

Dynamic compartmentalization of the pro-invasive transcription factor NHR-67 reveals a role for Groucho in regulating a proliferative-invasive cellular switch in *C. elegans*

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Abstract

A growing body of evidence suggests that cell division and basement membrane invasion are mutually exclusive cellular behaviors. How cells switch between proliferative and invasive states is not well understood. Here, we investigated this dichotomy *in vivo* by examining two cell types in the developing *Caenorhabditis elegans* somatic gonad that derive from equipotent progenitors, but exhibit distinct cell behaviors: the post-mitotic, invasive anchor cell and the neighboring proliferative, non-invasive ventral uterine (VU) cells. We show that the fates of these cells post-specification are more plastic than previously appreciated and that levels of NHR-67 are important for discriminating between invasive and proliferative behavior. Transcription of *nhr-67* is downregulated following post-translational degradation of its direct upstream regulator, HLH-2 (E/Daughterless) in VU cells. In the nuclei of VU cells, residual NHR-67 protein is compartmentalized into discrete punctae that are dynamic over the cell cycle and exhibit liquid-like properties. By screening for proteins that colocalize with NHR-67 punctae, we identified new regulators of uterine cell fate maintenance: homologs of the transcriptional co-repressor Groucho (UNC-37 and LSY-22), as well as the TCF/LEF homolog POP-1. We propose a model in which association of NHR-67 with the Groucho/TCF complex suppresses the default invasive state in non-invasive cells, which complements transcriptional regulation to add robustness to the proliferative-invasive cellular switch *in vivo*.

eLife assessment

This paper reports **valuable** data on a classic model for cell fate specification in the *C. elegans* gonad between cells with proliferative and invasive states. The authors present **convincing** data establishing that expression of the transcription factor NHR-67 is sufficient to drive an invasive fate, and that the alternative proliferative fate is associated with NHR-67 transcriptional down-regulation. While the data showing that NHR-67 forms punctae associated with transcriptional repressors in non-invasive cells is intriguing, they are, as the authors acknowledge, currently **incomplete** with respect to establishing a functional role for these NHR-67 punctae. The observations and extensive data will be of interest to developmental biologists studying transcriptional control of cell fate specification in animals, especially once issues around the functional significance of the punctae are resolved.

Introduction

Cellular proliferation and invasion are key aspects of development (reviewed in [Medwig & Matus, 2017](#) [↗](#)), and are also two of the defining hallmarks of cancer (reviewed in [Hanahan and Weinberg, 2000](#) [↗](#)). A growing body of evidence suggests that cell cycle progression and invasion through a basement membrane are mutually exclusive cellular behaviors in both development and disease states (reviewed in [Kohrman and Matus, 2017](#) [↗](#)). Switching between invasive and proliferative phenotypes has been observed in melanoma and recently in breast cancer ([Hoek et al., 2008](#) [↗](#); [Mondal et al., 2021](#) [↗](#)), but how these cell states are regulated in the context of development is not well understood. To investigate how this dichotomy in cellular behavior is controlled *in vivo*, we used *C. elegans*, leveraging its highly stereotypical development ([Sulston and Horvitz, 1977](#) [↗](#)), as well as its genetic and optical tractability. During development of the hermaphroditic reproductive system, the proximal granddaughters of the Z1 and Z4 somatic gonad progenitors, Z1.pp and Z4.aa, give rise to four cells that will adopt one of two cellular fates: a proliferative ventral uterine (VU) cell or the terminally differentiated, invasive anchor cell (AC) (**Figure 1A** [↗](#)) ([Kimble and Hirsh, 1979](#) [↗](#)). The distal cells of this competency group, Z1.ppa and Z4.aap, quickly lose their bipotentiality and become VU cells ([Seydoux et al., 1990](#) [↗](#)). In contrast, the proximal cells, Z1.ppp and Z4.aaa, undergo a stochastic Notch-mediated cell fate decision, giving rise to another VU cell and the post-mitotic AC (**Figure 1A,B** [↗](#)) ([Greenwald et al., 1983](#) [↗](#); [Seydoux and Greenwald, 1989](#) [↗](#)). Following fate specification, the AC undergoes invasive differentiation and breaches the underlying basement membrane, connecting the uterus to the vulval epithelium to facilitate egg-laying (**Figure 1B** [↗](#)) ([Sherwood and Sternberg, 2003](#) [↗](#)). The AC is the default cell fate of the somatic gonad, as disruption of Notch or Wnt signaling results in ectopic AC specification ([Phillips et al., 2007](#) [↗](#); [Seydoux and Greenwald, 1989](#) [↗](#)). Therefore, AC fate must be actively repressed.

Our previous work has shown that AC invasion is dependent on G₀ cell cycle arrest, which is coordinated by the pro-invasive transcription factor NHR-67 (NR2E1/TLX) (**Figure 1-figure supplement 1A** [↗](#)) ([Matus et al., 2015](#) [↗](#)). NHR-67 functions within a gene regulatory network comprised of four conserved transcription factors whose homologs have been implicated in several types of metastatic cancer ([Liang and Wang, 2020](#) [↗](#); [Milde-Langosch, 2005](#) [↗](#); [Nelson et al., 2021](#) [↗](#); [Wang and Baker, 2015](#) [↗](#)). We previously reported that NHR-67 is regulated by a feed-forward loop formed by EGL-43 (Evi1) and HLH-2 (E/Daughterless), which functions largely in parallel to a cell cycle-independent subcircuit controlled by FOS-1 (Fos) (**Figure 1-figure supplement 1A** [↗](#)) ([Medwig-Kinney et al., 2020](#) [↗](#)). EGL-43, HLH-2, and NHR-67 are reiteratively used within the Z lineage of the somatic gonad, in that they also function to independently

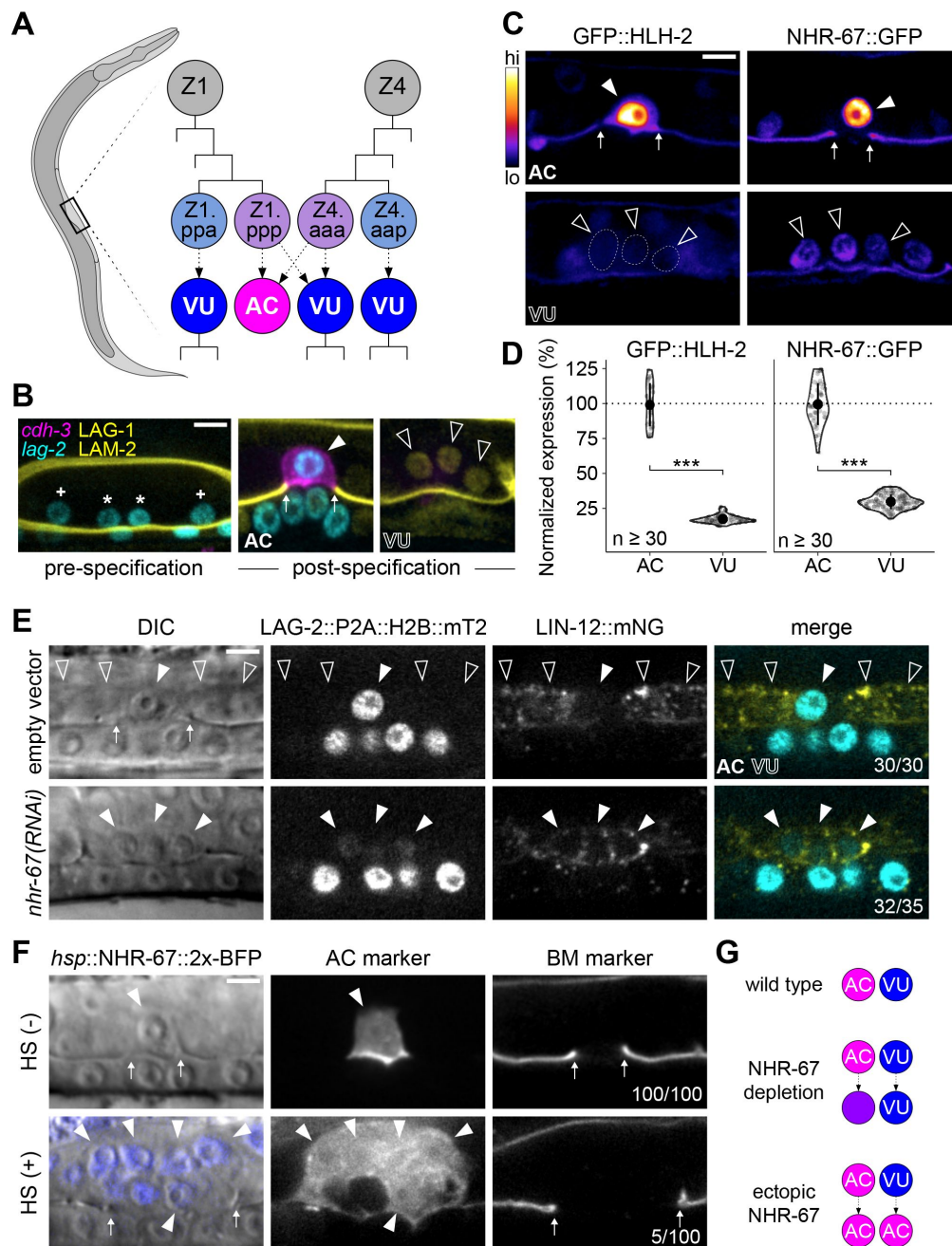


Figure 1

Invasive AC fate correlates to high levels of NHR-67.

