Breaking the *C. elegans* invasion/proliferation dichotomy

Michael A. Q. Martinez¹, Chris Z. Zhao¹, Frances E. Q. Moore¹, Callista Yee², Wan Zhang¹, Kang Shen², Benjamin L. Martin¹, David Q. Matus¹

¹Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY 11794, USA.

²Howard Hughes Medical Institute, Department of Biology, Stanford University, Stanford, CA 94305, USA.

*Author for correspondence (david.matus@stonybrook.edu)

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ABSTRACT

The acquisition of the post-mitotic state is crucial for the execution of many terminally differentiated cell behaviors during organismal development. However, the mechanisms that maintain the post-mitotic state in this context remain poorly understood. To gain insight into these mechanisms, we used the genetically and visually accessible model of *C. elegans* anchor cell (AC) invasion into the vulval epithelium. The AC is a terminally differentiated uterine cell that must exit the cell cycle and enter a post-mitotic state to initiate contact between the uterus and vulva through a cell invasion event. Here, we set out to identify the negative cell cycle regulators that maintain the AC in a post-mitotic, invasive state. Although our findings revealed a critical role for CKI-1 (p21CIP1/p27KIP1) in maintaining the post-mitotic state of the AC, loss of CKI-1 alone or in combination with other negative cell cycle regulators—including CKI-2 (p21CIP1/p27KIP1), LIN-35 (pRb/p107/p130), FZR-1 (Cdh1/Hct1), and LIN-23 (β-TrCP)—resulted in proliferating ACs that retained their invasive abilities. Upon examination of the gene regulatory network controlling AC invasion, we determined that proliferating, invasive ACs maintain pro-invasive gene expression. We therefore report that maintenance of the post-mitotic state is not necessary for AC invasion, breaking the previously established *C. elegans* invasion/proliferation dichotomy.
INTRODUCTION

The proper timing and coordination of terminal differentiation is critical for organismal development. In many cases, terminal differentiation requires cells to exit the cell cycle and enter a post-mitotic state (Soufi and Dalton, 2016). The general model for how terminally differentiated cells exit the cell cycle involves the repression of cyclin/CDK activity by negative cell cycle regulators such as CDK inhibitors (CKIs), retinoblastoma (Rb) family members, and E3 ubiquitin ligase complexes, including the anaphase-promoting complex/cyclosome (APC/C) and the Skp-Cullin-F-box protein (SCF) complex (Butitta and Edgar, 2007; Ruijtenberg and van den Heuvel, 2016). Despite this generally accepted model, the mechanisms by which terminally differentiated cells maintain their post-mitotic state remain unclear.

Exit from the cell cycle following terminal differentiation is considered essential for the execution of specialized cell behaviors. According to the “go versus grow” dichotomy, a mutually exclusive relationship exists between these cell behaviors and cell proliferation (Kohrman and Matus, 2017). However, accumulating evidence has challenged this dichotomy in both development and cancer. For instance, in the developing mouse retina, genetic ablation of Rb paralogs leads to fully differentiated neurons that retain the ability to form neurites and synapses while proliferating (Ajioka et al., 2007). Similar results have been observed in mouse inner ear hair cells following loss of Rb (Sage et al., 2005). Furthermore, recent evidence from two-dimensional and three-dimensional in vitro assays using skin cancer cells found that these cells can simultaneously invade and proliferate (Haass et al., 2014; Vittadello et al., 2020).

Here, we used C. elegans anchor cell (AC) invasion as a genetically dissectible in vivo model of developmental cell invasion. Since the AC must exit the cell cycle and enter a post-mitotic state upon invasive differentiation (Deng et al., 2020; Matus et al., 2015; Medwig-Kinney et al., 2020; Smith et al., 2022), we aimed to identify the negative regulators of the AC cell cycle that maintain the post-mitotic state. While our findings reveal a critical role for the CDK inhibitor CKI-1 (p21^{CIP1}/p27^{KIP1}) in maintaining the post-mitotic state of the AC, loss of CKI-1 alone or in combination with other negative cell cycle regulators resulted in proliferating ACs that retained their invasive abilities. Using two reporters of the gene regulatory network that controls AC invasion, we show that proliferating, invasive ACs maintain pro-invasive gene expression. Thus, our work demonstrates that maintenance of the post-mitotic state is not required for AC invasion, breaking the invasion/proliferation dichotomy in C. elegans.
RESULTS AND DISCUSSION

