Protein kinase VRK-1 regulates cell invasion and EGL-17/FGF signaling in

*Caenorhabditis elegans*

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Abstract

The Vaccinia-Related Kinases (VRKs) are highly conserved throughout the animal kingdom and phosphorylate several chromatin proteins and transcription factors. In early Caenorhabditis elegans embryos, VRK-1 is required for proper nuclear envelope formation. In this work we present the first investigation of the developmental role of VRKs by means of a novel C. elegans vrk-1 mutant allele. We found that VRK-1 is essential in hermaphrodites for formation of the vulva, uterus, utse, and for development and maintenance of the somatic gonad and thus the germ line. VRK-1 regulates anchor cell polarity and the timing of anchor cell invasion through the basement membranes separating vulval and somatic gonadal cells during the L3 larval stage. VRK-1 is also required for proper specification and proliferation of uterine cells and sex myoblasts. Expression of the Fibroblast Growth Factor-like protein EGL-17 and its receptor EGL-15 is reduced in vrk-1 mutants, suggesting that VRK-1 might act at least partially through activation of FGF signaling. Expression of a translational VRK-1::GFP fusion protein in the ventral nerve cord and vulva precursor cells restores vulva and uterus formation, suggesting both cell autonomous and non-autonomous roles of VRK-1.

Key Words: Anchor cell, Caenorhabditis elegans, Cell invasion, Cell polarity, Cell signaling, FGF, Uterus, Vaccinia Related Kinase, vrk-1, Vulva.
Introduction

Organ development requires sophisticated temporal and spatial control of gene expression and cell signaling to achieve the proper balance between cell proliferation and specification. The nematode Caenorhabditis elegans makes an excellent model organism for studying determination of cell fates and pattern formation during development. For instance, formation of the egg laying organs involves precisely orchestrating several conserved signaling cascades, such as the receptor tyrosine kinase (RTK)/Ras GTPase/MAPK, Notch and Wnt pathways (Sternberg, 2005).

Post-embryonic development of C. elegans occurs in 4 larval stages, L1-L4, each separated by a molting period. In hermaphrodites, formation of the somatic reproductive organs, the vulva, uterus and gonads, starts during the L1 larval stage and continues through L4 until adulthood (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977). During L1 and L2 larval stages, 6 of the 11 ventrally located epidermal Pn.p cells are specified as vulva precursor cells (VPCs) by LET-60 (Ras) and Wnt signaling acting on the Hox gene lin-39. During L3 larval stage, 3 central VPCs (P5.p-P7.p) receive an inductive signal from the anchor cell (AC), a specialized gonadal cell (Hill and Sternberg, 1992; Kimble, 1981). The AC expresses and secretes the epidermal growth factor (EGF)-like ligand LIN-3 that activates the EGF receptor LET-23 in the underlying VPCs. P6.p receives the highest level of LIN-3 and takes on a 1º fate, characterized by activation of the LET-60 signaling cascade (Sternberg and Horvitz, 1986). P6.p produces multiple ligands, such as DSL-1, LAG-2 and APX-1 (Chen and Greenwald, 2004; Shaye and Greenwald, 2002), which activate the LIN-12 (Notch) signaling pathway in the flanking P5.p and P7.p (Sternberg, 1988) and downregulate LET-60 signaling (Berset et al., 2001; Yoo et al., 2004). This results in the specification of a 2º fate of P5.p and P7.p (Sternberg, 1988; Yoo et al., 2004). The remaining 3 VPCs (P3.p, P4.p and P8.p) adopt a 3º fate and thus fuse with the syncytial epidermis hyp7. P5.p, P6.p and P7.p
undergo three rounds of division during the L3 and L4 larval stages to generate 22 vulval cells of seven different cell types. The vulval cells invaginate and undergo homotypic fusion to form seven distinct toroids. These toroids connect to the uterus, and evert as the hermaphrodite molts into adulthood (Sharma-Kishore et al., 1999). Formation of a connection between the vulva and the uterus is initiated in early L3 larval stage. The AC invades the basement membrane separating the gonad from the underlying developing vulva and attaches to two of the granddaughters of the central VPC, P6.pap and P6.ppa (Sherwood and Sternberg, 2003). The AC will stay at this position until the L4 larval stage when it fuses with 8 π cells of the uterus and laterally attaches to seam cells to form the utse syncytium (Newman et al., 1996). Invasion of the AC is initiated by an invasion cue emitted by the 1º fated vulva cells (Sherwood and Sternberg, 2003). This cue provides guidance to the AC for correct spatial and temporal invasion of the basement membrane, but its identity remains unknown. In addition, secretion of UNC-6 (Netrin) from the ventral nerve cord (VNC) is important for AC invasion (Ziel et al., 2009). An AC autonomous signaling cascade has been discovered, of which several important transducers have been characterized. *fos-I* encodes two isoforms of a basic region-leucine zipper transcription factor, FOS-1A and FOS-1B. Only FOS-1A activity is required for basement membrane removal and AC invasion (Sherwood et al., 2005). Known FOS-1A targets include CDH-3, a member of the cadherin superfamily, ZMP-1, a matrix metalloproteinase and EGL-43, a zinc finger protein (Hwang et al., 2007; Rimann and Hajnal, 2007; Sherwood et al., 2005). Simultaneous with the development of the vulva, a uterus lumen is formed that is flanked distally at each side by a spermatheca and gonadal arm. The gonad is a tube-shaped organ that starts at the spermatheca and extends towards the head or the tail end in L2/L3 larval stage (Kimble and Hirsh, 1979). The gonad makes a loop before reaching head or tail end and extends back towards the center of the animal, where both gonadal arms meet.
The Vaccinia-Related Kinase (VRK) family of proteins, an early branch from the casein kinase I family, is present in all metazoan kinomes (Klerkx et al., 2009; Nichols and Traktman, 2004). *C. elegans* and *Drosophila* have a single ortholog whilst the genomes of vertebrates contain 3 different VRK genes, *VRK1*-3. Human VRK1 phosphorylates several well-known transcription factors, such as tumor suppressor p53 (Lopez-Borges and Lazo, 2000), c-Jun (Sevilla et al., 2004a) and ATF2 (Sevilla et al., 2004b). Alteration of VRK1 activity prevents proper cell cycle progression (Valbuena et al., 2008) and has been linked to cancers (Santos et al., 2006). VRK3 acts negatively on ERK signaling through activation of VHR phosphatase (Kang and Kim, 2006). Purification of an activity that phosphorylates the tail of histone H2A led to the discovery of *Drosophila Nucleosomal Histone Kinase-1* (NHK-1), the fruit fly homolog of VRK (Aihara et al., 2004). Finally, biochemical studies and analysis of early *C. elegans* embryogenesis have identified the small chromatin associated protein BAF as a phosphorylation substrate for VRK-1 (Gorjanacz et al., 2007; Nichols et al., 2006). The nature and diversity of identified VRK substrates suggest that this family of protein kinases may play important roles in several developmental processes. Indeed, *nhk-1* mutations disrupt proper meiosis in *Drosophila*, which at the same time has prevented elucidation of phenotypes later in development (Cullen et al., 2005; Ivanovska et al., 2005).