(A) Schematic of *C. elegans* anchor cell (AC, magenta) and ventral uterine (VU, blue) cell fate specification from the Z1 and Z4 somatic gonad precursor cell lineages (p, posterior daughter; a, anterior daughter). (B) Micrographs depicting AC and VU cell differentiation over developmental time. AC/VU precursors express LAG-2 (H2B::mTurquoise), which eventually becomes restricted to the AC, whereas VU cells express LAG-1 (mNeonGreen) post-specification. The differentiated AC (*cdh-3p::mCherry::moeABD*) then invades through the underlying basement membrane (LAM-2::mNeonGreen). (C-D) Representative heat map micrographs (C) and quantification (D) of GFP-tagged HLH-2 and NHR-67 expression in the AC and VU cells at the time of AC invasion. (E) Expression of Notch (*lin-12::mNG*) and Delta (*lag-2::P2A::H2B::mT2*) following RNAi-induced knockdown of *nhr-67* compared to empty vector control. (F) Micrographs depicting the ectopic invasive ACs (*cdh-3p::mCherry::moeABD*, arrowheads) and expanded basement membrane (*laminin::GFP*, arrows) gap observed following heat shock induced expression of NHR-67 (*hsp::NHR-67::2x-BFP*) compared to non-heat shocked controls. (G) Schematic summarizing AC and VU cell fates that result from perturbations of NHR-67 levels. For all figures: asterisk (*), AC/VU precursor; plus (+), VU precursor; solid arrowhead, AC; open arrowhead, VU cell; arrows, basement membrane breach. Statistical significance determined by Student's t-test (* $p > 0.05$, ** $p > 0.01$, *** $p > 0.001$). Scale bars, 5 μ m.

regulate LIN-12 (Notch) signaling during the initial AC/VU cell fate decision (Medwig-Kinney et al., 2020). Despite its role in lateral inhibition between Z1.ppp and Z4.aaa, expression of LIN-12 is not absolutely required for VU cell fate (Sallee et al., 2015). Cell cycle state also cannot explain the difference between AC and VU cell fates, as arresting VU cells in G₀ through ectopic expression of CKI-1 (p21/p27) does not make them invasive (Smith et al., 2022). Thus, the mechanisms responsible for maintaining AC and VU cellular identities following initial cell fate specification remain unclear.

Maintenance of differentiated cell identity is essential for ensuring tissue integrity during development and homeostasis, and the inability to restrict phenotypic plasticity is now being recognized as an integral part of cancer pathogenesis (Hanahan, 2022). *In vitro* studies have identified several factors that safeguard differentiated cell identity (reviewed in Brumbaugh et al., 2019). Despite its largely autonomous modality of development, *C. elegans* has emerged as an ideal model system to study cell fate maintenance *in vivo*. There have been several reports of cell fate transformations that occur naturally, including two epithelial-to-neural transdifferentiation events (Jarriault et al., 2008; Riva et al., 2022), or following fate challenges (reviewed in Rothman and Jarriault, 2019). In such contexts, several epigenetic factors, including chromatin remodelers and histone chaperones, have been identified for their roles in restricting cell fate reprogramming (Hajduskova et al., 2019; Kagias et al., 2012; Kolundzic et al., 2018; Patel et al., 2012; Rahe and Hobert, 2019; Zuryn et al., 2014). However, in some cases, ectopic expression of a specific transcription factor is sufficient to overcome these barriers, as was first shown through pioneering work in mouse embryonic fibroblasts (Davis et al., 1987). Indeed, there are several examples in *C. elegans* where ectopic expression of single lineage-specific transcription factors induces cell fate transformations (Fukushige and Krause, 2005; Gilleard and McGhee, 2001; Horner et al., 1998; Jin et al., 1994; Kiefer et al., 2007; Quintin et al., 2001; Richard et al., 2011; Riddle et al., 2013; Tursun et al., 2011; Zhu et al., 1998). Moreover, *C. elegans* uterine tissue may be particularly amenable to fate transformations, as ectopic expression of a single GATA transcription factor, ELT-7, is sufficient to induce transorganogenesis of the somatic gonad into gut by reprogramming the mesodermally-derived tissue into endoderm (Riddle et al., 2016). Valuable insights have been made into how the function of fate-specifying transcription factors can be tuned through means such as autoregulation and dynamic heterodimerization (Leyva-Díaz and Hobert, 2019; Sallee et al., 2017). We are just beginning to understand how an additional layer of control over transcriptional regulators can be achieved through compartmentalization (Boija et al., 2018; Lim and Levine, 2021).

Here, in our endeavor to understand how AC and VU cellular fates are maintained, we identified two mechanisms that together modulate the invasive-proliferative switch in *C. elegans*. We found that high levels of NHR-67 expression are sufficient to drive invasive differentiation, and that *nhr-67* is transcriptionally downregulated in the non-invasive VU cells following the post-translational degradation of its direct upstream regulator, HLH-2. Additionally, we observed that remaining NHR-67 protein in the VU cells compartmentalizes into punctae that exhibit liquid-like properties including dynamic assembly, fusion, and dissolution over cell cycle as well as rapid recovery kinetics after photobleaching. These NHR-67 punctae colocalize *in vivo* with UNC-37 and LSY-22, homologs of the transcriptional co-repressor Groucho, as well as with POP-1 (TCF/LEF), which is likely mediated through a direct interaction between UNC-37 and the intrinsically disordered C-terminal region of NHR-67. Through functional perturbations, we demonstrate that UNC-37, LSY-22, and POP-1 contribute to repression of the default invasive state in VU cells. We propose a model in which NHR-67 compartmentalizes through its interaction with Groucho, which, combined with transcriptional downregulation of *nhr-67*, may suppress invasive differentiation.

NHR-67 expression levels are important for distinguishing AC and VU cell identity

Despite arising from initially equipotent cells, the differentiated AC and VU cells exhibit very distinct cellular behaviors. The AC terminally differentiates to invade the underlying basement membrane while the VU cells remain proliferative, undergoing several rounds of division before terminally differentiating. One potential explanation for this difference in cell behavior is asymmetric expression of pro-invasive transcription factors. To investigate this possibility, we examined endogenous expression levels of four transcription factors that function in the gene regulatory network coordinating AC invasion (EGL-43, FOS-1, HLH-2, and NHR-67) using GFP-tagged alleles (Medwig-Kinney et al., 2020 [↗](#)). While FOS-1 levels are enriched in the AC compared to the VU cells (Figure 1-figure supplement 1B,C [↗](#)), FOS-1 has no known role in cell cycle regulation, so we did not pursue this protein further (Medwig-Kinney et al., 2021 [↗](#)). EGL-43 was also not a promising candidate, as it is expressed in both cell types at comparable levels, with VU cells exhibiting approximately 89% of AC expression (Figure 1-figure supplement 1B,C [↗](#)). In contrast, HLH-2 exhibits significant asymmetry in expression, as VU cells express merely 17% of HLH-2 levels observed in the AC (Figure 1C,D [↗](#)). Previous studies have shown that dimerization-driven degradation of HLH-2 is responsible for its downregulation in the VU cells (Benavidez et al., 2022 [↗](#); Karp and Greenwald, 2003 [↗](#); Sallee and Greenwald, 2015 [↗](#)). NHR-67 exhibits a similar pattern of expression with over three-fold enrichment in the AC, consistent with prior observations of transgenic reporters (Figure 1C,D [↗](#)) (Verghese et al., 2011 [↗](#)). Given the known role of NHR-67 in regulating cell cycle and invasion, we hypothesized that its differential expression between the AC and VU cells could contribute to their distinct cellular behaviors.

To assess the potential role of NHR-67 in regulating uterine cell identities, we manipulated its expression levels. We found that strong depletion of NHR-67 through RNA interference (RNAi) treatment results in ACs adopting VU-like characteristics. During AC/VU cell fate specification, LIN-12 (Notch) normally becomes restricted to the VU cells while the Delta-like ligand LAG-2 (visualized by LAG-2::P2A::H2B::mTurquoise2 (Medwig-Kinney et al., 2022 [↗](#))) accumulates in the AC (Wilkinson et al., 1994 [↗](#)). Here, we observe that NHR-67 deficient ACs not only proliferated and failed to invade, as reported previously (Matus et al., 2015 [↗](#)), but also ectopically expressed membrane-localized Notch (visualized by LIN-12::mNeonGreen (Pani et al., 2022 [↗](#))) (Figure 1E [↗](#); Figure 1-figure supplement 2A,B [↗](#)). Notably, NHR-67-deficient ACs expressed both LIN-12 and LAG-2, potentially indicating an intermediate state between AC and VU cell fate (Figure 1E [↗](#); Figure 1-figure supplement 2A,B [↗](#)). Next, we ectopically expressed NHR-67 ubiquitously following AC/VU specification using a heat shock inducible transgene (*hsp::NHR-67::2x-BFP*) (Medwig-Kinney et al., 2020 [↗](#)) and observed the presence of multiple invasive ACs at a low penetrance (approximately 5%, $n > 50$), denoted by ectopic expression of an AC marker (*cdh-3p::mCherry::moeABD*) and expansion of the basement membrane gap (Figure 1F [↗](#)). As previous work has demonstrated that proliferative ACs cannot invade (Matus et al., 2015 [↗](#)), we concluded that these invasive ectopic ACs most likely arose from fate conversion of neighboring VU cells. Taken together, these pieces of evidence suggest that high and low levels of NHR-67 correlate to properties of AC and VU cell identities, respectively (Figure 1G [↗](#)).

