each pattern of molecular activity of a cell that can be visualized as a three dimensional surface, that looks like the topology of a certain terrain, which includes mountains that represent undifferentiated cells, flat valleys that represent differentiated cells, and barriers that separate the states that have the potential to differentiate into one type of cell from those that have the potential to differentiate into other kinds of cells. Additionally, it is possible to visualize the effect of mutations that change that landscape and analyze the changes.

Weinstein and Mendoza (2013) decided to encompass in another model only one cell and its extracellular microenvironment in order to include additional relevant molecules and interactions; specifically, they included Wnt signaling to simulate the mechanism that maintains VPC competence during L2, and the polarization of P5.p and P7.p, and also the signaling pathways involved in the control of cell fusion (Fig. 2.11). They formalized the model as a set of multivalued logic functions. With this model it is possible to simulate the dedifferentiation and trans-differentiation of the primary and secondary vulval fates that have been observed experimentally (Wang and Sternberg 1999). They also show that either a gradient of inductive signal or a delay between the inductive signal and the lateral signal are sufficient for the control of fate determination, they included Ras effector switching (Zand et al. 2011) as the mechanism that allows an isolated VPC to acquire the secondary fate, and they proposed that the self activation of *lin-39* requires MPK-1 mediated phosphorylation of LIN-39. In 2015, they published another model (Weinstein et al. 2015) to explore the dynamic effect of the mechanism for the cross-regulation

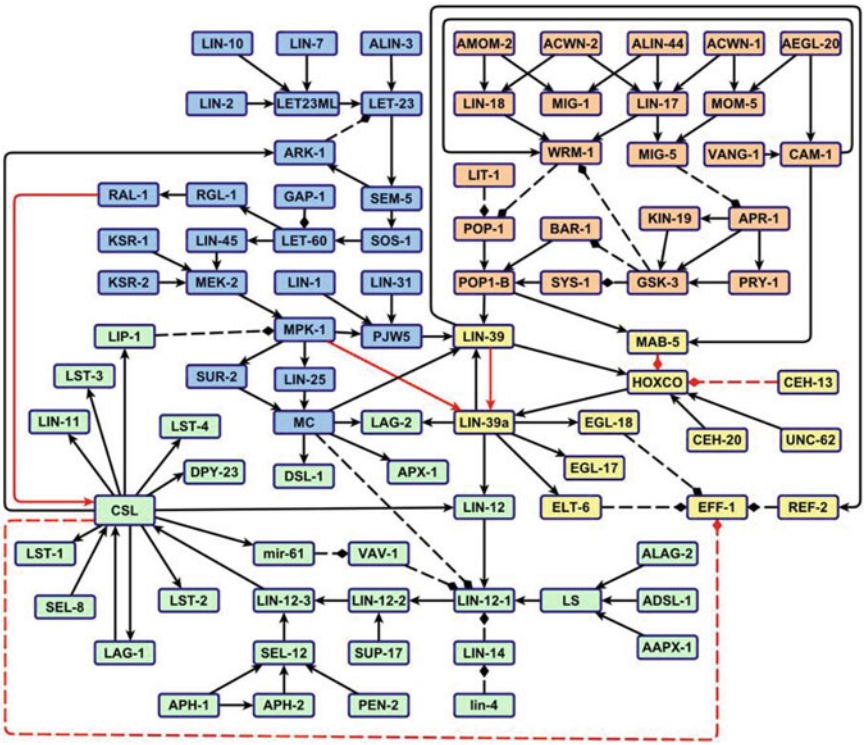

Fig. 2.11 The topology of the model proposed by Weinstein and Mendoza (Weinstein and Mendoza 2013): *Pointed*, *continuous arrows* represent activating interactions, and *blunt*, *discontinuous arrows* represent inhibitions. *Red arrows* represent predictions supported by the model. RTK/Ras/ERK is shown in *blue*, Wnt in *orange*, Notch in *green* and the Hox genes and other molecules involved in the control of cell fusion in *yellow*

between the cell cycle and the cell fate determination of VPCs (Nusser-Stein 2012). The model includes two modules. The first module is a simplified version of the network of molecules involved in VPC fate determination, including the mutual inhibition between RTK-Ras-ERK and Notch signaling pathways and effector switching. The second module includes the main molecules involved in the control of cell cycle progression. Both modules are formalized as a set of multivalued logic functions. The cell cycle module was also formalized as a set of ordinary differential equations to better validate cyclic behavior. The main finding of this work is that the interconnection between Notch signaling and the CDK/Cyclin complexes, functions to reset fate determination after each cell division. According to this model, there is no need for a sequential control of fate determination and existing sequential control may confer robustness to fate determination.