We have previously described a role for VRK-1 in nuclear envelope formation in early, fast-dividing *C. elegans* embryos (Gorjanacz et al., 2007). To elucidate the function of VRK-1 in post-embryonic development we have characterized a *vrk-1* mutant allele and found defects in formation of the reproductive organs. Our analysis has shown that VRK-1 is essential for formation of a normal vulva, uterus, and utse, and for development and maintenance of the somatic gonad. Mutation of *vrk-1* impinges on FGF signaling, which may partly explain the defects in vulval and uterine development.
Materials and methods

**Strains and alleles**

*C. elegans* nematode strains were cultured and handled under standard conditions (Stiernagle, 2006) at 20°C. We used strain N2 (Bristol) as wild-type reference. Other alleles and transgenes used in this study: AH142 zhIs4[pTB10 (P_{lip-1}::nls::gfp)] (Berset et al., 2001), GS384 fos-1(ar105) (Seydoux et al., 1993), MT2124 let-60(n1046) (Ferguson and Horvitz, 1985), NH2466 ayIs4[P_{egl-17}::nls::gfp] (Burdine et al., 1998), NH2447 ayIs2[P_{egl-15}::nls::gfp] (Harfe et al., 1998), NK409 qyls50[P_{cdh-3}::mCherry::moeABD] (Ziel et al., 2009), PD4810 ccIs4810[P_{inn-1}::lmn-1::gfp] (Liu et al., 2000), PS3352 syls50[P_{cdh-3}::gfp] (Inoue et al., 2002), PS3808 syls80[P_{inn-1}::nls::gfp] (Gupta and Sternberg, 2002), PS4308 syls107[P_{inn-1}::gfp] (Hwang and Sternberg, 2004), PS4384 syls113[P_{ost-1}::ost-1/SPARC::gfp] (Hwang et al., 2007), PS4411 syls123[P_{fos-1}::yfp::fos-1A], (Sherwood et al., 2005), PS5443 [P_{egl-43}::nls::yfp] (Hwang et al., 2007), VB1497 ayIs4; rrf-3(pk1426); sid-1(qt2) (Tiensuu et al., 2005). BN3 vrk-1(ok1181)mIn1[mls14 dpy-10(e128)] II was generated by backcrossing VC704 from the International *C. elegans* Gene Knockout Consortium to the wild-type N2 6 times. BN3 was further crossed with aforementioned strains (see Suppl. Table I).

**Microscopy**

Microscopy analysis including differential interference contrast (DIC) and fluorescent protein imaging was done mainly on a Leica SP2 confocal microscope with 100x 1.40 – 0.7 oil CS objective. Animals were mounted in a 5 μl drop of 10 mM levamisole on a 3% agarose pad, covered with a 24 mm x 24 mm coverslip, which was sealed with Vaseline.

**Software analysis and statistics**
Images were captured using Leica Confocal Software LAS AF. ImageJ was used to measure fluorescent intensities of individual nuclei or cells in single focal sections. Statistical analysis was performed using Microsoft Excel. Equal adjustment of control, mutant and RNAi images was done using Adobe Photoshop.

**RNAi**

For RNA interference by feeding, clones were obtained from the library described by (Kamath and Ahringer, 2003). RNAi was achieved by feeding L4 staged animals for 24 hours at 20°C on bacterial lawns expressing double-stranded RNA for the targeted gene. The empty pPD129.36 L4440 feeding vector was used as control. F1 offspring were evaluated when reaching the correct stage.

Knockdown of *vrk-1* expression in vulva precursor cells was performed by tissue-specific expression of a *vrk-1* RNA hairpin in VB1497 *ayIs4; rrf-3(pk1426); sid-1(qt2)* animals (Tiensuu et al., 2005). Plasmid pBN30 containing the *lin-31* promoter upstream of a 791 bp *vrk-1* exon 4 inverted repeat separated by intron 2 from the *eat-2* gene was injected together with plasmid pRF4 rol-6 into the germ line of VB1497 young adults. Transgenic animals were identified based on the dominant Rol phenotype.