NHR-67 is enriched in the AC through transcriptional regulation by HLH-2

Next, we investigated how NHR-67 expression levels become asymmetric between the AC and VU cells. We and others have previously shown that HLH-2 positively regulates NHR-67 expression in the context of the AC (Figure 1-figure supplement 1A [↗](#)) (Bodofsky et al., 2018 [↗](#); Medwig-Kinney et al., 2020 [↗](#)). If this regulatory interaction also exists in the context of the VU cells, it could explain why the relative expression pattern of NHR-67 in the AC and VU cells mirrors that of HLH-

2. In support of this hypothesis, we found that initial onset of HLH-2, which has shown to be asymmetric in Z1.pp and Z4.aa (Attner et al., 2019 [↗](#)), correlates to that of NHR-67 onset (**Figure 2-figure supplement 1A** [↗](#)). To test whether HLH-2 degradation is responsible for NHR-67 downregulation in the VU, we drove ectopic expression of HLH-2 using a transgene under the control of a heat shock inducible promoter (*hsp::HLH-2::2x-BFP*) (Medwig-Kinney et al., 2020 [↗](#)) and observed elevated NHR-67 expression in VU cells (43% increase; $n > 30$) (**Figure 2A,B** [↗](#)). To control against potential dimerization-driven degradation of HLH-2 in the VU cells, which the heat shock inducible transgene would still be susceptible to, we disrupted UBA-1, an E1 ubiquitin-activating enzyme that has recently been shown to be necessary for HLH-2 degradation in VU cells (Benavidez et al., 2022 [↗](#)). Following perturbation of UBA-1 through RNAi treatment, HLH-2 expression in the VU cells increased more than four-fold and NHR-67 expression increased by nearly 60% compared to the empty vector control (**Figure 2-figure supplement 1B-D** [↗](#)). Both experiments suggest that *nhr-67* expression in the VU cells is at least partially regulated by levels of HLH-2.

It has previously been proposed that the interaction between HLH-2 and *nhr-67* is direct. This is based on the identification of E binding motifs within a 276 bp region of the *nhr-67* promoter that is required for NHR-67 expression in the uterine tissue and encompasses the location of several hypomorphic mutations (*pf2*, *pf88*, *pf159*) (**Figure 2C** [↗](#)) (Bodofsky et al., 2018 [↗](#); Verghese et al., 2011 [↗](#)). We performed a yeast one-hybrid assay by generating a bait strain containing this *nhr-67* promoter region and pairing it with an HLH-2 Gal4-AD prey plasmid from an existing yeast one-hybrid library (Reece-Hoyes et al., 2005 [↗](#)). Yeast growth on the selective SC-HIS-TRP plates containing the competitive inhibitor 3-aminotriazole (3-AT) suggests that HLH-2 is capable of binding directly to this 276 bp region of the *nhr-67* promoter (**Figure 2D** [↗](#)). Together, these results support that direct transcriptional regulation of *nhr-67* by HLH-2 contributes to the asymmetry in NHR-67 expression between the AC and VU cells.

NHR-67 dynamically compartmentalizes in VU cell nuclei

Upon closer examination of GFP-tagged NHR-67, it became evident that the AC and VU cells not only exhibit differences in overall NHR-67 levels, but also in localization of the protein. While NHR-67 localization is fairly uniform throughout the AC nucleus (excluding the nucleolus), we often observed discrete punctae throughout the nuclei of VU cells (**Figure 3A,B** [↗](#)). These punctae were observed with NHR-67 endogenously tagged with several different fluorescent proteins, including GFP, mNeonGreen, mScarlet-I, and TagRFP-T (**Figure 3-figure supplement 1A,B** [↗](#)) and in the absence of tissue fixation methods that can cause artificial puncta (Irgen-Gioro et al., 2022 [↗](#)), suggesting that they are not an artifact of the fluorophore or sample preparation.

To characterize dynamics of these punctae during interphase states of the cell cycle, we paired GFP-tagged NHR-67 with a CDK activity sensor. The CDK activity sensor is comprised of a fragment of DNA Helicase B (DHB) fused to a fluorophore (2x-mKate2), expressed under a ubiquitous promoter (**Figure 3C** [↗](#)) (Adikes et al., 2020 [↗](#)). DHB contains a strong nuclear localization signal (NLS), flanked by four serine sites, as well as a weaker nuclear export signal (NES). As CDK activity increases over the cell cycle, the CDK sensor is translocated from the nucleus to the cytoplasm, allowing for correlation of its relative subcellular localization to cell cycle state (**Figure 3C** [↗](#)) (Adikes et al., 2020 [↗](#); Spencer et al., 2013 [↗](#)). Time-lapse microscopy revealed that the number of NHR-67 punctae was dynamic over the course of the cell cycle, with punctae first appearing shortly after mitotic exit in the G1 phase, and then reducing in number to two large punctae prior to nuclear envelope breakdown before disappearing (**Figure 3D,E** [↗](#)). We collected additional recordings with finer time resolution and captured fusion, or condensation, of punctae prior to their dissolution (representative of 6 biological replicates) (**Figure 3F** [↗](#)). These punctae also exhibit relatively rapid diffusion kinetics, as observed by fluorescence recovery following photobleaching ($t_{1/2} = 46$ seconds; $n = 8$) at a rate within the same order of magnitude as P granule proteins PGL-1 and PGL-3 (**Figure 3G,H** [↗](#)) (Putnam et al., 2019 [↗](#)).

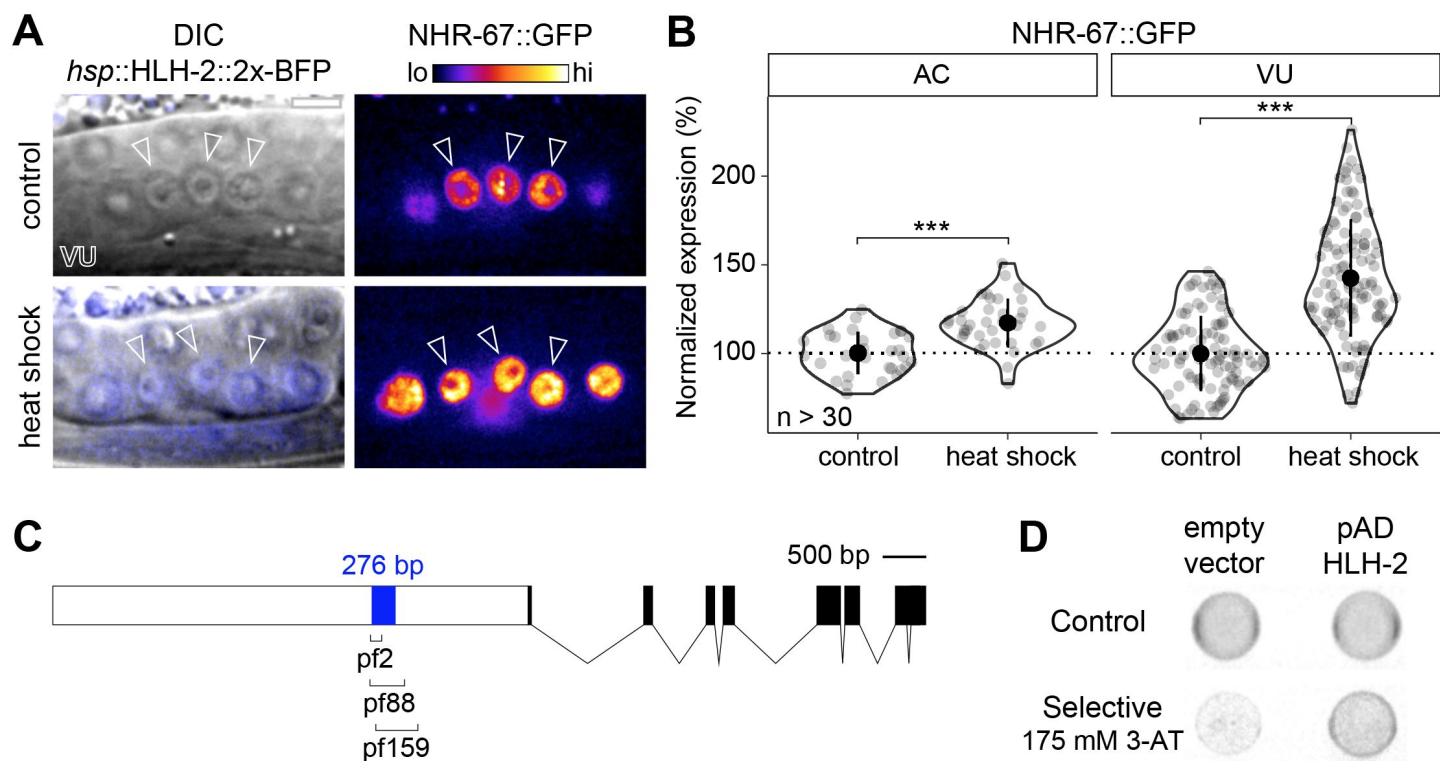


Figure 2

NHR-67 expression is downregulated in VU cells through direct transcriptional regulation by HLH-2.

(A-B) Representative heat map micrographs (A) and quantification (B) of NHR-67::GFP expression in VU cells following heat shock induced expression of HLH-2 (2x-BFP) compared to non-heat shocked controls. (C) Schematic of a 276 bp putative regulatory element within the promoter of *nhr-67* (Bodofsky et al., 2018), annotated with the location of three hypomorphic mutations (*pf2*, *pf88*, and *pf159*). (D) Yeast one-hybrid experiment pairing HLH-2 Gal4-AD prey with the 276 bp fragment of the *nhr-67* promoter as bait on SC-HIS-TRP plates with and without competitive inhibitor 3-AT (175 mM).