In summary, several diagrammatic and dynamic models of VPC fate determination, some that emphasize the importance of sequential control and others that

emphasize the importance of the gradients of the inductive signals, have contributed a lot to our understanding of the molecular process involved in the control of VPC differentiation.

2.5 Vulval Morphogenesis

Vulval morphogenesis involves three separate and coordinated processes: (i) Formation of the uterine-vulval connection, (ii) Migration of the vulval cells to the center of the developing vulva, invagination and formation of the vulval toroids, (iii) Attachment of the vulval muscles to the vulva and innervation (Fig. 2.1, before eversion), leading to the formation of a functional adult vulva (Sharma-Kishore et al. 1999; Lints and Hall 2009; Schindler and Sherwood 2013; Schmid and Hajnal 2015; Gupta et al. 2012).

2.5.1 Formation of the Uterine-Vulval Connection

First the basement membranes of the AC and the adjacent VPCs are attached together by HIM-4 (Hemicentin). Intracellular VAB-10A (plakin) and PAT-3/INA-1 (Integrin) fasten HIM-4 to the region, and promote hemicentin basement membrane linkage formation (Morrissey et al. 2014).

The AC initiates the degradation of the basement membrane separating it from the daughters of P6.p during the L3 stage. This cell invasion process depends on an AC autonomous signaling cascade, of which some important transducers have been identified; specifically, FOS-1A, one isoform of a leucine zipper transcription factor encoded by *fos-1* (Sherwood et al. 2005) and its known targets which include CDH-3, ZMP-1, EGL-43, HLH-2, MIG-6, HIM-4 and MIG-10B (Schindler and Sherwood 2011; Klerkx et al. 2009; Wang et al. 2014), (Fig. 2.12). VRK-1, which is expressed during L3 by P6.p, regulates AC invasion independently of *fos-1*. *vrk-1* inhibition is required by the AC to invade the granddaughters of P6.p, and *vrk-1* up regulates *egl-17* expression in P6.p (Klerkx et al. 2009) (Fig. 2.12). However, neither *egl-17(lf)* nor loss of function of its receptor, *egl-15(lf)* cause defects in AC invasion. The invasion cue (secreted by P6.p and attracting AC invasion) remains unknown. It is proposed that this cue from P6.p affects the formation and polarization of the membrane protrusion (invadosome) derived from the AC.

The ventral nerve cord (VNC) releases the ligand UNC-6/Netrin that regulates AC by directing its receptor UNC-40, and their effectors: the two Rac GTPases CED-10 and MIG-2, UNC-34 (Ena/VASP), UNC-115 (*ABLIM1*/limain), MIG-10B (lamellipodin), F-actin, $PI(4,5)P_2$, and HIM-4 (hemicentin) to the invasive cell membrane (Fig. 2.12) (Wang et al. 2014; Ziel et al. 2009). The *mig-10b* isoform regulates AC invasion through a mechanism that does not depend on UNC-6/UNC-40 signaling. The localization of UNC-40 and its effectors: MIG-10B,