**Transgenesis**

A *P_{vrk-1::VRK-1::GFP}* construct was made by PCR amplification and stitch technique. 870 bp of genomic *vrk-1* DNA upstream of the *vrk-1* start codon was used as promoter sequence. The *vrk-1* stop codon was replaced by GFP with a stop codon, followed by 1494 bp of *vrk-1* 3’UTR sequence. The construct was introduced into the pSTW destination vector containing an *unc-119* rescuing allele by Gateway cloning. The final plasmid was bombarded into *unc-119(ed3)* worms, which generated two integrated lines, YL255 vrIs13 and YL262 vrIs15. Of
these, $vrIs13$ shows the strongest expression of VRK-1::GFP and was used for the experiments shown in Figs. 3, 6 and Suppl. Figs. 1, 2. $vrIs15$ was used for the experiments shown in Suppl. Fig. 3.
Results

Mutation of vrk-1 interferes with vulval development

To investigate the role of vrk-1 in postembryonic development we characterized a novel vrk-1 allele, ok1181, isolated by the International C. elegans Gene Knockout Consortium. The wild-type vrk-1 gene encodes a 67 kDa protein of 610 amino acids that has an N-terminal serine/threonine protein kinase domain and a C-terminal basic-acidic-basic (BAB) motif (Aihara et al., 2004; Klerkx et al., 2009). The ok1181 allele deletes 1139 bp from the second intron to the fifth exon, corresponding to ~30% of the protein kinase domain and the entire BAB motif. To determine whether the C-terminus of the VRK-1 protein is expressed in ok1181 mutants, we cloned the ok1181 mRNA by RT-PCR. This revealed that exon 2 is spliced to exon 6 in ok1181 mutants, thus skipping exon 5, but maintaining the reading frame (Fig. 1A). Conceptual translation of the ok1181 mRNA predicts a protein of 31 kDa. However, we have not been able to detect any truncated VRK-1 protein by Western blot analysis of ok1181 extracts (data not shown), suggesting that the amount of mutant VRK-1 protein expressed in ok1181 animals is below the detection level of our antibodies.

Depletion of VRK-1 by RNAi leads to early embryonic death (Gorjanacz et al., 2007; Piano et al., 2002). Homozygous vrk-1(ok1181) animals produced by heterozygous vrk-1(ok1181) hermaphrodites (denoted hereafter as vrk-1 mutants) overcome this lethality presumably by maternal contribution and show no apparent defects until early L3 larval stage. The gonads in vrk-1 mutants extend and loop back but do not reach the fully extended stage (Fig. 1B). The germ line contains fewer and larger, abnormal nuclei (Fig. 1B). Upon reaching adulthood, the gonads are devoid of germ nuclei, resulting in 100% sterility (data not shown). In addition, vrk-1 mutants do not form a uterus lumen at the L4 stage (n>100) nor a uterus seam syncytium (utse) (Fig. 1C). When reaching adulthood, 75% (n>200) of the vrk-1
mutants have a strong protruding vulva (Pvl) phenotype (Fig. 1D), presumably as consequence of lacking utse formation (Hanna-Rose and Han, 1999).

**VRK-1 regulates timing of anchor cell invasion**

As the *vrk-1* mutants show a highly penetrant Pvl phenotype, we sought to investigate vulval development. To evaluate vulval induction we generated a *vrk-1* mutant strain expressing GFP in the anchor cell (AC) under control of the *lin-3* promoter. We observed no change in relative expression of the LIN-3 inductive signal in the AC (*n*=15) (Fig. 2A), suggesting that vulval induction is normal in *vrk-1* mutants. Furthermore, we lineage the VPC divisions in *vrk-1* mutants and found no defects in the first two rounds of division (*n*=10; data not shown). At late L4 however, 33% of *vrk-1* mutants were missing from 1 to 4 vulva cells (*n*=18; data not shown). We did not investigate if the reduced cell number was the consequence of underproliferation or cell death, but since this relative mild phenotype was less frequent than the Pvl phenotype, it cannot be the cause of the latter. We conclude that the AC induces the vulva normally and that the vulval lineage does not deviate dramatically from the wild-type. We next went on to investigate the behavior of the AC. In wild-type animals, AC invasion is always completed by the P6.p 4-cell stage in late L3 (Sherwood and Sternberg, 2003). The basement membranes can be seen by differential interference contrast (DIC) microscopy but for better visualization we generated a strain expressing OST-1/SPARC::GFP in the *vrk-1* mutant background. SPARC is an extracellular matrix-associated protein that is located on basement membranes (Fitzgerald and Schwarzbauer, 1998). As expected, control animals showed an interruption of the basement membranes right above the central vulva cells in late L3 (Fig. 2B). In 86% of the *vrk-1* mutants, however, the AC had not breached the basement membranes at this stage (*n*=29)(Fig. 2B). This AC invasion defect is a delay, as by the 8-cell stage in L4 all *vrk-1* mutants had breached basement membranes (data
not shown). Prior to invasion the AC is polarized, at least partially controlled by UNC-6 (Netrin) secreted from the VNC (Ziel et al., 2009). To investigate if AC polarization also depends on \textit{vrk-1}, we analyzed the localization of the actin-binding protein, moeABD fused to mCherry. In control animals, mCherry::moeABD accumulated at the basal side of the AC, facing the basement membranes (Fig. 2C, \textit{n}=50). Basal mCherry::moeABD accumulation was also observed in \textit{vrk-1} mutants, but 42\% of the mutants had additional apically localized mCherry::moeABD foci (Fig. 2C, \textit{n}=48). Thus, both AC polarity and timing of invasion are affected by the \textit{vrk-1} mutation.

Mutation of \textit{fos-1} leads to a strong inhibition of AC invasion (Sherwood et al., 2005). We therefore investigated if mutation of \textit{vrk-1} affected expression of FOS-1 and its downstream targets CDH-3 and EGL-43. Compared to control animals, we did not detect changes in expression of any of these genes in \textit{vrk-1} mutants (Suppl. Fig. 1). We do not rule out the possibility that VRK-1 functions in \textit{fos-1} signaling, but our data indicate that VRK-1 regulates AC invasion independently of this pathway.