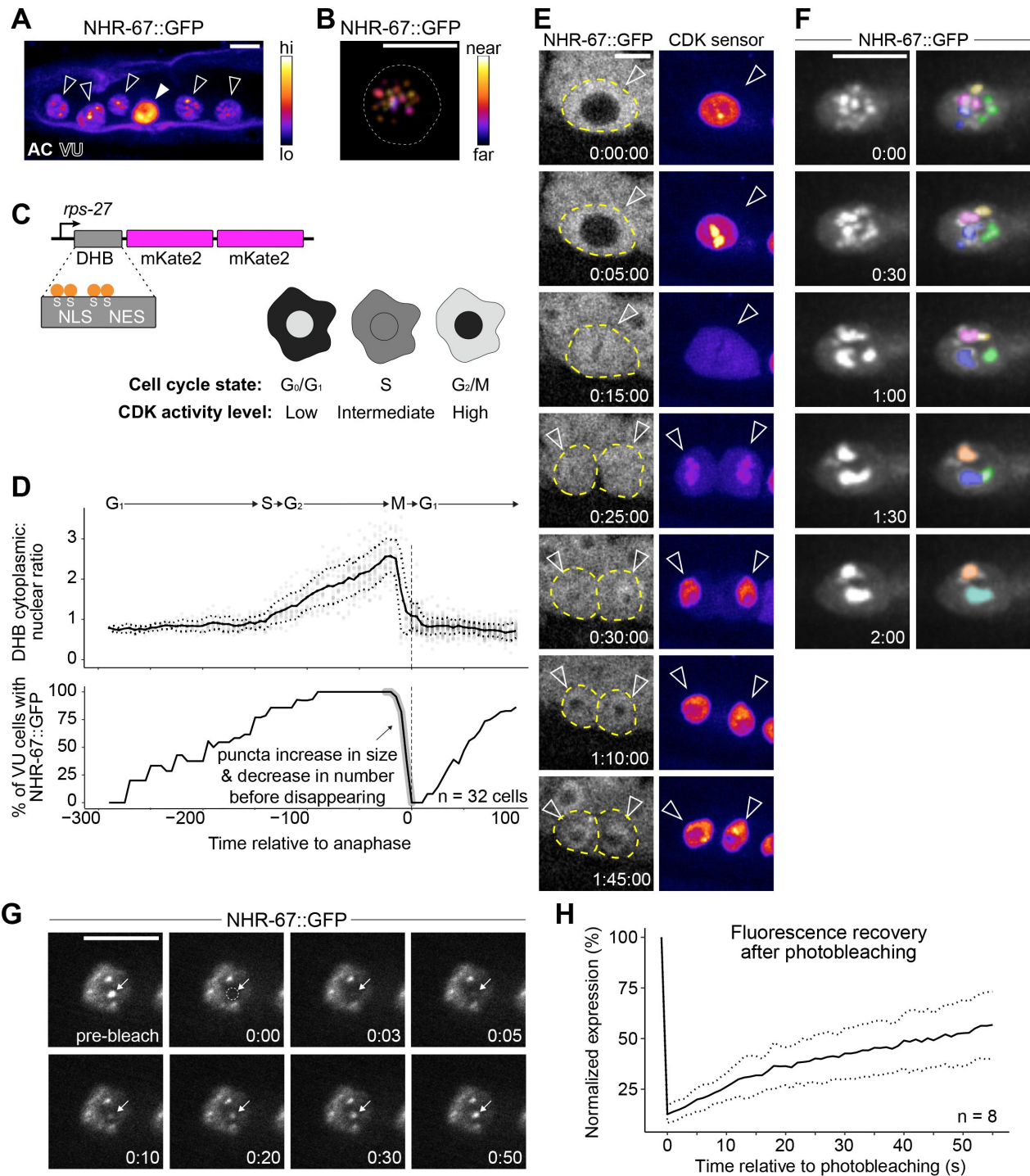


Figure 3

NHR-67 dynamically compartmentalizes in nuclei of VU cells.

(A) Heat-map maximum intensity projection of NHR-67::GFP showing protein localization in the AC and VU cells. (B) Spatial color coded projection of NHR-67::GFP punctae in VU, with nuclear border indicated with a dotted line. (C) Schematic of DNA Helicase B (DHB) based CDK sensor and its dynamic localization over the cell cycle. (D) Graphs depicting CDK activity levels and corresponding cell cycle state (top), and percentage of cells exhibiting NHR-67::GFP punctae (bottom) over time, aligned to anaphase. (E) Representative time-lapse of NHR-67::GFP over the course of a cell cycle, with cell membranes indicated with dotted lines. (F) Time-lapse depicting NHR-67::GFP punctae fusion prior to cell division. Bottom panels are pseudo-colored. (G-H) Quantification (G) and representative images (H) depicting fluorescence recovery of NHR-67::GFP following photobleaching of individual punctae (arrow).

Groucho homologs UNC-37 and LSY-22 associate with NHR-67 punctae and contribute to VU cell fate

Next, we tested the extent to which NHR-67 punctae colocalize with homologs of other proteins known to compartmentalize in nuclei by pairing GFP-and mScarlet-I-tagged NHR-67 with other endogenously tagged alleles. As NHR-67 is a transcription factor, we speculated that its punctae may represent clustering around sites of active transcription, which would be consistent with data showing RNA Polymerase II and the Mediator complex can compartmentalize with transcription factors (Cho et al., 2018). To test this hypothesis, we co-visualized NHR-67 with a GFP-tagged allele of *ama-1*, the amanitin-binding subunit of RNA polymerase II (Hills-Muckey et al., 2021) and failed to observe significant colocalization between NHR-67 and AMA-1 punctae (Manders' overlap coefficient, $M = 0.066$) compared to negative controls where a single channel was compared to its 90-degree rotation ($M = 0.108$) (Figure 4A,B). Another possibility considered is that NHR-67 punctae could correlate to chromatin organization, as heterochromatin has been shown to be compartmentalized in the nucleus (Larson et al., 2017; Strom et al., 2017). However, we did not observe significant colocalization of NHR-67 with the endogenously tagged HP1 heterochromatin proteins (Patel and Hobert, 2017) HPL-1 ($M = 0.076$) or HPL-2 ($M = 0.083$) (Figure 4A,B). We next tested if NHR-67 colocalizes with the transcriptional co-repressor Groucho, as Groucho had recently been shown to compartmentalize in the nuclei of cells in *Ciona* (Treen et al., 2021). The *C. elegans* genome encodes one Groucho homolog, UNC-37, which we acquired an mNeonGreen-tagged allele of (Ma et al., 2021), and a Groucho-like protein, LSY-22, which we tagged with TagRFP-T (Figure 4-figure supplement 1). We observed significant colocalization of NHR-67 punctae with both LSY-22 ($M = 0.686$) and UNC-37 ($M = 0.741$), comparable to colocalization measures in heterozygous NHR-67::mScarlet-I/NHR-67::GFP animals ($M = 0.651$), which were used as positive controls (Figure 4A,B). This evidence suggests that NHR-67 punctae do not localize to sites of active transcription or chromatin compaction, but instead associate with transcriptional co-repressors.

Since the AC is the default state of the AC/VU cell fate decision (Seydoux and Greenwald, 1989), we hypothesized that the punctae including NHR-67, UNC-37, and LSY-22 may function in repressing invasive differentiation. To test this hypothesis, we depleted UNC-37 and LSY-22 utilizing the auxin inducible degron (AID) protein degradation system, in which a protein of interest is tagged with an AID that is recognized by TIR1 in the presence of auxin and ubiquitinated by the SCF E3 ubiquitin ligase complex (Figure 4C) (Martinez et al., 2020; Zhang et al., 2015). We re-tagged LSY-22 with mNeonGreen::AID (Figure 4-figure supplement 1) and acquired a BFP::AID-tagged allele of *unc-37* (Kurashina et al., 2021). Each AID-tagged allele was paired with a transgene encoding *Arabidopsis thaliana* TIR1 (*A_tTIR1*) that was co-expressed with a nuclear-localized mCherry::HIS-11. Following auxin treatment, we observed ectopic expression of an AC marker (*cdh-3p::mCherry::moeABD*) in 28% of LSY-22::AID animals and 59% of UNC-37::AID animals ($n = 64$ for both) (Figure 4D,E). These results are consistent with phenotypes we observed in genetic backgrounds with *unc-37* hypomorphic (*unc-37(e262wd26)*) and null (*unc-37(wd17wd22)*) mutant alleles (Figure 4-figure supplement 2). It is likely that dual depletion of UNC-37 and LSY-22 would result in a higher penetrance of ectopic ACs given their partial redundancy in function (Flowers et al., 2010), but animals possessing both AID-tagged alleles were not viable when paired with the *A_tTIR1* transgene.