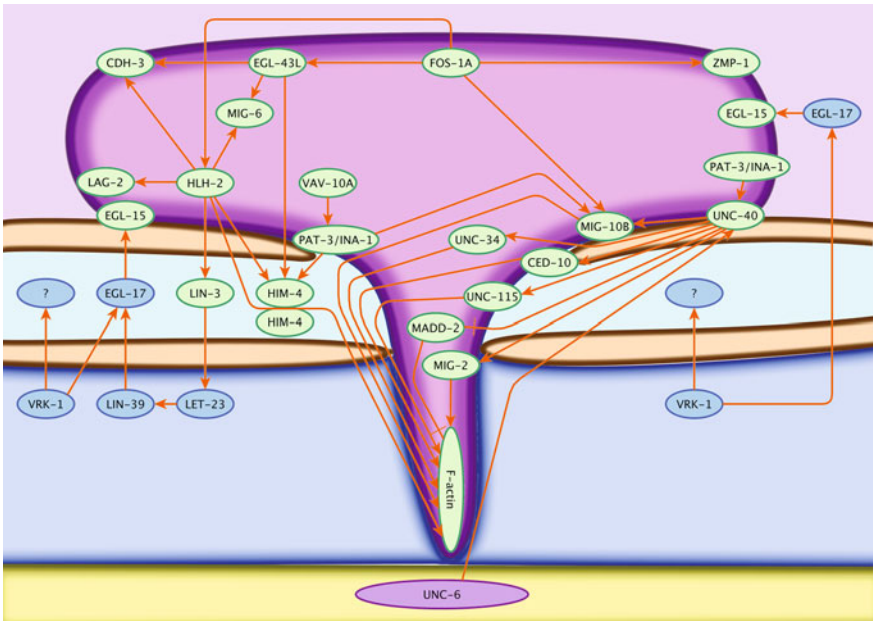

Fig. 2.12 A model summarizing the molecular mechanism involved in anchor cell invasion: The AC is shown in purple and the molecules expressed within the AC that are required for invasion in *light green*, P6.pap, P6.ppa and the molecules expressed in those cells and involved in AC invasion in *light blue*, the ventral nerve cord and UNC-6 that is secreted by the ventral cord neurons in *light purple*

MIG-2, UNC-34 and UNC-115 on the invasive cell membrane also depends on the function of the integrin heterodimer receptor INA-1/PAT-3 that has a scaffolding role at the invasive membrane (Wang et al. 2014).

The *Caenorhabditis elegans* homolog of the Opitz syndrome gene, madd-2/Mid1 represses the intrinsic invasive capacity of the AC and prevents the formation of ectopic protrusions, one of the main functions of UNC-6/Netrin and the vulval guidance cues may be overcoming the inhibitory activity of MADD-2 locally. Notably, the ectopic protrusions that form in the absence of MADD-2 function compete with the forming invadosome for factors; such as regulators of actin polymerization and as a result decrease the overall efficiency AC invasion (Morf et al. 2013).

During the L3 larval stage the AC expresses LAG-2 and induces the six adjacent VU cells to differentiate into π (pi) cells via Notch signaling (Newman et al. 1995), instead of becoming ρ (rho) cells and then the six π cells divide to form 12 cells. After the AC invades between the P6.p cell descendants, the AC and the eight nearest π cells express *aff-1* and fuse to form the utse syncytium (the hymen) (Sapir et al. 2007). The AC nucleus is necessary within the utse syncytium for proper utse development and the following genes: *fos-1, cdh-3, him-4, egl-43, zmp-1* and *mig-10,* that are required for AC invasion, also promote utse cell growth towards the

seam cells (Ghosh and Sternberg 2014). The uterine toroids and the sex myoblasts also affect utse development (Ghosh and Sternberg 2014).

The π cells that do not fuse with the AC to form the utse cell are induced by LIN-3 secreted by vulF that activates RTK/Ras/ERK signaling in the four π cells, which are the nearest to the developing vulva, transforming them into uv1 cells (Chang et al. 1999). Both the expression of LIN-3 in vulF cells, and the correct localization of uv1 cells depend on EGL-38 function (Chang et al. 1999). The gene *egl-38* is expressed in both uv1 cells and vulF cells, and *egl-38*(+) function is necessary for prospective uv1 cells to respond to the LIN-3 signal, or to otherwise acquire the uv1 fate (Rajakumar and Chamberlin 2007). The uv1 cells help connect the uterus with the vulva by forming adherens junctions with the utse and the vulF vulval cells (Lints and Hall 2009). The uv1 cells are the most likely source of the neurotransmitter tyramine that plays a specific role in the inhibition of egg laying (Alkema et al. 2005). It is important to note that the vulF cells must express LIN-3 during uv1 cell differentiation, if vulva development and somatic gonad development are not well coordinated because of a mutation such as the loss of function of *lin-28* (Moss et al. 1997; Euling and Ambros 1996), the connection between the vulva and uterus does not form correctly and that causes an *Egl* phenotype.