Background

The *C. elegans* egg-laying system requires a direct connection between the uterus and the vulva. In the mid-L3 larval stage, a terminally differentiated post-mitotic uterine cell, called the anchor cell (AC), initiates the uterine-vulval connection by breaching the basement membrane (BM) that separates these tissues (Sherwood and Sternberg, 2003) (Fig. 1A, left). AC invasion occurs in synchrony with the divisions of P6.p, the underlying 1º-fated vulval precursor cell (VPC). By the P6.p 4-cell stage, BM dissolution is complete (Sherwood and Sternberg, 2003). Robust control of the AC cell cycle appears to be required for invasive differentiation, as loss of the post-mitotic state results in a complete loss of invasive capacity (Matus et al., 2015) (Fig. 1A, right). The SWI/SNF chromatin remodeling complex, specifically the evolutionarily conserved BAF assembly, along with three conserved transcription factors—EGL-43 (EVI1), HLH-2 (E/Daughterless), and NHR-67 (TLX/Tailless)—have all been implicated in the regulation of AC invasion because of their critical role in maintaining the post-mitotic state (Deng et al., 2020; Matus et al., 2015; Medwig-Kinney et al., 2020; Smith et al., 2022). However, the direct negative regulators of the AC cell cycle are largely unknown.

**Uterine-specific depletion of CKI-1 rarely causes AC proliferation**

In a genetic background where only the uterine cells are sensitive to RNAi (Hagedorn et al., 2009), loss of either EGL-43 or NHR-67 leads to AC proliferation without BM invasion in over 60% of animals (Matus et al., 2015; Medwig-Kinney et al., 2020). Conversely, uterine-specific RNAi-mediated depletion of CKI-1 (p21\(^{CIP1}\)/p27\(^{KIP1}\)), a canonical negative regulator of the *C. elegans* cell cycle (Feng et al., 1999; Fukuyama et al., 2003; Hong et al., 1998), rarely results in this phenotype, occurring in about 3% of animals (Matus et al., 2015).

In an attempt to increase the penetrance of the proliferative AC phenotype (≥2 AC), we first built a new RNAi construct for *cki-1* using the highly efficient RNAi vector, T444T (Sturm et al., 2018). Unlike the standard RNAi vector, L4440 (Timmons and Fire, 1998), T444T includes a pair of T7 terminator sequences to prevent transcription of the vector backbone. We then tested the T444T-based *cki-1(RNAi)* construct in a uterine-specific RNAi strain (*rrf-3(pk1426); rde-*)
1(ne219); fos-1ap::rde-1) with fluorescent reporters to visualize the AC cell membrane and BM. Though 5% of animals exposed to this construct had ≥2 ACs (Fig. S1A), this result only represents a 2% increase in the proliferative AC phenotype when compared to the original cki-1(RNAi) experiment (Matus et al., 2015). In each of these animals, BM invasion was observed. This finding could be attributed to either a late RNAi effect or reestablishment of the post-mitotic, invasive state following AC proliferation. Alternatively, this result raises the intriguing possibility that ACs can simultaneously invade and proliferate, which we explore in the subsections below.

Systemic depletion of CKI-1 causes a moderate increase in AC proliferation

To better visualize the dichotomy between AC invasion and proliferation, we generated a strain for three-color imaging of the AC, cell cycle, and BM (Fig. 1A-C). Specifically, this strain contains an endogenous transcriptional reporter of lag-2 (Delta), LAG-2::P2A::H2B::mTurquoise2, to mark the AC nucleus (Medwig-Kinney et al., 2022), a ubiquitous CDK activity sensor, DHB::2xmKate2, to visualize and quantify cell cycle state (Adikes et al., 2020), and an endogenous translational reporter of lam-2, LAM-2::mNeonGreen, to label laminin, a core BM component (Jayadev et al., 2019). Conveniently, the histone-based lag-2 transcriptional reporter also functions as a P6.p lineage tracker, facilitating the developmental staging of AC invasion (Fig. 1A,C). The CDK activity sensor is a fluorescent fragment of human DNA helicase B (DHB), which is predominantly nuclear in post-mitotic cells, such as the wild-type AC, and becomes increasingly cytoplasmic in proliferating cells as they progress through the cell cycle (Fig. 1B,C). Thus, the ratio cytoplasmic-to-nuclear of DHB fluorescence provides a visual and quantitative readout of CDK activity, and more generally, cell cycle progression (Adikes et al., 2020; Martinez and Matus, 2022; Spencer et al., 2013; van Rijnberk et al., 2017).