\textbf{Postembryonic expression of VRK-1}

In \textit{C. elegans}, many genes are part of operons, which are groups of genes expressed from a common promoter (Blumenthal, 2005). \textit{vrk-1} is the last gene in an operon of eight genes. However, significant numbers of operon genes are thought to also have an internal promoter sequence (Huang et al., 2007). To analyze this latter possibility, we generated transgenic strains using 870 bp directly upstream of the \textit{vrk-1} translational start codon, followed by the \textit{vrk-1} ORF fused in-frame with GFP and terminating with 1.4 kb of \textit{vrk-1} 3’UTR. We obtained two stably expressing, integrated lines, showing very similar expression patterns. Nuclear expression of VRK-1::GFP was observed in neurons and hypodermal cells in the head, VNC and tail of \textit{C. elegans} larvae (Suppl. Fig. 2). We also detected strong
expression in all VPCs (Fig. 3A; Suppl. Fig. 2; \(n>50\)). At the L3 larval stage expression was highest in the primary fated vulva cell P6.p and its descendants (Fig. 3A; \(n=28\)). At L4 stage, VRK-1::GFP was expressed at equal levels in all 22 nuclei (Fig. 3C, Suppl. Figs. 2, 3). During division of VPCs, VRK-1::GFP showed an identical dynamic localization as previously reported in early embryos (Gorjanacz et al., 2007). VRK-1::GFP was nuclear during interphase but relocated to the nuclear periphery immediately before cell division (Fig. 3A, 4-cell stage late). During mitosis the protein was bound to chromatin (data not shown; see (Gorjanacz et al., 2007)). One of the two transgenic strains showed additional but very weak VRK-1::GFP expression in some uterine cells (data not shown). We have never observed expression in the AC with any of the two strains. Consistent with our results, two other laboratories have generated strains expressing GFP driven by the sequence directly upstream of the \(vrk-1\) ORF and also reported expression in the vulva (McKay et al., 2003; Vazquez-Manrique et al., 2007). Although immunofluorescence experiments using \(\alpha\)-VRK-1 antibodies revealed that endogenous VRK-1 is expressed in the germ line and in early embryos ((Gorjanacz et al., 2007); data not shown), none of the two VRK-1::GFP strains showed signal in the germ line. However, many transgenes in \textit{C. elegans} are silenced in the germ line, which likely affects the \(P_{vrk-1}\)::VRK-1::GFP transgene (Kelly et al., 1997).

To investigate if the VRK-1::GFP expression pattern reflects the site of action of endogenous VRK-1, we performed tissue-specific knockdown and rescue experiments. We rationalized that if VRK-1 acts in the VPCs to regulate AC behavior and utse formation, depletion of VRK-1 specifically from these cells should reproduce the Pvl phenotype observed in \(vrk-1\) mutants. Following the approach of Tiensuu and colleagues, we used the \textit{lin-31} promoter to drive expression of a \textit{vrk-1} RNA hairpin in VPCs of animals with sensitized RNAi cellular response (caused by the \textit{rrf-3(pk1426)} allele) but incapable of systemic RNAi (caused by the \textit{sid-1(qt2)} allele) (Tiensuu et al., 2005). The \textit{lin-31} promoter is
active in Pn.p cells, including VPCs, but not in the gonad (Tan et al., 1998). Observing two independent mosaic transgenic lines, we found that 19.7% of the transformed animals developed Pvl and/or Egl phenotypes within 48h of reaching the adult stage (Fig. 1D; \( n=212 \)). This represents a statistical difference when compared to non-transformed \( rrf-3(pk1426); sid-1(qt2) \) animals, of which only 4.0% developed an Egl phenotype within 48h of reaching the adult stage (\( n=101, p<0.001 \) by Chi-square Test). In contrast to \( vrk-1 \) mutants, but consistent with the cell-specificity of the \( lin-31 \) promoter, the transgenic animals were fertile.

We next tested if expression of VRK-1::GFP can rescue the defects observed in the \( vrk-1 \) mutant. Indeed, expression of VRK-1::GFP in the vulva cells rescued the AC invasion delay (100%, \( n=12, p=2.3\times10^{-7} \) by Fisher’s Exact Test) (Fig. 3B, Suppl. Fig. 3A) and re-established formation of the uterine lumen and utse (100%, \( n=29, p=3.0\times10^{-22} \) by Fisher’s Exact Test) (Fig. 3C; Suppl. Fig. 3B). In adult \( vrk-1 \) mutants, VRK-1::GFP expression reduced the Pvl phenotype to 24% (\( n=50, p=2.4\times10^{-13} \) by Fisher’s Exact Test), whilst, as expected, the germline defects were not rescued. Importantly, these experiments demonstrate the functionality of the fusion protein and suggest that sequences immediately upstream and downstream of the \( vrk-1 \) ORF direct VRK-1::GFP expression in a manner reminiscent of endogenous \( vrk-1 \). To complement our observation that FOS-1 expression is \( vrk-1 \)-independent (Suppl. Fig. 1), we investigated if \( fos-1 \) is required for VRK-1 expression. Similarly to \( vrk-1 \) mutants, disruption of FOS-1 activity prevents utse formation (Sherwood et al., 2005); however, we did not observe any changes in VRK-1::GFP expression in vulva cells in \( fos-1 \) mutants (Suppl. Fig. 1).

**Defects in uterine precursor cells**

We have previously described an essential role for VRK-1 in nuclear envelope formation in rapidly dividing \( C. elegans \) embryos (Gorjanacz et al., 2007). The defects in
vulval and uterine formation could therefore be related to an abnormal nuclear structure in post-mitotic vulva and uterine cells. To investigate in detail the morphology of the nuclear envelope, we analyzed the localization of LMN-1::GFP in vrk-1 mutants. LMN-1 (Lamin) is a component of the nuclear envelope, underlying the nuclear membrane and providing stability to the nuclear envelope (Liu et al., 2000). In early embryos depleted of VRK-1 by RNAi, LMN-1 does not localize to the nuclear rim and nuclear morphology is disrupted (Gorjanacz et al., 2007). However, LMN-1::GFP showed a smooth, uniform nuclear envelope staining in the vulva cells of both control (n=10) and vrk-1 mutant (n=18) animals, suggesting that vulva cell nuclei are assembled correctly in the absence of wild-type VRK-1 activity (Fig. 4A, arrowheads). In contrast, the uterine cells displayed severe nuclear envelope defects in all vrk-1 mutant animals (Fig. 4A, arrows). The abnormal LMN-1::GFP distribution in the uterine cells prompted us to have a closer look at the development of the uterus. We sought to investigate whether the π cells, specialized uterine cells some of whose progeny will fuse with the AC to form the utse syncytium, are born and have the right cell fate (Newman et al., 1996). In wild-type animals, LIN-11, a LIM homeodomain transcription factor, was expressed in all 12 (6 left and 6 right) π cells (Fig. 4B) (Newman et al., 1999). In vrk-1 mutants, 75% (n=12) of the animals completely lacked expression of LIN-11 in uterine cells (Fig. 4B) while the remaining 25% had a reduced number of P_{lin-11}::GFP positive cells (data not shown). Thus, VRK-1 is necessary for specification of uterine π cells. Importantly, uterine defects in vrk-1 mutants were rescued completely by expression of VRK-1::GFP specifically in vulva cells (Fig. 3C), arguing that VRK-1 expression is not required in uterine cells and that VRK-1 acts cell non-autonomously.