In *egl-26* mutant animals, vulF cells adopt an abnormal morphology that causes the formation of a thick layer of vulval tissue at the apex of the vulva that blocks the passage from the uterus to the vulva. EGL-26 is expressed in vulE cells and is localized at the apical membrane that contacts vulF cells. It is likely that vulE cells use EGL-26 to instruct morphological changes in the neighboring vulF cells (Hanna-Rose and Han 2002) and EGL-26 membrane localization is necessary for its function (Estes et al. 2007). When *egl-26* is mutated, vulF cells retain their expected pattern of gene expression, the polarity of vulF cells is normal, vulF–uterine cell–cell signaling capabilities are maintained and the AC invasion is not affected. All this suggests that *egl-26* mutations specifically affect vulF cell shape (Estes et al. 2007).

2.5.2 *Migration of the Vulval Cells Towards the Center of the Developing Vulva, Invagination and Formation of Seven Stacked Vulval Toroids*

The migration of the vulval cells towards the center of the developing vulva, the formation of the seven vulval toroids and the invagination of the vulva, all lead to the formation of a channel that connects the uterus to the exterior of the worm. These three related processes require the vulval cells to remodel their cytoskeleton and undergo shape changes (Sharma-Kishore et al. 1999).

Few known molecular pathways connect fate specification and morphogenesis. One such pathway, involves primary fate cells, including the granddaughters of

P6.p where LIN-39 activates the transcription of *vab-23*, which promotes *smp-1* transcription (Pellegrino et al. 2011; Pellegrino and Hajnal 2012). The semaphorin protein encoded by *smp-1* is transported to the apical membrane, where it is required for the migration of vulval cells towards the center of the developing vulva. All vulval cells express the plexin ortholog *plx-1* that encodes the receptor for SMP-1 and PLX-1 expressing cells migrate towards SMP-1 expressing cells. After a vulval cell contacts an SMP-1 expressing cell, the signal-receiving cell begins expressing *smp-1* and transporting SMP-1 to the lumen facing membrane. SMP-1 expression and migration towards the center of the developing vulva are propagated to adjacent vulval cells (Dalpé et al. 2005). Once a cell that expresses PLX-1 reaches an SMP-1 expressing cell, the cell that expresses PLX-1 stops migrating, preventing vulval cells from sliding past each other (Liu et al. 2005). The effectors of PLX-1/SMP-1 include the Rac-like GTPases CED-10 and MIG-2, and their GEF UNC-73 (Schindler and Sherwood 2013).

While the vulval cells migrate towards the center of the developing vulva, the cells on each side send projections towards the same type of vulval cells at the other side of the developing vulva, forming a series of concentric rings (toroids) that stack and generate a tubular structure (Fig. 2.2) (Sharma-Kishore et al. 1999; Dalpé et al. 2005; Liu et al. 2005). After the formation of the rings, some of the cells within the rings fuse in the following order: vulD, vulA, vulC, vulF and vulE. vulD is a binucleate ring and the pair of cells within vulB1 and vulB2 rings remain unfused, the other rings are tetranucleate (Sharma-Kishore et al. 1999). Two fusogens (fusion proteins) mediate cell fusion in the vulval rings; specifically, EFF-1 is expressed by vulA (where it causes vulA cells to fuse on each side before forming rings, before LIN-39 is expressed and inhibits EFF-1). EFF-1 is required for intratoroidal fusions in vulC vulE and vulF cells (Pellegrino et al. 2011; Shemer and Podbilewicz 2002). The second fusion protein AFF-1 is needed in the precursors of vulA and vulD cells to fuse them and generate stable toroidal syncytia (Sapir et al. 2007; Alper and Podbilewicz 2008).

It is not clear how *eff-1* and *aff-1* expression is controlled in the different vulval rings; *aff-1* expression is activated by FOS-1 which is expressed in all vulval cells (Sapir et al. 2007), LIN-29 is a candidate *aff-1* expression regulator in VPCs because LIN-29 is needed for *aff-1* expression in seam cells (Friedlander-Shani and Podbilewicz 2011). However LIN-29 is not required to express *aff-1* in π or utse cells (Sapir et al. 2007). Neither pharyngeal pm8 nor intestinal valve vpi1 expressed *aff-1*::GFP in *lag-1* mutants, which suggests that the LIN-12/LAG-1/SEL-8 transcriptional complex also positively regulates *aff-1* expression in the digestive tract that also forms toroidal structures (Rasmussen et al. 2008). The expression of *eff-1* is negatively regulated by VAB-23 in the granddaughters of P6.p (Pellegrino et al. 2011), and Notch signaling inhibits *eff-1* expression in the digestive tract. Therefore it is likely that it also inhibits *eff-1* expression in the granddaughters of P5.p and P7.p (Rasmussen et al. 2008). After the L3/L4 molt, when the vulval rings form, we do not know how the expression of *eff-1* is regulated.