With the three-color imaging strain at our disposal, we first wanted to determine the penetrance of the proliferative AC phenotype following RNAi-mediated depletion of CKI-1 in a wild-type background. Unexpectedly, 100% of control(RNAi) and cki-1(RNAi) animals showed a post-mitotic, invasive AC (Fig. S1B,C; Table 1). We next introduced the rrf-3(pk1426) null allele (Simmer et al., 2002), which on its own confers RNAi hypersensitivity in many somatic tissues, including the uterus (Matus et al., 2010). While nearly all rrf-3(pk1426); control(RNAi) animals contained a post-mitotic, invasive AC (Fig. 1D,E), there was one animal where a BM gap was not observed below a post-mitotic AC (Table 1). In comparison, 24% of rrf-3(pk1426); cki-1(RNAi) animals possessed ≥2 ACs, which correlated with a significant increase in AC-specific
CDK activity when compared to rrf-3(pk1426); control(RNAi) animals (0.25 ± 0.06 (n = 30) vs. 0.73 ± 0.56 (n = 55)). In almost half of those animals with ≥2 ACs, BM invasion was observed (Table 1). Taken together, these data demonstrate that systemic depletion of CKI-1 enhances the penetrance of the proliferative AC phenotype.

Unfortunately, we were unable to assess the effect of the cki-1(gk132) null allele on AC proliferation (The C. elegans Deletion Mutant Consortium, 2012), as it leads to embryonic lethality in 100% of homozygotes (Buck et al., 2009) (Fig. S2A). Instead, we crossed the cdc-14(he141) null allele into the three-color imaging strain (Saito et al., 2004). cdc-14 encodes a dual-specificity phosphatase that positively regulates CKI-1 activity in a number of larval tissues (Clayton et al., 2008; Roy et al., 2011; Roy et al., 2014; Saito et al., 2004), likely by preventing its phosphorylation-dependent degradation (Saito et al., 2004). Nonetheless, 100% of cdc-14(he141) animals had a post-mitotic, invasive AC, even in the presence of cki-1(RNAi) (Fig. S2B,C; Table 1), suggesting that CDC-14 is not a regulator of CKI-1 in the AC or that its function is redundant with that of another phosphatase.

In the following subsection, we introduce viable null or hypomorphic alleles that impact other negative regulators of the cell cycle (cki-2, lin-35, fzr-1, and lin-23) to determine their role in maintaining the post-mitotic state of the AC.

**CKI-1 is critical for maintaining the post-mitotic state of the AC**

Only a handful of factors negatively regulate the somatic cell cycle during C. elegans larval development (Kipreos and van den Heuvel, 2019). These include the CDK inhibitors CKI-1 and CKI-2 (p21<sup>cip1/p27<sup>kip1</sup></sup> (Buck et al., 2009; Hong et al., 1998), the sole Rb family member LIN-35 (pRb/p107/p130) (Boxem and van den Heuvel, 2001; Boxem and van den Heuvel, 2002), the APC/C co-activator FZR-1 (Cdh1/Hct1) (Fay et al., 2002), and the F-box/WD-repeat protein LIN-23 (β-TrCP) (Kipreos et al., 1996; Kipreos et al., 2000; Nayak et al., 2002). Although they all ensure normal cell cycle exit either generally (CKI-1 and LIN-23), redundantly (LIN-35 and FZR-1), or tissue specifically (CKI-2), their role in maintaining the post-mitotic state is poorly understood.

To determine their role in the AC, we generated four separate three-color imaging strains. Three of these strains include putative null alleles, i.e., cki-2(ok2105), lin-35(n745), or lin-23(e1883)

The fourth strain harbors fzr-1(ku298), a hypomorphic allele, as FZR-1 function is required for viability (Fay et al., 2002). However, regardless of their genotype, live imaging and subsequent quantification of CDK activity demonstrated that they all possessed a post-mitotic, invasive AC (Fig. 2; Table 1).

These results were consistent with what we found after depleting CKI-1 by RNAi in a wild-type background of the three-color imaging strain (Fig. S1B,C; Table 1). We next explored whether we could induce AC proliferation by depleting CKI-1 in each mutant background. Following cki-1(RNAi) treatment, we observed ≥2 ACs in 13%, 15%, 35%, and 81% of cki-2(ok2105), lin-35(n745), fzr-1(ku298), and lin-23(e1883) animals, respectively (Fig. 2). Compared to mutant controls, this corresponded to a significant increase in AC-specific CDK activity (0.14 ± 0.03 (n = 30) vs. 0.55 ± 0.50 (n = 39), 0.15 ± 0.04 (n = 31) vs. 0.77 ± 0.52 (n = 54), 0.17 ± 0.05 (n = 32) vs. 0.64 ± 0.57 (n = 55), and 0.13 ± 0.03 (n = 34) vs. 1.22 ± 0.72 (n = 111), respectively).