TCF/LEF homolog POP-1 associates with NHR-67 punctae and contributes to VU cell fate post-specification

While UNC-37 and LSY-22 appear to be important for maintenance of normal uterine cell fates, both genes are broadly expressed and exhibit comparable levels (<10% difference) between the AC and VU cells (Figure 5A,C; Figure 5-figure supplement 1A,B); therefore, we hypothesized that another factor must be involved. It had previously been reported that the sole TCF/LEF homolog in *C. elegans*, POP-1, forms a repressive complex with UNC-37 in the early embryo to

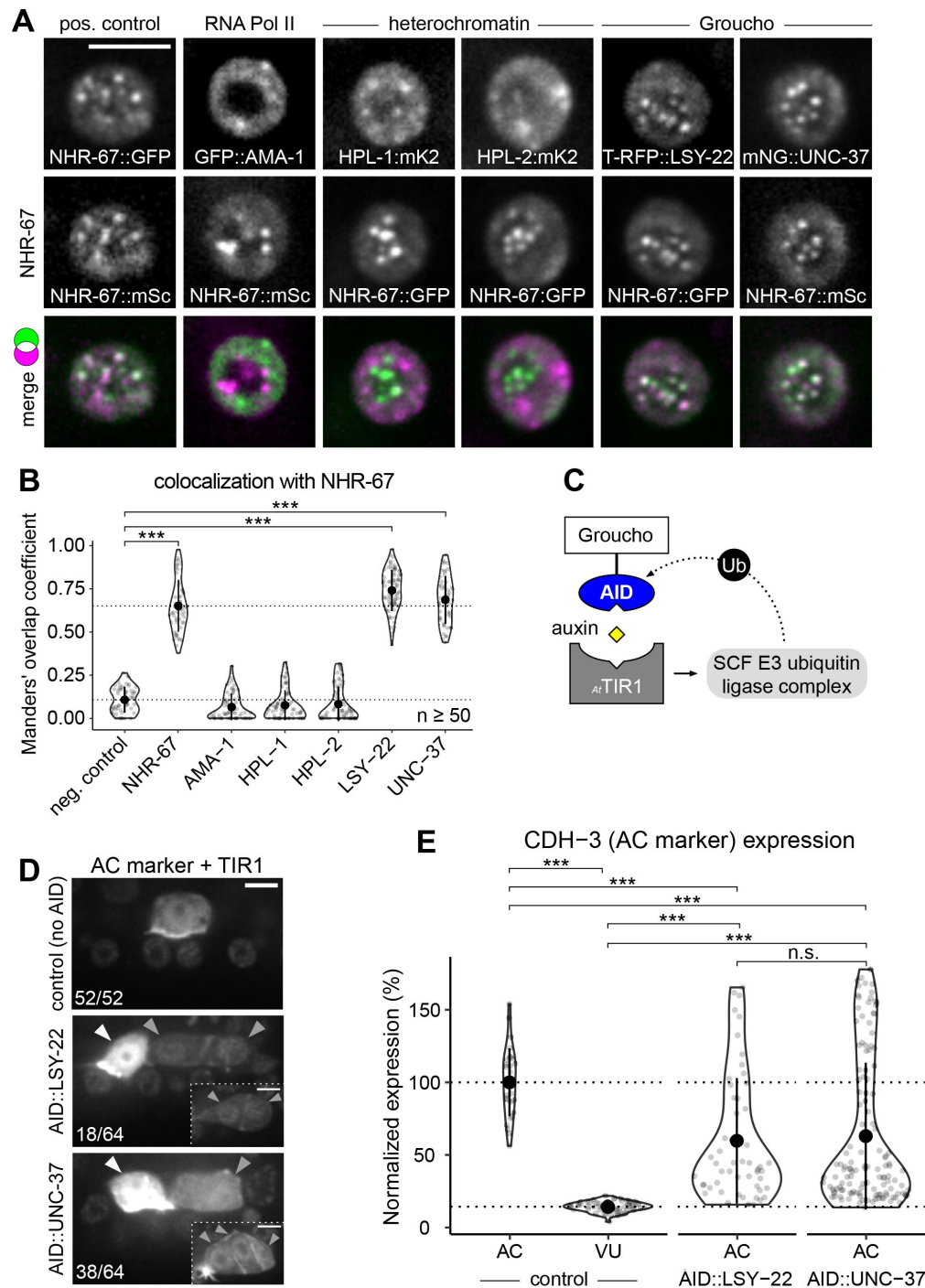


Figure 4

Groucho homologs LSY-22 and UNC-37 colocalize with NHR-67 punctae and contribute to maintenance of VU cell fate.

(A) Co-visualization of NHR-67 with RNA Polymerase II (GFP::AMA-1), HP1 heterochromatin proteins (HPL-1::mKate2 and HPL-2::mKate2), and Groucho homologs (TagRFP-T::LSY-22 and mNeonGreen::UNC-37) in VU cells using endogenously tagged alleles. (B) Quantification of colocalization, with plot reporting Manders' overlap coefficients compared to negative controls (90 degree rotation of one channel) and positive controls. (C) Schematic of the auxin inducible degron (AID) system, where *AtTIR1* mediates proteasomal degradation of AID-tagged proteins in the presence of auxin. (D) Representative images of phenotypes observed following individual AID-depletion of UNC-37 and LSY-22 compared to control animals without AID-tagged alleles. All animals compared here are expressing TIR1 ubiquitously (*rpl-28p::AtTIR1::T2A::mCherry::HIS-11*) and an AC marker (*cdh-3p::mCherry::moeABD*). Insets depict different z planes of the same image. (E) Quantification of AC marker (*cdh-3p::mCherry::moeABD*) expression in ectopic ACs resulting from AID-depletion of UNC-37 and LSY-22 compared to control AC and VU cells.

restrict expression of the endoderm-determining gene, *END-1* (Calvo et al., 2001). Additionally, POP-1 has a known role in development of the somatic gonad, as perturbing its function results in ectopic ACs (Siegfried and Kimble, 2002). Examination of an eGFP-tagged *pop-1* allele (van der Horst et al., 2019), showed significant enrichment in the VU cells (>20%) compared to the AC (Figure 5B,C; Figure 5-figure supplement 1A,B). We also observed that endogenous POP-1 forms punctae in the nuclei of VU cells, which had previously been observed during interphase in non-Wnt signaled embryonic cells (Maduro et al., 2002). These POP-1 punctae colocalize with NHR-67 ($M = 0.547$), although to a lesser degree than UNC-37 and LSY-22, likely because the strong POP-1 fluorescence outside of punctae made them more difficult to segment (Figure 5D,E). Additionally, *nhr-67(RNAi)* treatment resulted in a significant increase in AC expression of eGFP::POP-1 compared to empty vector controls (225%, $n > 30$), a pattern we observed following depletion of other transcription factors (Medwig-Kinney et al., 2020) and chromatin modifiers (Smith et al., 2022) required for AC arrest and invasion (Figure 5F,G; Figure 5-figure supplement 2A,B). This negative regulation of POP-1 by NHR-67 may explain why the proteins have opposite patterns of enrichment.

It has previously been suggested that POP-1 may be functioning as an activator in the VU precursors Z1.ppa and Z4.aap based on the relative expression of a POP-1 transgene (Sallee et al., 2015). This view is largely dependent on the notion that high levels of POP-1 correlate to repressive function and low levels are conducive for activator roles (Shetty et al., 2005). In contrast, we did not find evidence of transcriptional activation by POP-1 in the AC/VU precursors nor their differentiated descendants using an established POPTOP (POP-1 and TCF optimal promoter) reporter, which contains seven copies of POP-1/TCF binding sites and the *pes-10* minimal promoter (Figure 5H; Figure 5-figure supplement 3A,B) (Green et al., 2008). The growing consensus regarding the Wnt/ β -catenin asymmetry pathway is that relative levels of POP-1 and β -catenin are more important than absolute protein levels of POP-1 (Phillips and Kimble, 2009). Our proposed model of POP-1 acting as a repressor in the proximal gonad is consistent with the finding that SYS-1 (β -catenin) expression is restricted to the distal gonad early in somatic gonad development and is not detectable in the AC or VU cells (Figure 5-figure supplement 3C) (Phillips et al., 2007; Sallee et al., 2015). It is also supported by recent evidence suggesting that UNC-37/LSY-22 mutant alleles phenocopy *pop-1* knockdown, which produces ectopic distal tip cells (Bekas and Phillips, 2022).

One aspect that makes studying the repressive role of POP-1 in cell fate maintenance challenging is that its activator function is required for distal cell fate specification in the somatic gonad earlier in development. Loss of either POP-1 and SYS-1 results in a Sys (symmetrical sister cell) phenotype, where all somatic gonad cells adopt the default proximal fate and thereby give rise to ectopic ACs (Siegfried and Kimble, 2002; Siegfried et al., 2004). This likely occluded previous identification of the potential repressive role of POP-1 in maintaining VU cell fates. To achieve temporal control over POP-1 expression to tease apart its roles, we sought to use the AID system, but insertion of the degron into the *pop-1* locus disrupted the protein's function. Instead, we paired eGFP-tagged POP-1 with a uterine-specific anti-GFP nanobody (Smith et al., 2022; Wang et al., 2017). The anti-GFP nanobody is fused to ZIF-1 and serves as an adapter, recognizing GFP-tagged proteins and promoting their ubiquitination by the Cullin2-based E3 ubiquitin ligase, which ultimately targets them for degradation via the proteasome (Figure 6-figure supplement 1A) (Wang et al., 2017). This anti-GFP nanobody, visualized by nuclear expression of mCherry, was not detectable prior to or even shortly after the AC/VU cell fate decision, which allowed us to bypass disruption of initial cell specification (Figure 6-figure supplement 1B). While this method only produced a mild knockdown of POP-1 in the VU cells, we still observed the ectopic AC phenotype at low penetrance (7%, $n = 60$) (Figure 6-figure supplement 1C). To achieve stronger depletion, we used RNAi for further POP-1 perturbations.