During a screen for mutations that affect the invagination of the *C. elegans* vulva, Herman et al. (1999) discovered that *sqv-1* to *sqv-8* (squashed vulva) loss of

function cause a partial collapse of the vulval lumen and an elongation of the central vulval cells. The space between the vulval cells and the cuticle is considerably smaller in the Sqv mutants than in the wild type. These mutations do not prevent the formation of vulval toroids.

The cell surface and surrounding extracellular matrix is composed of a large repertoire of glycans attached to both proteins and lipids. Glycosaminoglycans (GAGs) are long unbranched polymers with specific repeating disaccharides; one sugar is usually a uronic acid (e.g., glucuronic acid) and the other is either N-acetylglucosamine or N-acetylgalactosamine. All *sqv* genes have been shown to control the biosynthesis of the glycosaminoglycans chondroitin or heparan sulphate and probably function in the Golgi apparatus (Bulik et al. 2000; Hwang and Horvitz 2002a, b; Hwang et al. 2003a, b).

The regulation of UDP-glucuronic acid production in a specific subset of vulval cells helps determine the shape of the vulva (Hwang and Horvitz 2005b) and defective glycosaminoglycan formation in *sqv* mutants might lead to collapse of the vulval structure (Bulik and Robbins 2002). Invagination is initiated in the *sqv* mutants but the lumen does not increase in volume. One model that explains how the *sqv* genes work assumes that the proteoglycans, that bind large amounts of water, are secreted into the vulval lumen, creating osmotic pressure that expands the lumen. Another possibility is that loss of chondroitin sulphate in the *sqv* mutants increases adhesion between vulval cells, thereby preventing expansion of the vulval space (Herman et al. 1999).

Notch and RTK/Ras/ERK signaling differentially regulate the force-generating actin myosin network to shape the vulva. Unphosphorylated LIN-1 activates the expression of the RHO kinase LET-502 in the vulA, vulB1, vulB2, vulC and vulD toroids, while Notch signaling inhibits the phosphorylation of LIN-1. LET-502 induces actomyosin-mediated contraction of the apical lumen in the vulA-vulD toroids, thereby generating a dorsal pushing force. In contrast, RTK/Ras/ERK signaling inhibits LET-502 RHO kinase expression in the vulE and vulF toroids by phosphorylating LIN-1 to prevent toroid contraction and allow the AC to expand the dorsal vulval lumen (Farooqui et al. 2012).

In summary, LIN-39 activates the expression of VAB-23. VAB-23 activates the expression of SMP-1 and vulval cells that express SMP-1 attract adjacent vulval cells that express PLX-1. EFF-1 is needed for the intratoroidal fusions of vulC vulE and vulF cells and AFF-1 is needed for the intratoroidal fusions of vulA and vulD cells. The SQV genes are necessary to allow the volume of the vulval lumen to increase. Additionally, RTK/Ras/ERK signaling inhibits LET-502 RHO kinase expression and inhibits the contraction of the vulE and vulF toroids. In contrast, Notch signaling causes the contraction of vulA-vulD toroids by allowing the expression of LET-502. Thus, compared to the earlier stages of vulva development that are very well characterized, we only have a partial outline of the molecular players required for morphogenesis of the vulva.

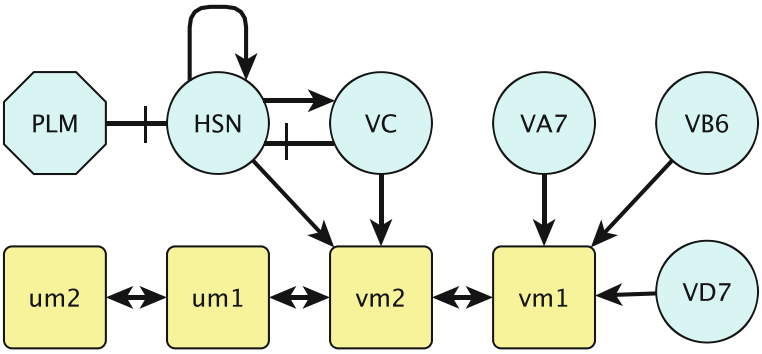

Fig. 2.13 The neural circuit involved in egg laying control: *pointed arrows* represent activating interactions and *blunt arrows* represent inhibitory interactions. The vulval and uterine muscle cells are shown in *yellow*, the PLM sensory neuron is shown as a *blue octagon*, and the motor neurons are shown as *blue circles*