Additionally, we observed an increase in the percentage of proliferating ACs that underwent BM invasion (Table 1). Overall, these findings, together with our cki-1(RNAi) results in RNAi-sensitive backgrounds (Fig. 1D,E; Fig. S1A), highlight the critical role of CKI-1 in maintaining the post-mitotic state of the AC.

**Maintenance of the post-mitotic state is not required for AC invasion**

To further investigate the lack of AC invasion defects following robust cell cycle induction (Table 1), we collected time-lapse movies of cycling ACs from pre-invasion to post-invasion using a variant of the three-color imaging strain (Fig. 3; Fig. S3). Included in this strain is an AC-specific F-actin probe, cdh-3p::mCherry::moesinABD, which we paired with a ubiquitous CDK activity sensor, DHB::2xmTurquoise2, and an endogenous lam-2 translational reporter, LAM-2::mNeonGreen. We also added the fzr-1(ku298) hypomorphic allele rather than the lin-23(e1883) null allele, as fzr-1(ku298) animals were easier to propagate and had the next highest penetrance of the proliferative AC phenotype in the presence of cki-1(RNAi) (Fig. 2C,G; Table 1).

Prior to time-lapse imaging, we captured static images of the invasive AC in fzr-1(ku298); control(RNAi) animals (Fig. 3A), which allowed us to obtain a baseline level of CDK activity values (0.31 ± 0.05 (n = 23)). This baseline allowed us to selectively record the activity of pre-
invasive ACs in fzr-1(ku298); cki-1(RNAi) animals that had already started cycling. Remarkably, we collected five time-lapse datasets in which single or multiple cycling, pre-invasive ACs showed an enrichment of the F-actin probe at the invasive membrane (Hagedorn et al., 2013) (1.74 ± 0.36 (n = 7)), followed by successful invasion of the underlying BM (Fig. 3B; Movie 1). In one of these datasets, we observed the division of a pre-invasive AC, with both daughter cells re-entering the cell cycle before invading (Fig. S3). Altogether, these observations led us to conclude that maintenance of the post-mitotic state is not required for AC invasion.

The pro-invasive GRN is functionally intact in proliferating, invasive ACs

After finding that direct disruption of the cell cycle leads to proliferating, invasive ACs, we investigated whether these ACs maintain a functional pro-invasive gene regulatory network (GRN) (Deng et al., 2020; Medwig-Kinney et al., 2020). This GRN consists of a cell cycle-dependent and cell cycle-independent arm. We predicted that gene expression in both arms would remain unchanged in proliferating, invasive ACs.

To test our hypothesis, we quantified endogenous levels of NHR-67, which is the distal transcription factor in the cell cycle-dependent arm and is upstream of CKI-1. Additionally, we quantified transcriptional levels of the matrix metalloproteinase (MMP) gene, zmp-1, which is one of the downstream effectors of the cell cycle-independent arm. We quantified these levels in two separate fzr-1(ku298) strains, each carrying most, if not all, of the original three-color imaging alleles in the presence of cki-1(RNAi). In one strain, we inserted an mNeonGreen tag into the C-terminus of the endogenous nhr-67 locus using CRISPR/Cas9 (Paix et al., 2015). In the other strain, we exchanged the endogenous lag-2 transcriptional reporter, LAG-2::P2A::H2B::mTurquoise2, for a zmp-1 transcriptional reporter transgene, zmp-1p::CFP (Inoue et al., 2002). Supporting our prediction, NHR-67::mNeonGreen expression decreased by only 17% in proliferating, invasive ACs (Fig. 4A,B), whereas zmp-1p::CFP expression decreased by 73% in proliferating, invasive ACs (Fig. 4C,D). However, given that depletion of GRN transcription factors results in proliferating, non-invasive ACs and a complete loss of zmp-1 transgene expression (Hwang et al., 2007; Matus et al., 2015; Medwig-Kinney et al., 2020; Rimann and Hajnal, 2007; Sherwood et al., 2005), these data indicate that the pro-invasive GRN is functionally intact in proliferating, invasive ACs (Fig. 4E).