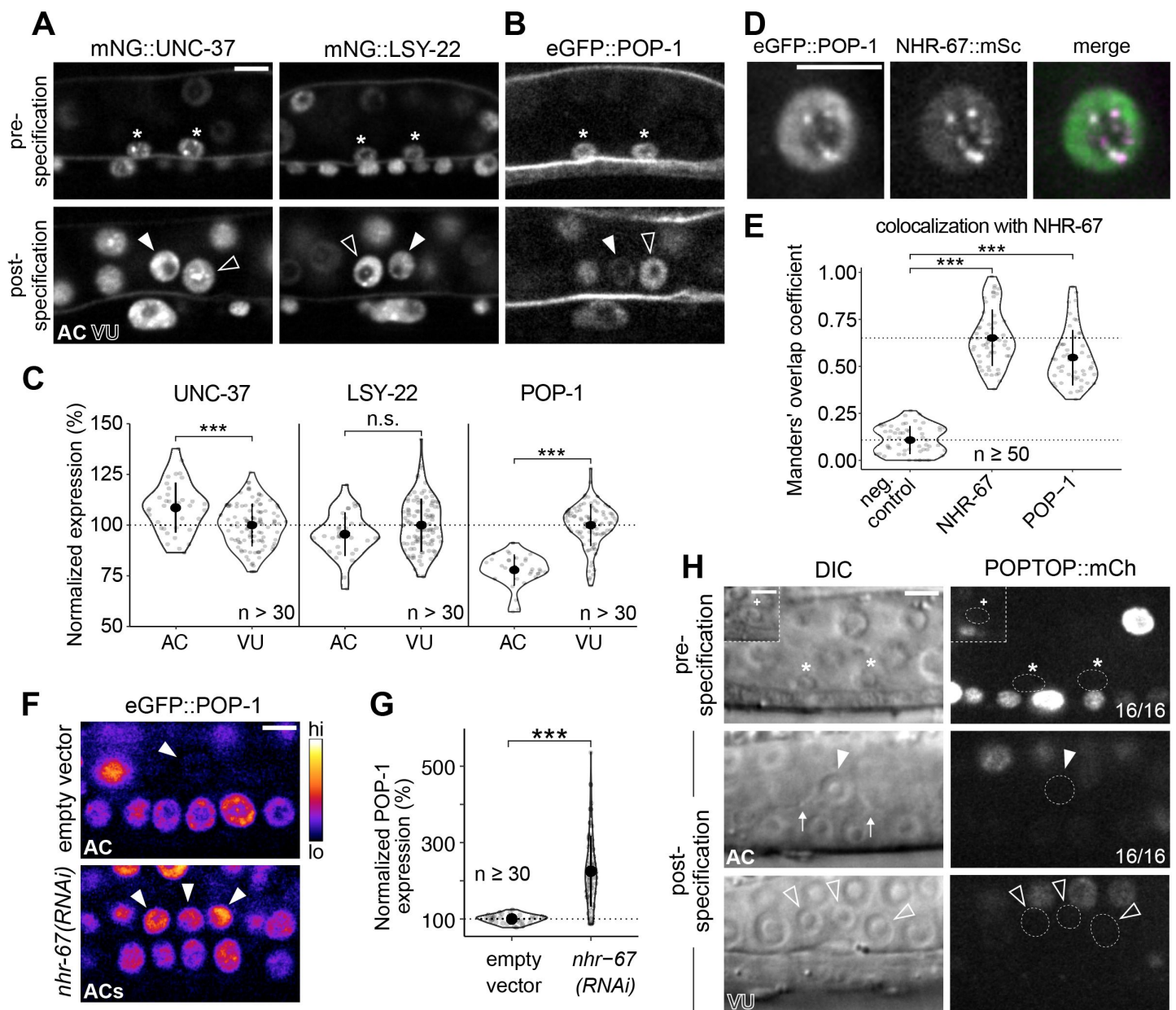


Figure 5

POP-1 is enriched in VU cells and colocalizes with NHR-67 punctae.

(A-B) Expression of mNeonGreen::UNC-37 and mNeonGreen::LSY-22 (A) and eGFP::POP-1 (B) in the AC/VU precursors pre-specification (left), as well as in the AC and VU cells post-specification (right). (C) Quantification of UNC-37, LSY-22, and POP-1 expression at the time of AC invasion. (D) Co-visualization of NHR-67::mScarlet-I and eGFP::POP-1 in the VU. (E) Quantification of POP-1 and NHR-67 colocalization, with plot reporting Manders' overlap coefficient compared to negative and positive controls. (F) Representative micrographs showing expression of POPTOP, a synthetic *pop-1*-activated reporter construct, in wild-type ACs, VU cells, and their precursors. Insets depict different z planes of the same image. (G-H) Micrographs (G) and quantification (H) of eGFP-tagged POP-1 expression in proliferative ACs following RNAi depletion of *nhr-67* compared to empty vector control.

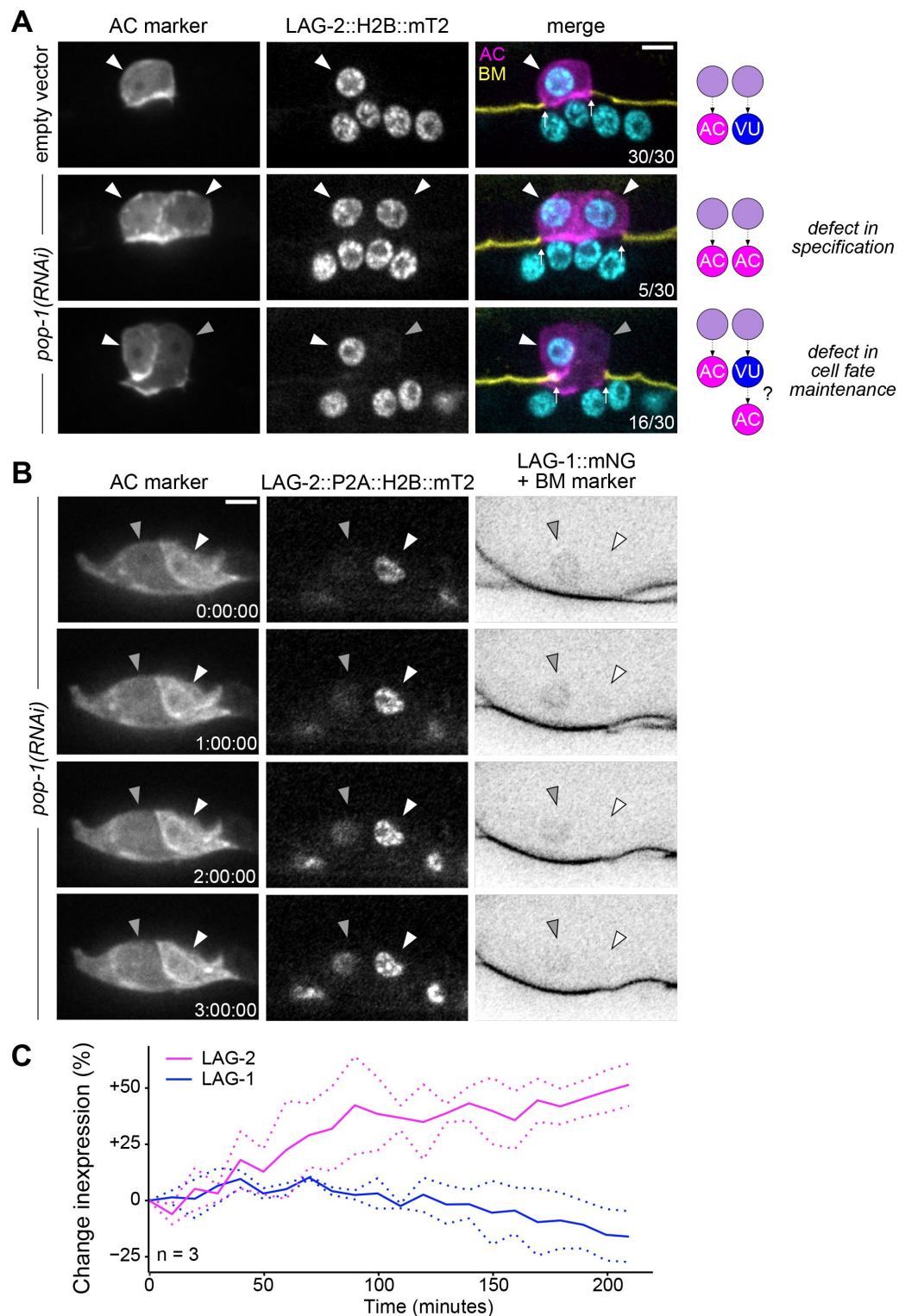


Figure 6

Ectopic ACs arise through VU-to-AC cell fate transformation.

(A) Representative images of ectopic AC (*cdh-3p::mCherry::moeABD*; *LAG-2::P2A::H2B::mTurquoise2*) phenotypes observed following RNAi depletion of POP-1. Schematics (right) depict potential explanations for observed phenotypes. (B) Expression of AC markers and a VU cell marker (*LAG-1::mNeonGreen*, inverted to aid visualization) in *pop-1(RNAi)* treated animals over time. (C) Quantification of LAG-2 (magenta) and LAG-1 (blue) expression in transdifferentiating cells produced by *pop-1(RNAi)* over time.

To interrogate the phenotypic consequences of POP-1 perturbation, we utilized a strain expressing two markers of AC fate (*cdh-3p::mCherry::moeABD* and *LAG-2::P2A::H2B::mTurquoise2*). Following treatment with *pop-1(RNAi)*, we observed several animals with two or more bright *cdh-3/lag-2+* ACs, consistent with known phenotypes caused by cell fate misspecification in the somatic gonad (17%, *n* = 30) (**Figure 6A**). We also observed animals with invasive cells that express AC markers at different levels (53%, *n* = 30), suggesting that the cells did not adopt AC fate at the same time (**Figure 6A**). To test whether the subset of dim *cdh-3/lag-2+* ACs are the result of VU-to-AC cell fate conversion, we visualized AC and VU fates simultaneously using the AC markers previously described along with an mNeonGreen-tagged allele of *lag-1* (CSL), a protein downstream of Notch signaling whose expression becomes restricted to the VU cells following AC/VU cell fate specification. Following treatment with *pop-1(RNAi)*, we found that a subset of ectopic ACs co-express AC markers and LAG-1, likely indicating an intermediate state between the two cell types (**Figure 6–figure supplement 2**). To visualize this process live, we used time-lapse microscopy and were able to capture ectopic ACs gradually upregulating LAG-2 (+51%, *n* = 3) and downregulating LAG-1 (-16%, *n* = 3) over time (**Figure 6B,C**), consistent with VU-to-AC cell fate conversion.