**VRK-1 regulates EGL-17/Fibroblast Growth Factor signaling**
Several interconnected signaling pathways are crucial for proper vulval and uterine formation (Sternberg, 2005). To investigate involvement of VRK-1 in receptor tyrosine kinase (RTK)/Ras/MAP kinase (MAPK) signaling, we measured the activity of the *egl-17* promoter. EGL-17, a fibroblast growth factor (FGF)-like protein, is expressed in P6.p and its descendants (Burdine et al., 1998). Both in control and *vrk-1* animals, *P_{egl-17}::GFP* expression was higher in P6.p descendants than in neighboring P5.p and P7.p descendants (Fig. 5B). By measuring fluorescent intensity in P6.p descendants we found that *vrk-1* mutants (*n*=61) had a 4.6-fold reduction in *P_{egl-17}::GFP* expression compared to control animals (*n*=72; *p*<0.001 by Student’s *t*-test) (Fig. 5B). To confirm that the reduced *P_{egl-17}::GFP* expression reflected a decrease in endogenous EGL-17 activity, we investigated the behavior of the sex myoblasts (SMs) in *vrk-1* mutants. During L2 larval stage two SMs, SML and SMR, migrate from the posterior part of the body to the centre close to P6.p to establish the vulval and uterine muscles (Sulston and Horvitz, 1977). The SMs divide three times to generate eight vulval and eight uterine muscle cells. The behavior of the SMs depends on EGL-17 and its receptor EGL-15, an FGF receptor tyrosine kinase, which activates LET-60 (Ras) in the SMs (Burdine et al., 1998; DeVore et al., 1995). Monitoring a *P_{egl-15}::GFP* reporter strain revealed that *vrk-1* mutants (*n*=68) expressed 45% less GFP in SML and SMR as compared to control animals (*n*=84; *p*<0.001 by Student’s *t*-test) (Fig. 5C). SMs migrated normally but failed to proliferate properly. During early-mid L3 stage the SMs divided once in both control animals (*n*=20) and *vrk-1* mutants (*n*=11) but nuclear morphology was abnormal in SM daughter cells in *vrk-1* mutants and cell division had ceased (Suppl. Fig. 4, *n*=14). In conclusion, two components of EGL-17/FGF signaling both show significantly lower expression in the *vrk-1* mutant.

Hyperactivation of Ras signaling by the *let-60(n1046)* gain-of-function allele causes formation of ectopic multivulvae (Muv; (Ferguson and Horvitz, 1985)) while inhibition of LET-60 signaling reduces EGL-17 expression (Cui and Han, 2003). Since mutation of *vrk-1*
reduced expression of \( \text{P}_{\text{egl-17}}::\text{GFP} \) we investigated if \( \text{vrk-1}(ok1181) \) was able to suppress the \( \text{let-60}(n1046) \) Muv phenotype. However, \( \text{let-60}(n1046); \text{vrk-1}(ok1181) \) double mutants had a slightly increased frequency of Muv animals (Suppl. Table II). This suggests that VRK-1 either acts upstream of LET-60 or that VRK-1’s effect on EGL-17/EGL-15 signaling is independent of LET-60. LET-60-independent regulation of the EGL-17/EGL-15 pathway has been proposed previously (DeVore et al., 1995; Sundaram, 2006).

Specification of the 2º cell fate in P5.p and P7.p involves both a graded LIN-3 signal from the AC and lateral induction via ligands produced by the 1º cell-fated P6.p (see Introduction). The observation that \( \text{vrk-1} \) mutants had a reduced expression of \( \text{P}_{\text{egl-17}}::\text{GFP} \), a commonly used marker for the 1º cell fate, prompted us to investigate commitment of P5.p and P7.p to the 2º cell fate. Expression of the phosphatase \( \text{LIP-1} \) in P5.p and P7.p and their descendants requires activated LIN-12 (Notch) signaling while only very weak expression is detected in the P6.p lineage (Berset et al., 2001). The \( \text{vrk-1} \) mutation did not cause detectable changes in expression of \( \text{P}_{\text{lip-1}}::\text{GFP} \) (Fig. 5C, \( n=17 \) control and 14 \( \text{vrk-1} \) animals), suggesting that P6.p in the absence of VRK-1 still expresses sufficient inducers of LIN-12 and that VRK-1 is not required in P5.p and P7.p to regulate LIN-12.

**VRK-1 as a sumoylation target during vulval development**

Understanding VRK-1’s role in vulval and uterine development requires not only the identification of the signaling pathways affected by VRK-1 but also a characterization of how VRK-1 is regulated. To identify genes regulating VRK-1 expression in VPCs, we analyzed by RNAi 13 candidates with reported expression and/or function in vulval formation. By this approach we have identified \( \text{smo-1} \) as a negative regulator of VRK-1 expression. Depletion of SMO-1 by RNAi resulted as expected in high embryonic lethality ((Kamath et al., 2003); data not shown). Compared to control RNAi animals, 88% of larvae surviving the \( \text{smo-1} \) RNAi
showed a dramatic increase in expression of VRK-1::GFP (Fig. 6A, n=24). *smo-1* encodes the small ubiquitin-related modifier protein SUMO and is required for morphogenesis of the gonad, vulva and uterus (Broday et al., 2004). Through processes similar to ubiquitination, sumoylation involves E1, E2, and E3 enzyme activities. Since interfering with the activities of E1-E3 blocks sumoylation and thereby phenocopies depletion of SUMO we next analyzed the expression of VRK-1::GFP in larvae depleted for UBA-2, a subunit of the SUMO activating E1 enzyme (Poulin et al., 2005). Similar to the observation in *smo-1*(RNAi) animals, depletion of UBA-2 led to an increase in expression of VRK-1::GFP in 85% of the animals observed (Fig. 6B, n=20). Together, these findings demonstrate an involvement of sumoylation in VRK-1 protein synthesis and/or degradation. However, despite several attempts, we have been unable to generate α-VRK-1 antibodies of sufficient quality to investigate if VRK-1 itself is sumoylated.