Conclusions
In this study, we establish a critical role for CKI-1 in maintaining the post-mitotic state of the AC. However, without knowing the null phenotype of cki-1, it remains difficult to ascertain the redundancies, if any, in the AC cell cycle machinery (Matus et al., 2015). In C. elegans, there are two CDK inhibitors: CKI-1 and CKI-2. Recent work has shown that cki-2 becomes upregulated in terminally differentiated vulval cells that are devoid of cki-1 expression (Portegijs, 2019). This same compensatory relationship may exist in the AC. This could in part explain the enhanced penetrance of the proliferative AC phenotype following loss of CKI-1 in rrf-3(pk1426) and cki-2(ok2105) null mutant backgrounds (Fig. 1D,E; Fig. 2A,E). Interestingly, we observed a synergistic increase in the number of proliferating ACs in lin-23(e1883); cki-1(RNAi) animals (Fig. 2D,H). Perhaps complete loss of CKI-1, CKI-2, and LIN-23 is required to achieve a fully penetrant ≥2 AC phenotype. We also do not know the null phenotype of fzr-1. Therefore, it may have a more significant role in maintaining the post-mitotic state of the AC. However, use of the fzr-1(ku298) hypomorphic allele sufficiently triggered cell cycle entry in post-mitotic neurons (The et al., 2015). Lastly, as LIN-35 functions in both canonical cell cycle regulation and negative regulation of RNAi pathways (Boxem and van den Heuvel, 2001; Boxem and van den Heuvel, 2002; Lehner et al., 2006; Wang et al., 2005), we cannot rule out the possibility that the lin-35(n745) null allele is simply enhancing the efficiency of cki-1(RNAi) (Fig. 2B,F). These ambiguities could be addressed in future studies by creating and/or combining conditional null alleles using the auxin-inducible degron (AID) system (Ashley et al., 2021; Hills-Muckey et al., 2021; Martinez et al., 2020; Negishi et al., 2021; Sepers et al., 2022; Xiao et al., 2023; Zhang et al., 2015) or an analogous approach (Nance and Frøkjær-Jensen, 2019).

Finally, we demonstrate that AC invasion can occur irrespective of cell cycle state (Fig. 3), which breaks the dichotomy between invasion and proliferation in C. elegans (Matus et al., 2015). We evaluated the functionality of the GRN that controls AC invasion (Deng et al., 2020; Medwig-Kinney et al., 2020), specifically examining the expression of two components: NHR-67 and zmp-1 (Fig. 4). These components provide readouts of the cell cycle-dependent and cell cycle-independent arm, respectively. We observed a considerable reduction in zmp-1 expression in proliferating, invasive ACs, but not in NHR-67 expression. The presence of reduced zmp-1 expression in these cells suggests that cell cycle progression downregulates the production of MMPs independently of the GRN components that transcriptionally regulate them (Medwig-Kinney et al., 2020). However, MMPs are not necessary for AC invasion (Kelley et al., 2019). In the absence of MMPs, the AC adapts by expanding its invasive F-actin network and...
physically forcing its way through the underlying BM in an ATP-dependent manner. Given that cell invasion and proliferation are both energy-intensive processes, future investigation is warranted into whether they can occur simultaneously in the absence of MMPs. To our knowledge, this is the first example in development where an invasively differentiated cell can perform its specialized function while proliferating. Although evidence suggests that tumor cells must alternate between proliferative and invasive states (Kohrman and Matus, 2017; Mondal et al., 2022), additional research targeting the cell cycle in invasive tumor cells is required to determine the necessity of this phenotypic switch.

**MATERIALS AND METHODS**

**Strains**

All strains were grown and maintained following standard procedures (Brenner, 1974). A complete list of strains used in this study is available in Table S1.

**Alleles**

The following alleles were used in this study: *qyls102[fos-1ap::rde-1]* (Hagedorn et al., 2009); *LG I bmd156[rps-27p::DHB::2xmKate2]* (Adikes et al., 2020), *bmd294[rps-27p::DHB::2xTurquoise2]* (this study), and *lin-35(n745)* (Lu and Horvitz, 1998); *LG II bmd168[rps-27p::DHB::2xmKate2]* (Adikes et al., 2020), *lin-23(e1883)* (Kipreos et al., 2000), *cki-1(gk132)* (The C. elegans Deletion Mutant Consortium, 2012), *cdc-14(he141)* (Saito et al., 2004), *fzr-1(ku298)* (Fay et al., 2002), *cki-2(ok2105)* (The C. elegans Deletion Mutant Consortium, 2012), and *rrf-3(pk1426)* (Simmer et al., 2002); *LG IV qyls10[lam-1p::lam-1::GFP]* (Ziel et al., 2009), *qyls225[cdh-3p::mCherry::moesinABD]* (Matus et al., 2015), and *nhr-67(wy1787[nhr-67::mNeonGreen])* (this study); *LG V lag-2(bmd202)[lag-2::P2A::H2B::mTurquoise2]* (Medwig-Kinney et al., 2022), *rde-1(ne219)* (Tabara et al., 1999), and *syls67[zmp-1p::CFP]* (Inoue et al., 2002); *LG X lam-2(qy20[lam-2::mNeonGreen])* (Jayadev et al., 2019) and *qyls24[cdh-3p::mCherry::PLCδPH]* (Ziel et al., 2009).