2.5.3 *Migration, Attachment and Innervation of Muscle Cells*

The neural circuit involved in the control of egg laying is among the simplest in *C. elegans*. Each time the worm lays eggs, the vulval muscles contract, causing a transient opening of the vulva that allows eggs to be expelled. The neural circuit is composed of the four uterine muscles (um1 and um2), the four vulval muscles (vm1 and vm2) and two sets of neurons (two hermaphrodite-specific motor neurons (HSNs) and six ventral chord neurons VC1-VC6) (Lints and Hall 2009; White 1986).

The vulval muscle cells are derived from two sex myoblast (SM) cells, which are located at the posterior of the animal during the L1 stage and migrate anteriorly during L2 and L3 to flank the developing gonad near the vulva region (Sulston and Horvitz 1977). Following migration, the SM cells each divide three times in the L3 stage to generate 16 cells. Of these muscles, the um cells, induce contractions that move eggs through the uterus. The other eight cells, four vm1 and four vm2, extend processes in a diagonal configuration that contact the vulval lips and control opening during mating and egg laying. The four vm2 cells connect between the uterus and vulF and the ventral body wall; the four vm1 cells connect between vulC and vulD toroids and the ventral body wall (Lints and Hall 2009).

The neural circuit involved in egg laying control (Fig. 2.13) is connected as follows: The uterine muscles and the vulval muscles are electrically coupled with each other; HSNs directly excite the vm2 s and VC motor neurons. The VC neurons excite the vm2 vulval muscles and inhibit the HSNs. The motor neurons VA7, VB6 and VD7 excite vm1 cells, but their role in egg laying control is not known (Lints and Hall 2009; White 1986). Notably, the HSNs are active in the absence of synaptic input, suggesting that autonomous HSN activity may control egg laying

(Zhang et al. 2008). Additionally, body touch excites the posterior sensory neurons that transduce touch stimuli (PLM) and may inhibit egg laying, in part by interfering with HSN calcium oscillations (Zhang et al. 2008).

Summary of the molecular mechanisms involved in the control of vulval morphogenesis: During the larval stage L3 the AC invades between P6.pap and P6.ppa in a process mediated by *fos-1,* and the AC induces the six adjacent VU cells to differentiate into π (pi) cells via Notch signaling, then the six π (pi) cells divide, after that the AC and the eight nearest π cells express *aff-1* and fuse to form the utse syncytium. The four-π cells nearest to the vulva do not fuse with the AC to form the utse cell; instead they are induced by LIN-3 secreted by vulF cells to become uv1 cells. Vulva development and somatic gonad development must be coordinated to allow the connection between the vulva and uterus to form correctly and avoid an *Egl* phenotype.

Proximal vulval toroids express *smp-1* to attract more distal vulval cells that express *plx-1*. Once the cells are near the center of the developing vulva EFF-1 is needed for the intratoroidal fusions of vulC vulE and vulF cells and AFF-1 is needed for the intratoroidal fusions of vulA and vulD cells.

Two sex myoblast (SM) cells migrate anteriorly during L2 and L3 towards the vulva region. Then, the SM cells each divide three times in the L3 stage to generate 16 cells, eight become uterine muscle (um1 and um2) cells, and eight become vulval muscle cells (vm1 and vm2). The uterine muscles and the vulval muscles are electrically coupled with each other; the HSN neurons directly excite the vm2 vulval muscles and VC motor neurons, and HSN neurons may autonomously excite themselves. VC neurons excite the vm2 vulval muscles and inhibit the HSNs. Additionally; body touch may inhibit HSN excitation through the sensory neuron PLM.

In summary, the cellular and molecular mechanisms of vulval morphogenesis are just emerging and involve concerted regulation of multiple signaling pathways, cell migration, cell fusion and invasion of the vulval primordium by the anchor cell.