**Discussion**

Studies on formation of the *C. elegans* vulva have contributed significantly to understanding cell behavior, cell-cell interactions and signaling pathways in organogenesis. In this study we have demonstrated that the Vaccinia-Related Kinase VRK-1 plays an essential role in development of the *C. elegans* egg-laying organs. Animals carrying a *vrk-1* mutant allele that deletes approximately half of the ORF displayed defects in anchor cell (AC) invasion and formation of the vulva, utse and uterus, resulting in a strong protruding vulva (Pvl) phenotype.
**VRK-1 regulates AC polarity and invasion**

The AC breached the basement membranes separating it from the epithelial vulva precursor cells (VPCs) significantly later in *vrk-1* mutants. The transcription factor FOS-1A is a key regulator of AC invasion but an uncharacterized FOS-1A-independent pathway is also important (Sherwood and Sternberg, 2003). Our observation that neither FOS-1 itself nor FOS-1 targets CDH-3 and EGL-43 showed altered expression in *vrk-1* mutants and that FOS-1 was not required for VRK-1 expression, suggest that VRK-1 acts FOS-1-independently. AC invasion is regulated by an unknown cue from the VPCs (Sherwood and Sternberg, 2003) and by secretion of UNC-6 (Netrin) from the ventral nerve cord (VNC) (Ziel et al., 2009). UNC-6 regulates polarized accumulation of its receptor UNC-40 and F-actin at the basal side of the AC (Ziel et al., 2009). While basal accumulation of F-actin was still observed in the AC of *vrk-1* mutants, additional F-actin foci were induced apically, suggesting that proper AC apical-basal polarity is dependent on VRK-1. Future studies should address if VRK-1 regulates AC polarity via UNC-6/UNC-40. The invasion cue emitted by the central vulva cells towards the AC to direct the breaching of the basement membranes has remained unknown but we postulate that VRK-1 could have a function in the emission of this cue. Sequences immediately upstream of the VRK-1 ORF induced expression of VRK-1::GFP in the VPCs and cells of the VNC, but not in the AC, yet the transgene rescued the AC invasion delay completely. In addition, depletion of VRK-1 specifically from VPCs phenocopied the vulval defects observed in *vrk-1* mutants. This suggests that VRK-1 activity in the cells underlying the AC is necessary for proper temporal regulation of AC invasion (Fig. 6C). Given the implication of cell invasion across basement membranes in a multitude of biological processes, we speculate that VRKs may have widespread roles during animal development and disease conditions, including cancer metastasis (Santos et al., 2006; Sherwood, 2006).
VRK-1 is required for specification and proliferation of uterine and muscle cells

Downregulation of EGL-17 (FGF) and EGL-15 (FGFR) expression upon mutation of \textit{vrk-1} implies that VRK-1 is likely an activator of, or a signal transducer in this Receptor Tyrosine Kinase (RTK) pathway. This correlates with the observed upregulation of VRK-1 in the central 1º fated vulva cell and its progeny during L2/L3 larval stage and suggests an important role for VRK-1 in these cells. EGL-17 expression in VPCs is controlled by the LIN-39 Hox transcription factor, which in turn is positively regulated by Wnt and LET-60 (Ras) signaling (Cui and Han, 2003; Sternberg, 2005). We found that VRK-1 does not appear to be required for hyperactivated LET-60 to induce multivulvae formation. Although this implies that VRK-1 is not an essential component of LET-60 signaling in ectopic VPCs, it does not exclude a role of VRK-1 in LET-60 signaling in P6.p and its descendants. Likewise, while inhibition of LET-60 signaling leads to a decrease in EGL-17 expression (Burdine et al., 1998), hyperactivation of LET-60 does not increase EGL-17 expression in P6.p (data not shown; (Berset et al., 2001)). During vulval formation, RTK signaling is also activated in the sex myoblasts (SMs) in response to secretion of EGL-17-from the central VPCs and the gonads (Burdine et al., 1998; DeVore et al., 1995; Thomas et al., 1990). Our observation that proliferation of SMs was impaired in \textit{vrk-1} mutants is likely to be caused at least partially by decreased EGL-17/EGL-15 signaling. Conversely, hyperactivation of EGL-15 prevents differentiation of SM descendants without affecting SM proliferation (Sasson and Stern, 2004). Taken together, these observations suggest that FGF signaling has to be precisely controlled to achieve proper balance between proliferation and differentiation during development of \textit{C. elegans} vulval and uterine muscles.

Reduced VRK-1 activity led to failure of uterine π cell specification. Absence of π cells implied that the utse structure at the junction between the vulva and the uterus was not
formed. Together, this provides an explanation for the observed Pvl phenotype in *vrk-1* mutants as when the vulva everts, the utse (Newman et al., 2000) and presumably also the vulval muscles are required for providing mechanical support to prevent abnormal eversion. Importantly, specific depletion of VRK-1 from VPCs was sufficient to induce the Pvl phenotype, whereas expression of VRK-1::GFP in the VPCs and VNC rescued utse and uterine development and suppressed the Pvl phenotype. Thus, similar to VRK-1’s role in AC invasion, the effect of *vrk-1* mutation on π cells and SMs appears to be cell non-autonomous (Fig. 6C).