**Generation of the transgenic bmd294 allele**
Both pWZ186 (Addgene plasmid #163641) and a plasmid containing 2xmTurquoise2 were double digested with Bsu36I and NgoMIV to excise 2xmKate2 and 2xmTurquoise2, respectively. pMAM038 (rps-27p::DHB::2xmTurquoise2) was cloned by T4 ligation using the backbone from pWZ186 and 2xmTurquoise2 as an insert. After sequence confirmation, pMAM038 was used as a repair template for insertion into the genome at a safe harbor site on chromosome I corresponding to the MosSCI insertion site, ttTi4348 (Frøkjær-Jensen et al., 2012). pAP082 was used as the sgRNA plasmid for chromosome I insertion by CRISPR/Cas9 (Pani and Goldstein, 2018). Young adults were transformed using standard microinjection techniques, and integrants were identified through the SEC method (Dickinson et al., 2015).

**Generation of the endogenous wy1787 allele**

A repair template containing 30xlinker-mNeonGreen, with homology at the 5' and 3' ends to the C-terminus of the *nhr-67* locus, was PCR amplified from pJW2171 (Addgene plasmid #163095) and concentrated using a PCR purification kit (Qiagen). 3 µl of 10 µM stock tracrRNA (IDT) was incubated with 0.5 µl of a 100 µM crRNA (IDT) for 5 min at 95°C followed by 5 min at 25°C. Immediately following incubation, the tracrRNA:crRNA mixture was incubated with 0.5 µl of Alt-R Cas9 protein (IDT) for 10 min at 37°C. The repair template and co-injection marker (pRF6) were combined with the mixture to a final concentration of 200 ng/µl and 50 ng/µl, respectively, for a final injection mix volume of 10 µl. Adult worms possessing no more than a single row of eggs were transformed using standard microinjection techniques. Roller F1 progeny were singled out, and F2 progeny were genotyped for insertions (Paix et al., 2015). The sequence of the PCR primers and guide are available in Tables S2 and S3, respectively.

**RNAi**

All RNAi experiments were performed by first synchronizing animals at the L1 larval stage using the bleaching technique (Porta-de-la-Riva et al., 2012). These animals were then fed bacteria expressing the T444T-based RNAi construct for *cki-1* until the mid-L3 larval stage (P6.p four-cell stage), when AC invasion normally occurs (Sherwood and Sternberg, 2003). Control animals were fed bacteria carrying the empty T444T vector.

**Generation of a T444T-based RNAi construct for cki-1**
A T444T-based RNAi construct targeting cki-1 was generated by inserting a 552 bp synthetic DNA fragment, based on the cki-1 cDNA sequence from WormBase (Davis et al., 2022), into the T444T vector (Addgene plasmid #113081) using the BglII and XhoI restriction sites (Sturm et al., 2018). The synthetic DNA was purchased from IDT as a gBlock with homology arms to T444T for Gibson assembly. The sequence of the synthetic DNA is available in Table S4.

Image acquisition

Images were acquired using a spinning disk confocal microscope supported by Nobska Imaging. This confocal system consists of a Hamamatsu ORCA EM-CCD camera mounted on an upright Zeiss Axio Imager.A2, equipped with a Borealis-modified Yokogawa CSU-10 spinning disk scanning unit with 6 solid state (405, 440, 488, 514, 561, and 640 nm) lasers and a Zeiss Plan-Apochromat 100x/1.4 oil DIC objective.

For static imaging, animals were anesthetized by placing them into a drop of M9 on a 5% agarose pad containing 7 mM sodium azide and securing them with a coverslip. Time-lapse imaging was performed using a modified version of a previously published protocol (Kelley et al., 2017), as described in Adikes et al. (2020).