2.6 Insight into the Evolution of Vulva Development

The knowledge about *C. elegans* vulva development together with the ease with which many related species can be cultured under laboratory condition make the vulva a superb model for the study of the evolution of development (Sommer 2005). Vulval development has been studied and compared in many varieties of *C. elegans* (Pénigault and Félix 2011a; Delattre and Félix 2001), and at least 51 rhabditid species (Kiontke et al. 2007). In this section we review how some characteristics vary between the rhabditids. When possible we also address the molecular changes that may cause those differences, and how those traits are affected by natural selection, sexual selection and genetic drift.

2.6.1 Variation in the Size of the Vulval Competence Group

One of the vulval development traits that vary between different nematode species is the size and composition of the vulval competence group. A cell forms part of the vulval competence group if it normally acquires a vulval fate or it is capable of replacing an ablated cell that normally acquires a vulval fate.

All the nematodes studied so far have 12 Pn.p cells of which P5.p, P6.p and P7.p always acquire vulval fates and form part of the vulval competence group. In some species P3.p, P4.p and P8.p form part of the vulval competence group (Kiontke et al. 2007). At least two cellular mechanisms may remove cells from the vulval equivalence group; specifically, fusion with hyp7 during L1 and programmed cell death.

In *C. elegans*, the competence of P3.p and P4.p depends on the concentration of EGL-20 and CWN-1, two Wnt ligands expressed near the posterior end of the worm that are secreted to form a gradient (Pénigault and Félix 2011a, b). The concentration of the Wnt ligands around P3.p is enough to allow it to be competent in half of all N2 worms, in other varieties of *C. elegans* the frequency of P3.p competence varies between 15 and 59 % (Delattre and Félix 2001), with an average for the species as a whole of 33 % (Pénigault and Félix 2011a).

In many of the closely related *Caenorhabditis* species, such as *C. briggsae, C. remanei* and *C. brenneri*, the frequency of P3.p competence is lower than 24 %, while in other *Caenorhabditis* species such as C. *japonica* and *C. drosophilae*, the frequency of P3.p competence is almost 100 % (Pénigault and Félix 2011a). In *C. briggsae,* sometimes the competence of P4.p is lost (Braendle et al. 2010), in the rhabditids belonging to the genus *Oscheius* P3.p does not form part of the vulval competence group the frequency of P4.p and P8.p competence varies between species, and is highly polymorphic in different strains of *O. tipulae* (Delattre and Félix 2001; Felix 2006). The diplogastrid nematode *Pristionchus pacificus* has a very reduced vulval competence group formed by P5.p, P6.p and P7.p, and its non-vulval epidermal cells P(1-4,9-11).p undergo programmed cell death (Sommer and Sternberg 1996).

In summary it is likely that the vulval competence group of the stem rhabditid, was composed of 5 cells (P4.p-P8.p). In the basal *Caenorhabditis* species it grew to 6 cells (P3.p-P8.p) and in some *Caenorhabditis* species it has shrunk back to 5, In the basal diplogastrid it shrunk to 4 cells (P3.p-P8.p) and then it shrunk again in *P. pacificus* and other species to include 3 cells (P5.p-P7.p) (Kiontke et al. 2007).

Environments where sometimes P5.p, P6.p or P7.p is lost may confer a higher fitness to a larger vulval competence group. However, the short lifespan of *C. elegans* and other related nematodes might prevent the selection of mechanisms that allow the worms to regenerate. Healing mechanisms may require more flexibility and reversibility in cell fate specification and that may cause developmental problems.

2.6.2 Reproductive Barriers

The process of speciation begins when two different populations of one species are separated by physical barriers such as the separation of an island from a continent and then they adapt to their environment through natural selection. When one environment includes many niches that require different adaptations; once the population includes several varieties of individuals, reproductive barriers between the varieties may appear and then, the varieties become species that evolve separately (Mendelson et al. 2007).