IDR of NHR-67 facilitates protein-protein interaction with UNC-37

Given that UNC-37, LSY-22, and POP-1 phenocopy each other with respect to AC/VU fates and all three colocalize with NHR-67 punctae, we next sought to further characterize the interactions among these proteins. Previous work has either directly identified or predicted protein-protein interactions among POP-1, UNC-37, and LSY-22 (Boxem et al., 2008; Calvo et al., 2001; Flowers et al., 2010; Reece-Hoyes et al., 2005; Simonis et al., 2009; Zhong and Sternberg, 2006). Using a yeast two-hybrid assay with UNC-37 Gal4-AD prey, we confirmed that UNC-37 directly interacts with both POP-1 and LSY-22 after observing yeast growth on the selective SC-HIS-TRP-LEU plates containing 3-AT (**Figure 7–figure supplement 1**). Using the same technique, we found that NHR-67 binds directly to UNC-37, as previously predicted (Li et al., 2004; Simonis et al., 2009), but found no evidence of it directly interacting with LSY-22 or POP-1 (**Figure 7–figure supplement 1**).

To further characterize the protein-protein interaction between NHR-67 and UNC-37, we assessed the protein structure of NHR-67 using AlphaFold, an artificial intelligence-based protein structure prediction tool (Jumper et al., 2021; Varadi et al., 2022), and PONDR, a predictor of intrinsic disorder (Peng and Zhang, 2006). Both identify an intrinsically disordered region (IDR) at the C-terminus of NHR-67 (**Figure 7A,B**). IDRs are low complexity domains that lack fixed three-dimensional structure and have been shown to support dynamic protein-protein interactions (Chong et al., 2018). To determine if the IDR of NHR-67 is important for facilitating its interaction with UNC-37, we repeated the yeast two-hybrid experiment using UNC-37 Gal4-AD prey, pairing it with different fragments of the NHR-67 protein: full-length, without its IDR (Δ IDR), and its IDR alone (**Figure 7C,D**). Yeast growth on the selective SC-HIS-TRP-LEU plates containing the competitive inhibitor 3-aminotriazole (3-AT) demonstrates that the 108 amino acid IDR sequence of NHR-67 is necessary and sufficient to bind with UNC-37 (**Figure 7C,D**).

Thus, we propose the following potential model of *C. elegans* uterine cell fate maintenance based on the data presented here and known roles of Groucho and TCF proteins in regulating transcription. First, transcription of *nhr-67* is directly regulated by HLH-2, resulting in its enrichment in the AC compared to the VU cells. In the AC, where NHR-67 levels are high and POP-1 is repressed, NHR-67 is free to activate genes promoting invasive differentiation. In the VU cells, where NHR-67 levels are low and POP-1 levels are high, POP-1 assembles with LSY-22, UNC-37, and NHR-67, either directly repressing NHR-67 targets or sequestering NHR-67 away from its targets (**Figure 7E**). It is possible that POP-1 negatively regulates NHR-67 at the transcriptional level as well, as the *nhr-67* promoter contains 7 putative TCF binding sites (Zacharias et al., 2015).

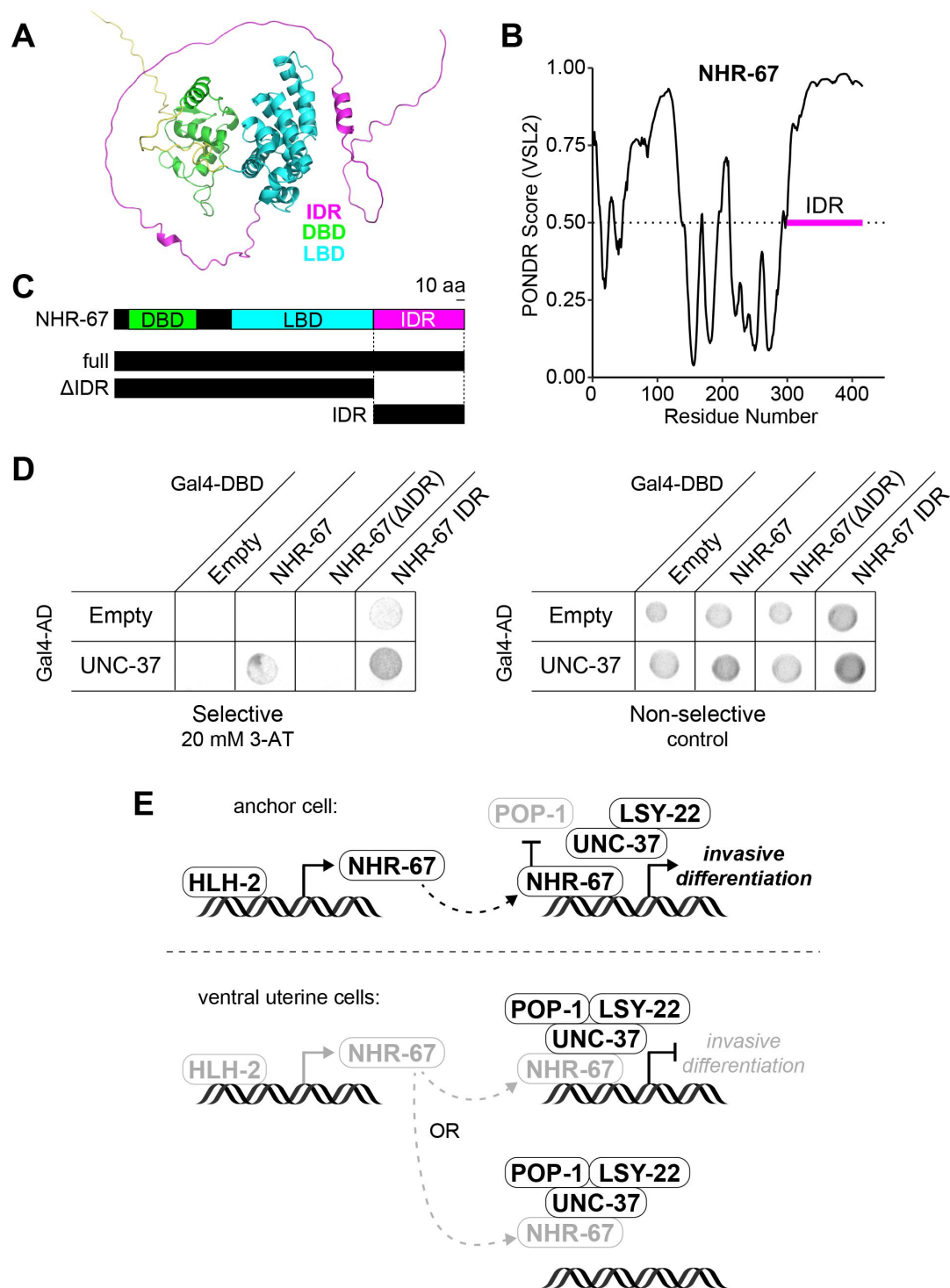


Figure 7

NHR-67 binds to UNC-37 through IDR-mediated protein-protein interaction.

(A) Predicted structure of NHR-67 generated by AlphaFold. (B) Measure of intrinsic disorder of NHR-67 using the PONDR VSL2 prediction algorithm. (C) Schematic of NHR-67 protein coding sequences used for Yeast two-hybrid experiments with reference to its intrinsically disordered region (IDR, magenta), DNA binding domain (DBD, green), and ligand binding domain (LBD, cyan). Scale bar, 10 amino acids. (D) Yeast two-hybrid experiment shows pairing of UNC-37 with either full-length NHR-67 or the IDR alone allows for yeast growth in the presence of competitive inhibitor 3-AT (20 mM). (E) Possible models of the roles of NHR-67, UNC-37, LSY-22, and POP-1 in maintenance of AC and VU cell fate. In the ventral uterine cells, the association of NHR-67 with the Groucho/TCF complex may result in repression of NHR-67 targets (top) or sequestration of NHR-67 away from its targets (bottom).

In summary, here we provide evidence that activity of the pro-invasive transcription factor, NHR-67, is simultaneously regulated by two distinct processes, which together modulate the proliferative-invasive switch in *C. elegans*. We show that NHR-67 is a potent fate-specifying transcription factor, in that its expression is sufficient for invasive differentiation of ACs in the somatic gonad. The compartmentalization of NHR-67 in the VU cells could serve as a potential mechanism to suppress its function in activating the pro-invasive program. We also discovered that NHR-67 forms nuclear foci in non-invasive cells, which exhibit liquid-like properties, indicated by observations of their condensation, dissolution, and relatively rapid recovery from photobleaching, similar to what has been described with P granules (Brangwynne et al., 2009 [↗](#)). These NHR-67 punctae associate with Groucho homologs, UNC-37 and LSY-22, likely through a direct protein-protein interaction with UNC-37 mediated by the C-terminal IDR of NHR-67. We postulate that this association leads to protein condensation, as has recently been described in *Ciona* embryos (Treen et al., 2021 [↗](#)). Furthermore, repression of the default invasive state appears to be dependent on expression of the TCF/LEF homolog POP-1, which clarifies our understanding of the dual roles this protein plays during development of the somatic gonad. It is also interesting to note that the dynamic punctae formed by POP-1 in non-Wnt signaled cells was first described 20 years ago (Maduro et al., 2002 [↗](#)), but their function are only now being appreciated in light of recent advances in our understanding of the formation of higher order associations in the nucleus.