Imaging processing and analysis

The acquired images were processed using ImageJ/Fiji (Schneider et al., 2012). DHB ratios were quantified as previously described (Adikes et al., 2020). NHR-67::mNeonGreen and zmp-1::CFP fluorescence were quantified as previously described (Medwig-Kinney et al., 2020). AC invasion was defined as the complete loss of fluorescent BM signal underneath the AC. Plots were generated using Prism software, and figures, including cartoons, were created using a combination of Adobe Photoshop and Illustrator.

Statistics

A power analysis was performed to determine the number of animals (N) needed per experiment (Pollard et al., 2019). The figure legends specify the measures of central tendency, error bars, numeric P-values, and statistical tests used.
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Data availability

All relevant data can be found within the article and its supplementary information.

Competing interests

D.Q.M. is a paid employee of Arcadia Science.

Author contributions


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**C. elegans invasion/proliferation dichotomy**

A breached BM leads to a wild-type AC, while an intact BM results in a proliferating AC.

**CDK activity sensor**

CDK activity sensor involves CDK activity, which is measured as cytoplasmic DHB divided by nuclear DHB.

**DIC LAM-2::mNG LAG-2::P2A::H2B::mT2 cki-1(RNAi) rrf-3(pk1426)**

**rnf-3(pk1426)**

Control (RNAi) gives 0.0, whereas cis-1 (RNAi) gives ≥2 AC (8/34).

**DHB cytoplasmic:nuclear ratio**

The ratio is plotted against control (RNAi) and cis-1 (RNAi).
Figure 1. A moderate increase in AC proliferation is observed upon systemic depletion of CKI-1. (A) Schematic of the *C. elegans* invasion/proliferation dichotomy. (B) Schematic of the CDK activity sensor, DHB, which is phosphorylated by CDK and translocates from the nucleus to the cytoplasm in response to CDK activity during cell cycle progression. The equation used to quantify CDK activity is shown. (C) DIC (left) and confocal images merging LAM-2::mNeonGreen with LAG-2::P2A::H2B::mTurquoise2 (middle) and DHB::2xmKate2 (right) from the P6.p 1-cell stage to the P6.p 4-cell stage are shown. Arrowheads and brackets indicate the position of the AC and P6.p cells, respectively. The scale bar represents 5 µm. (D) Confocal images merging LAM-2::mNeonGreen with LAG-2::P2A::H2B::mTurquoise2 (left) and DHB::2xmKate2 (right) from the P6.p 4-cell stage are shown. Arrowheads indicate the position of the AC(s) in *rrf-3(pk1426)* animals following control(RNAi) and *cki-1(RNAi)* treatment. The scale bar represents 5 µm. (E) Plot displays median and IQR of DHB::2xmKate2 ratios in the AC(s) of *rrf-3(pk1426)* animals following control(RNAi) and *cki-1(RNAi)* treatment (N ≥ 30 animals per treatment). Statistical significance was determined by a Mann-Whitney test (P < 0.0004). See also Figure S1, S2.
**Figure 2.** CKI-1 is critical for maintaining the post-mitotic state of the AC. (A-D) Confocal images merging LAM-2::mNeonGreen with LAG-2::P2A::H2B::mTurquoise2 (left) and DHB::2xmKate2 (right) from the P6.p 4-cell stage are shown. Arrowheads indicate the position of the AC(s) in cki-2(ok2105) (A), lin-35(n745) (B), fzr-1(ku298) (C), and lin-23(e1883) (D) animals following control(RNAi) and cki-1(RNAi) treatment. Scale bars represent 5 µm. (E-H) Plots display median and IQR of DHB::2xmKate2 ratios in the AC(s) of cki-2(ok2105) (E), lin-35(n745) (F), fzr-1(ku298) (G), and lin-23(e1883) (H) animals following control(RNAi) and cki-1(RNAi) treatment (N ≥ 30 animals per treatment). Statistical significance was determined by a Mann-Whitney test (P < 0.0001).
Table 1. Genetic analysis of negative cell cycle regulators during AC invasion.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>% invasion</th>
<th>% no invasion</th>
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<tr>
<td></td>
<td></td>
<td>1 AC</td>
<td>≥2 AC</td>
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<tr>
<td>wild type</td>
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<td>0</td>
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<tr>
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<td>100</td>
<td>0</td>
</tr>
<tr>
<td>lin-35(n745)</td>
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<td>0</td>
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<tr>
<td>fzr-1(ku298)</td>
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<td>0</td>
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<tr>
<td>lin-23(e1883)</td>
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<tr>
<td>wild type</td>
<td>cki-1(RNAi)</td>
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<td>13</td>
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<td>lin-23(e1883)</td>
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<td>19</td>
<td>74</td>
</tr>
</tbody>
</table>