Mating between different species of *Caenorhabditis* nematodes causes the sterilization of maternal individuals. The sperm cells from other species induce sterility and shorten the lifespan of maternal individuals by invading the ovary, and occasionally, other tissues. When males from a species with females breed with hermaphrodites from another species the damage tends to be stronger (Ting et al. 2014). The damage caused to hermaphrodites by inter-species mating suggests that adaptations that prevent inter species mating would increase the fitness of hermaphrodites, although males from different *Caenorhabditis* nematode species readily mate with maternal individuals from other species (Ting et al. 2014). Mating is a very complex behavior (Sherlekar and Lints 2014) and differences in the shape and location of the vulva may function as reproductive barriers in rhabditid nematodes.

According to the ring hypothesis in vulva development (Kolotuev and Podbilewicz 2008; Kiontke et al. 2007) the direction of the last division of the VPCs determines the number of rings that form the vulva: longitudinal divisions lead to the formation of two rings; transverse, oblique, or no divisions lead to the formation of one ring. Very little is known about the molecular mechanism involved in the control of the plane of the last division of the VPCs in *C. elegans* (see Sect. 2.4.3), and studying this molecular mechanism in different species may be very informative in the future.v

In all rhabditids studied so far, the vulA cells never form more than one ring because even if they divide longitudinally, the two daughter cells fuse before they get near enough to the center of the developing vulva to produce an additional ring. Also, in all rhabditids studied so far, vulD, vulE and vulF cells divide transversally, or do not divide at all and produce one ring each. The number of rings produced by vulB and vulC varies. The total number of rings varies between 6 and 8 (Kolotuev and Podbilewicz 2004; Podbilewicz 2008).

Another trait that may function as a reproductive barrier between species is the location of the vulva. *C. elegans,* and most other rhabditids are didelphic (have two ovaries) and their vulva is located in the middle of the body. Monodelphy has evolved separately six times. In most cases where monodelphy has evolved, a more posterior location of the vulva has also evolved and a more posterior vulva has not evolved in any known didelphic species. Additionally, monodelphy may favor the evolution of a posterior vulva, and a posterior vulva may favor the evolution of gonad-independent vulva induction (Kiontke et al. 2007).

In summary, the variation in both the number of rings that form the vulva, and its location along the anterior-posterior axis have the potential to function as reproductive barriers between different rhabditids.

2.6.3 Induction by Wnt Signaling

The vulva of *C. elegans* and other closely related *Caenorhabditis* species, such as *C. briggsae, C. remanei* and *C. brenneri* is induced by *lin-3,* a member of the epidermal growth factor (EGF) family that is expressed in the gonadal anchor cell (Hoyos et al. 2011; Félix 2012). *Pristionchus pacificus* vulva formation relies on continuous gonadal induction secreted by the AC and other gonadal cells that lasts more then 10 h (Sigrist and Sommer 1999).

In *C. elegans*, one of the main targets of vulval induction is the homeotic gene *lin-39* (Yi and Sommer 2007). In *C. elegans* maintains the competence of VPCs by controlling cell fusion, while in *P. pacificus, lin-39* maintains the competence of VPCs by activating *pax-3* and inhibiting programed cell death (Yi and Sommer 2007). However *lin-39* is required for vulval induction in *C. elegans* (Clark et al. 1993; Clandinin et al. 1997; Maloof and Kenyon 1998) but not in *P. pacificus* (Sommer et al. 1998). It has been suggested that in *P. pacificus* Wnt signaling and not Ras signaling induces vulva formation, the evidence supporting this it that the loss of function of *bar-1* and other genes that form part of the Wnt signaling pathway cause a very penetrant Vul phenotype and so far no evidence for EGF/RAS signaling in *P. pacificus* vulva induction exists (Tian et al. 2008). The molecular mechanism of vulval induction in *P. pacificus* is still not known as well as that of *C. elegans.*

The function of the gene *egl-17* is required to attract the sex myoblasts to their precise final positions in both *C. elegans and P. pacificus* (Photos et al. 2006). In *C. elegans lin-39* activates the transcription of *egl-17* (Cui and Han 2003), and *vab-23* (Pellegrino et al. 2011; Pellegrino and Hajnal 2012), those are two of the main interactions linking cell fate specification and morphogenesis in *C. elegans.* If in *P. pacificus lin-39* is not required for induction, how are VPC differentiation and vulval morphogenesis linked in this organism?

The main goal in studying vulval development is to understand how the molecular mechanisms that direct the formation of this organ work to develop a functional vulva. After 40 years of research, the vulva still has many open questions to analyze (Schmid and Hajnal 2015).

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