**Regulation of VRK-1 expression**

The rescuing capacity of the VRK-1::GFP transgene combined with tissue-specific RNAi experiments suggested that the behavior of the transgene reflected the expression pattern of endogenous *vrk-1*. Moreover, using essentially the same regulatory sequences upstream of the VRK-1 ORF to drive expression of GFP only also directed expression in the vulva (McKay et al., 2003; Vazquez-Manrique et al., 2007). While this does not exclude an important role of the upstream operon promoter in inducing VRK-1 expression in certain tissues and/or during certain developmental stages, these observations support the notion that many operons may carry functionally relevant internal promoters (Huang et al., 2007).

Protein sumoylation, the conjugation of the small protein SUMO to a substrate, is a post-translational modification that typically affects the subcellular localization or protein stability of the substrate, thereby changing its biological activity (Geiss-Friedlander and Melchior, 2007). Inhibition of sumoylation by *smo-1* or *uba-2* RNAi caused a strong increase in expression of VRK-1::GFP. VRK-1 may either be a direct sumoylation substrate or negatively regulated by sumoylated proteins. Proteins involved in vulval and uterine formation that are known to be sumoylated are transcription factors LIN-1 (Leight et al.,
2005) and LIN-11 (Broday et al., 2004). Sumoylation of LIN-11 is required for its function in cell differentiation, and is thus essential for the formation of the uterus (Broday et al., 2004). Sumoylation of the ETS transcription factor LIN-1 is essential for transcriptional repression of genes that promote vulval cell fates (Leight et al., 2005). Downregulation of LIN-1 or LIN-11 by RNAi does not affect expression of VRK-1::GFP, indicating that these transcription factors do not regulate vrk-1 (data not shown). We conclude that VRK-1 expression is negatively affected by sumoylation but further work is required to determine if VRK-1 itself is sumoylated. Furthermore, our observations increase the number of proteins, which are both regulated by sumoylation and act in vulval and uterine development, strengthening the link between these processes. Interestingly, sumoylation of mammalian c-Fos reduces its activity (Bossis et al., 2005), opening the possibility that sumoylation may also influence FOS-1-dependent processes in the *C. elegans* AC.

During early embryogenesis, VRK-1 regulates the localization of chromatin-binding protein BAF-1 in a cell cycle and phosphorylation-dependent manner (Gorjanacz et al., 2007). The *vrk-1* mutant allele characterized in this work retains all kinase motifs but lacks most parts outside the kinase domain, presumably rendering the mutant protein unable to interact with some or all of its binding partners. Determination of whether VRK-1’s kinase activity is required for vulval and uterine development requires further studies. *Drosophila* and mammalian VRKs phosphorylate several transcription factors and affect chromatin modifications (Klerkx et al., 2009). So far, none of the *C. elegans* orthologs for these VRK substrates has been brought in relation to vulval or uterine development, nor AC invasion. However, future information about VRKs in *C. elegans* and other organisms will doubtlessly provide insight to the role of this emerging family of protein kinase in formation of *C. elegans* reproductive organs in particular and organ development in general.
Acknowledgments

This work was funded by grants from the Spanish Ministry of Science (RYC-2003-001521, BFU-2004-01096, BFU-2007-60116) to PA. In addition, we wish to acknowledge EMBO and the Boehringer Ingelheim Foundation for support to EK and Junta de Andalucía for institutional support. PWS is an investigator with the Howard Hughes Medical Institute. We are grateful to C. Ayuso for excellent technical assistance. We wish to thank D. Sherwood and S. Tuck for providing strains, C. R. Dombecki for corrections on the manuscript and two anonymous reviewers for constructive criticism. Some nematode strains used in this work were provided by the International *C. elegans* Gene Knockout Consortium and the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).
References


**Figure legends**

Fig. 1. *vrk-1* mutants show defects in germline, uterine and vulval development. (A)
Schematic representation of the *vrk-1* gene. Purple: *vrk-1* promoter used in this work; blue boxes: *vrk-1* exons; light blue boxes: *vrk-1* kinase domain; yellow box: BAB domain; shaded grey: 3’UTRs; red bar: region deleted in *ok1181*; dashed lines: mRNA splicing pattern. In *ok1181* mRNA, exon 2 is spliced to exon 6. (B) Gonad arms of control and *vrk-1* mutant late L4 larvae outlined in white. The *vrk-1* mutant gonad is smaller and contains abnormally enlarged cells (arrowheads). Boxed regions are shown at higher magnification to the left. (C) At late L4, the control larva has developed vulval (V) and uterine (U) lumens and uterine-seam cell connection (utse; arrowhead). The *vrk-1* mutant has developed a vulval lumen, but the uterine lumen and the utse are missing. (D) Control adult animal shows a normally everted vulva whilst mutation of *vrk-1* or RNAi-mediated depletion of VRK-1 specifically in vulva cells induces a protruding vulva (Pvl) phenotype (arrowheads). Scale bars, 10 µm.

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Fig. 4. VRK-1 is required for specification of uterine precursor cells. (A) In control and *vrk-1* mutant animals, vulva cells have a normal nuclear appearance visualized by expression of LMN-1::GFP (arrowheads). In contrast, nuclei of uterine precursor cells (arrows) have an aberrant structure in the *vrk-1* mutant. (B) In the control animal, 6 π cells on each side of the body are visualized by expression of GFP from the *lin-11* promoter (arrowhead points to π cells on one side). In most *vrk-1* mutants, no GFP expressing cells are observed. Scale bars, 5 µm.

Fig. 5. VRK-1 stimulates FGF signaling. (A, B) The average fluorescent intensity of P*egl-17*::GFP in the descendents of P6.p (A) and of P*egl-15*::GFP in the sex myoblasts at the 1-cell stage (B) is reduced in *vrk-1* mutants as compared to control animals (p<0.001, Student’s *t*-test). A.U. Arbitrary Units. (C) Expression of GFP driven by the *lip-1* promoter in P5.p and P7.p, and to a lesser extent in P6.p, is similar in control and *vrk-1* mutant animals. Scale bars, 5 µm.