AC invasion was scored at the P6.p 4-cell stage of vulval development, the stage when AC invasion normally occurs, using AC/VPC, BM, and cell cycle fluorescent reporters.
**A**

- **fzr-1(ku298)**
- *cdh-3p::mCh::moeABD*
- LAM-2::mNG
- DHB::2xmT2

**B**

- **fzr-1(ku298)**
- *cdh-3p::mCh::moeABD*
- LAM-2::mNG
- DHB::2xmT2

- **130 min**
  - pre-invasion
- **205 min**
  - initiation of invasion
- **310 min**
  - post-invasion
**Figure 3. Maintenance of the post-mitotic state is not required for AC invasion.** (A) Static images of cdh-3p::mCherry::moesinABD (left), LAM-2::mNeonGreen (middle), and DHB::2xmTurquoise2 (right) from the P6.p 4-cell stage are shown. Arrowheads indicate the position of the AC in a fzr-1(ku298); control(RNAi) animal. The scale bar represents 5 µm. (B) Time-lapse images of cdh-3p::mCherry::moesinABD (left), LAM-2::mNeonGreen (middle), and DHB::2xmTurquoise2 (right) from pre-AC invasion to post-AC invasion are shown. Arrowheads indicate the position of multiple invading ACs in a fzr-1(ku298); cki-1(RNAi) animal. The scale bar represents 5 µm. See also Movie 1 and Figure S3.
Movie 1. Time-lapse imaging confirms that cycling ACs can invade. A timelapse of two cycling ACs from pre-invasion to post-invasion is shown. The AC cell membrane, BM, and CDK activity are visualized using an F-actin probe (cdh-3p::mCherry::moesinABD), an endogenous lam-2 translational reporter (LAM-2::mNeonGreen), and a ubiquitous CDK activity sensor (DHB::2xmTurquoise2). The time points were acquired every 5 min for 350 min with a step size of 1 µm. The scale bar represents 5 µm.
A  
\[ fzr-1(ku298) \]

\[ \text{LAG-2::P2A::H2B::mT2} \quad \text{NHR-67::mNG} \quad \text{LAM-2::mNG} \quad \text{DHB::2xmK2} \]

B  
\[ fzr-1(ku298) \]

\[ \text{NHR-67::mNG} \]

\[ \text{control(RNAi)} \quad \text{cki-1(RNAi)} \]

C  
\[ \text{zmp-1p::CFP} \quad \text{LAM-2::mNG} \quad \text{DHB::2xmK2} \]

D  
\[ fzr-1(ku298) \]

\[ \text{zmp-1p::CFP} \]

\[ \text{control(RNAi)} \quad \text{cki-1(RNAi)} \]

E  
\[ \text{GRN (-)} \quad \text{proliferating AC} \quad \text{intact BM} \quad \text{GRN (+)} \quad \text{GRN TF depletion} \quad \text{BM} \quad \text{cell cycle perturbation} \quad \text{GRN (+)} \quad \text{proliferating AC} \quad \text{breached BM} \]
Figure 4. Proliferating, invasive ACs retain a functionally intact pro-invasive GRN. (A) Confocal images of LAG-2::P2A::H2B::mTurquoise2 (left), NHR-67::mNeonGreen (middle), LAM-2::mNeonGreen (middle), and DHB::2xmKate2 (right) from the P6.p 4-cell stage are shown. Arrowheads indicate the position of the AC(s) in fzr-1(ku298) animals following control(RNAi) and cki-1(RNAi) treatment. The scale bar represents 5 µm. (B) Plot displays mean and SD of normalized NHR-67::mNeonGreen intensity in the AC(s) of fzr-1(ku298) animals following control(RNAi) and cki-1(RNAi) treatment (N ≥ 30 animals per treatment). (C) Confocal images of zmp-1p::CFP (left), LAM-2::mNeonGreen (middle), and DHB::2xmKate2 (right) from the P6.p 4-cell stage are shown. Arrowheads indicate the position of the AC(s) in fzr-1(ku298) animals following control(RNAi) and cki-1(RNAi) treatment. The scale bar represents 5 µm. (D) Plot displays mean and SD of normalized zmp-1p::CFP intensity in the AC(s) of fzr-1(ku298) animals following control(RNAi) and cki-1(RNAi) treatment (N ≥ 31 animals per treatment). (E) Whereas depletion of pro-invasive GRN transcription factors (TFs) results in proliferating, non-invasive ACs (left), direct perturbation of the AC cell cycle results in proliferating, invasive ACs that maintain pro-invasive gene expression (right).