Fig. 6. Sumoylation regulates VRK-1 expression. (A) Expression of VRK-1::GFP in *smo-l(RNAi)* larvae is highly up-regulated as compared to control RNAi larvae. At microscope
settings for which fluorescent intensity reached saturation in the smo-1(RNAi) larvae, the VRK-1::GFP fluorescent signal is barely detectable in the control. Insert shows an image of the same control animal acquired using higher microscope sensitivity (0.3x magnification relative to other panels). (B) Expression of VRK-1::GFP in larvae depleted for UBA-2 is up-regulated as compared to control RNAi treatment. Scale bars, 5 µm. (C) Model of VRK-1’s role in vulval and uterine development. See Discussion for details. Pn.p, vulva precursor cells; VNC, ventral nerve cord cell; AC, anchor cell; π, uterine π cells; SM, sex myoblast.

Suppl. Fig. 1. FOS-1 and its targets are expressed normally in vrk-1 mutants. Expression of P
fos-1a
::FOS-1A::YFP (A), P
cdh-3
::GFP (B) and P
egl-43
::NLS::YFP (C) in the AC (arrowhead) is unaffected in vrk-1 mutants (n=16 (A); 19 (B); 10 (C)) as compared to control animals (n=13 (A); 15 (B); 10 (C)). (D) Similarly, expression of P
vrk-1
::VRK-1::GFP is independent of fos-1 (n= 22 control animals; 10 fos-1(ar105) animals). Scale bars, 5 µm.

Suppl. Fig. 2. Expression of P
vrk-1
::VRK-1::GFP at the L4 larval stage in neurons and hypodermal cells in the head (left), tail (right) and ventral (down) area of the worm. Strong expression is observed in the developing vulva (insert). Scale bar, 40 µm.

Suppl. Fig. 3. vrk-1 mutant phenotypes are rescued by low expression of VRK-1::GFP from the integrated transgene vrIs15. (A) In control animals (left), the AC has invaded the basement membrane by the P6.p 4-cell stage (arrowheads). In the vrk-1 mutant (middle), the basement membrane has not been invaded yet by the P6.p 4-cell stage. This delay is rescued by expression of VRK-1::GFP in the vulva cells from the vrk-1 promoter (right; arrowheads). Scale bar, 5µm. (B) The vrk-1 mutant at L4 stage does not have a uterine lumen nor does it
form an utse. Expression of VRK-1::GFP in the vulva cells rescues the uterine lumen (U) and utse (arrowheads). Scale bar, 10µm.

Suppl. Fig. 4. VRK-1 is required for sex myoblast divisions. Control and *vrk-1* mutant L3-L4 larvae were staged based on Pn.p cell divisions and morphology. Sex myoblasts and their descendents were identified based on \( P_{egi ti}::GFP \) expression. Shown are myoblasts from either the left or the right side of the body. In the *vrk-1* mutants sex myoblasts divided once while three rounds of division were observed in control animals. Scale bars, 10 µm.
### Suppl. Table I. Strains generated for this study

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</tr>
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<td>BN124</td>
<td>(\text{unc-119(ed3)} III; \text{vrIs13[\text{P}_{\text{vrk-1}}::\text{vrk-1::gfp unc-119(+)}}])</td>
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</tbody>
</table>

YL255  \(\text{unc-119(\text{ed3}) III; vrIs13[\text{P}_{\text{vrk-1}}::\text{vrk-1::gfp unc-119(+)}}]\)
Suppl. Table II. Mutation of vrk-1 enhances LET-60 (Ras) gain-of-function phenotype.

<table>
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<th>Muv^b (average ± S.D.^c)</th>
</tr>
</thead>
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<tr>
<td>vrk-1(ok1181)</td>
<td>&gt;100</td>
<td>0±0%</td>
</tr>
<tr>
<td>let-60(n1046)</td>
<td>432</td>
<td>70.7±9.6%</td>
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<tr>
<td>let-60(n1046); vrk-1(ok1181)</td>
<td>316</td>
<td>85.0±8.7%^*</td>
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</table>

Homozygous L4 and young adults were mounted on microscope slides and observed with a 63x objective. ^aNumber of animals analyzed. ^bAverage Multivulva phenotype observed in four independent experiments at 20°C. ^cStandard deviation. ^*Statistically different from let-60(n1046) (p<0.001 by Chi-square test).
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Suppl. Fig. 1. FOS-1 and its targets are expressed normally in *vrk-1* mutants. (A-C) Expression of P*fos-1a*:FOS-1A::YFP (A), Pcdh-3::GFP (B) and Pegl-43::NLS::YFP (C) in the AC (arrowhead) is unaffected in *vrk-1* mutants as compared to control animals. (D) Similarly, expression of P*vrk-1*:VRK-1::GFP is independent of *fos-1*. Scale bars, 5 μm.
Suppl. Fig. 2. Expression of Pvrk-1::VRK-1::GFP at the L4 larval stage in neurons and hypodermal cells in the head (left), tail (right) and ventral (down) area of the worm. Strong expression is observed in the developing vulva (insert). Scale bar, 40 μm.
Suppl. Fig. 3. vrk-1 mutant phenotypes are rescued by low expression of VRK-1::GFP from the integrated transgene vr/s15. (A) In control animals (left), the AC has invaded the basement membrane by the P6.p 4-cell stage (arrowheads). In the vrk-1 mutant (middle), the basement membrane has not been invaded yet by the P6.p 4-cell stage. This delay is rescued by expression of VRK-1::GFP in the vulva cells from the vrk-1 promoter (right; arrowheads). Scale bar, 5μm. (B) The vrk-1 mutant at L4 stage does not have a uterine lumen nor does it form an utse. Expression of VRK-1::GFP in the vulva cells rescues the uterine lumen (U) and utse (arrowheads). Scale bar, 10μm.
Suppl. Fig. 4. VRK-1 is required for sex myoblast divisions. Control and vrk-1 mutant L3-L4 larvae were staged based on Pn.p cell divisions and morphology. Sex myoblasts and their descendents were identified based on Pegl-15::GFP expression. Shown are myoblasts from either the left or the right side of the body. In the vrk-1 mutants sex myoblasts divided once while three rounds of division were observed in control animals. Scale bars, 10 μm.