With regard to protein compartmentalization in the nucleus, most research has been through the lens of transcriptional activation through RNA Polymerase II and the mediator complex (Boija et al., 2018 [↗](#); Cho et al., 2018 [↗](#); Sabari et al., 2018 [↗](#)) or repression through HP1 heterochromatin proteins (Larson et al., 2017 [↗](#); Strom et al., 2017 [↗](#)). Here we report the second observed case of compartmentalization of Groucho proteins (Treen et al., 2021 [↗](#)), which may suggest that Groucho proteins have evolutionarily conserved roles that require this type of subnuclear organization.

Still, as this is one of the first studies into compartmentalization of transcriptional repressors *in vivo*, there is much left to learn. For example, it is unknown whether DNA binding is necessary for nuclear puncta formation. The interaction between UNC-37 and NHR-67 does not appear to depend on DNA binding, as the C-terminal IDR region of NHR-67 (excluding its zinc finger domains) was sufficient for binding with UNC-37 *in vitro*, but it is possible that DNA binding is needed for oligomerization *in vivo*. Furthermore, it remains unclear if suppression of invasive differentiation is achieved by simply sequestering the pro-invasive transcription factor NHR-67 away from its transcriptional targets or through direct repression of transcription. If the latter, another question that arises is how the repressive complex gets recruited to specific genomic sites, since POP-1 and NHR-67 are both capable of binding to DNA, and whether repression is achieved through competition with transcriptional activators or recruitment of histone deacetylases. Direct targets of NHR-67 have not yet been discovered, which makes it difficult to investigate this specific aspect of the repressive mechanism at present. We see this as a promising avenue of future study as technologies advance, allowing for transcriptional profiling and target identification in specific tissues or cells (Gómez-Saldivar et al., 2020 [↗](#); Katsanos and Barkoulas, 2022 [↗](#)).

In this work, we have also identified several perturbations (i.e., increasing levels of NHR-67, decreasing levels of UNC-37/LSY-22) that result in incompletely penetrant transdifferentiation phenotypes and/or intermediate cell fates. We foresee these being ideal cell fate challenge backgrounds in which to perform screens to identify regulators of cellular plasticity, as has been done in other contexts (Rahe and Hobert, 2019 [↗](#)). Additionally, these induced fate transformations can be paired with tools to visualize and manipulate cell cycle (Adikes et al., 2020 [↗](#)) to determine if any cell cycle state is particularly permissive for cell fate plasticity. While G1 arrest has been shown to enhance conversion of human fibroblasts to dopaminergic neurons (Jiang et al., 2015 [↗](#)), mitosis is required for the natural K-to-DVB transdifferentiation event in *C.*

C. elegans (Riva et al., 2022 [↗](#)). As control of proliferation and invasion, as well as maintenance of differentiated cellular identities, are important for both homeostatic and disease states, it is our hope that this work will shed light on how cells switch between these states in the context of cancer growth and metastasis.

Materials and methods

C. elegans strains, culture, and nomenclature

Methods for *C. elegans* culture and genetics were followed as previously described (Brenner, 1974 [↗](#)). Developmental synchronization for experiments was achieved through alkaline hypochlorite treatment of gravid adults to isolate eggs (Porta-de-la-Riva et al., 2012 [↗](#)). L1 stage animals were plated on nematode growth media plates and subsequently cultured at 20°C or 25°C. Heat shock-inducible transgenes were activated by incubating animals on plates sealed with Parafilm in a 33°C water bath for 2-3 hours. In the text and in figures, promoter sequences are designated with a “p” following the gene name and gene fusions are represented by a double-colon (::) symbol.

CRISPR/Cas9 injections

New alleles and single-copy transgenes were generated by homology directed repair using CRISPR-based genome engineering. mScarlet::AID and mNeonGreen::AID were inserted into the C-terminus of the *nhr-67* locus by injecting adult germlines with Cas9 guide-RNA ribonucleoprotein complexes and short single-stranded oligodeoxynucleotide donors, as previously described (Ghanta and Mello, 2020 [↗](#)). Successful integration was identified through screening for fluorescence and by PCR. The *lsy-22* locus was edited by injecting a Cas9 guide RNA plasmid and repair template plasmid containing a self-excising cassette with selectable markers to facilitate screening (Dickinson et al., 2015 [↗](#); Dickinson and Goldstein, 2016 [↗](#); Huang et al., 2021 [↗](#)). Repair templates used to tag *lsy-22* with TagRFP-T::AID and mNeonGreen::AID were generated by cloning ~750-850 bp homology arms into pTNM063 and pDD312, respectively (Ashley et al., 2021 [↗](#); Dickinson et al., 2015 [↗](#)). All guide and repair sequences used can be found in Supplementary File 1.

Existing alleles

The GFP-tagged alleles of the pro-invasive transcription factors (*egl-43*, *fos-1*, *hlh-2*, and *nhr-67*) and the TagRFP-T::AID-tagged *nhr-67* allele were generated in preceding work (Medwig-Kinney et al., 2021 [↗](#), 2020 [↗](#)). Recent micropublications describe the P2A::H2B::mTurquoise2-tagged *lag-2* and mNeonGreen-tagged *lin-12* alleles used in this study (Medwig-Kinney et al., 2022 [↗](#); Pani et al., 2022 [↗](#)). The eGFP-tagged *pop-1* allele and POPTOP reporter were previously published (Green et al., 2008 [↗](#); van der Horst et al., 2019 [↗](#)), as were the AID::BFP and mNeonGreen tagged alleles of *unc-37* (Kurashina et al., 2021 [↗](#); Ma et al., 2021 [↗](#)). GFP-tagged *ama-1* (Hills-Muckey et al., 2021 [↗](#)) as well as mKate2-tagged *hpl-1* and *hpl-2* (Patel and Hobert, 2017 [↗](#)) were also disseminated in prior publications. The single-copy transgenes expressing the CDK sensor and TIR1 variants under ubiquitously expressed ribosomal promoters (*rps-27* and *rpl-28*, respectively) as well as the tissue-specific GFP-targeting nanobody are described in previous work (Adikes et al., 2020 [↗](#); Hills-Muckey et al., 2021 [↗](#); Smith et al., 2022 [↗](#); Wang et al., 2017 [↗](#)) and are located at neutral genomic sites, ttTi4348 or ttTi5605 (Frøkjær-Jensen et al., 2013 [↗](#)). The same is true for the heat shock inducible constructs for HLH-2 and NHR-67 (Medwig-Kinney et al., 2020 [↗](#)). The cadherin (*cdh-3*) anchor cell reporter and basement membrane (laminin) markers have already been characterized (Keeley et al., 2020 [↗](#); Matus et al., 2010 [↗](#)). The following mutant alleles were obtained from the *Caenorhabditis* Genetics Center: *unc-37(e262wd26)* and *unc-37(wd17wd22)* (Pflugrad et al., 1997 [↗](#)),

the latter of which was maintained using the chromosome I/III balancer *hT2* (McKim et al., 1993). The genotypes of all strains used in this study can be found within the Key Resources Table.

Auxin inducible protein degradation

The auxin inducible degron (AID) system was used for strong depletion of proteins of interest (Zhang et al., 2015). AID-tagged alleles were paired with the *Arabidopsis thaliana* F-box protein, transport inhibitor response 1 (*AtTIR1*), and treated with the water-soluble auxin 1-Naphthaleneacetic acid (K-NAA) at 1 mM concentration (Martinez et al., 2020). Auxin was added to nematode growth media plates according to previously published protocols (Martinez and Matus, 2020), which were then seeded with OP50 *E. coli*. To achieve robust depletion, synchronized L1 stage animals were directly plated on auxin plates.

RNA interference

The RNAi clones targeting *pop-1* and *uba-1* and the corresponding empty vector control (L4440) were obtained from the Vidal library (Rual et al., 2004). The RNAi constructs targeting the pro-invasive transcription factors (*egl-43*, *fos-1*, *hlh-2*, and *nhr-67*) and chromatin modifiers (*pbrm-1*, *swn-4*, and *swn-8*) are derived from the highly efficient RNAi vector T444T (Sturm et al., 2018) and were generated in preceding work (Medwig-Kinney et al., 2020; Smith et al., 2022). To avoid known AC/VU cell fate specification defects caused by *hlh-2* perturbations, synchronized animals were grown on OP50 until the L2 stage when they were then shifted to *hlh-2* RNAi plates.

Live-cell imaging

With the exception of the FRAP experiments shown in **Figure 3**, all micrographs were collected on a Hamamatsu Orca EM-CCD camera mounted on an upright Zeiss AxioImager A2 with a Borealis-modified CSU10 Yokagawa spinning disk scan head (Nobska Imaging) using 405 nm, 440 nm, 488 nm, 514 nm, and 561 nm Vortran lasers in a VersaLase merge and a Plan-Apochromat 100×/1.4 (NA) Oil DIC objective. MetaMorph software (Molecular Devices) was used for microscopy automation. Several experiments were scored using epifluorescence visualized on a Zeiss AxioCam MRM camera, also mounted on an upright Zeiss AxioImager A2 and a Plan-Apochromat 100×/1.4 (NA) Oil DIC objective. For static imaging, animals were mounted into a drop of M9 on a 5% Noble agar pad containing approximately 10 mM sodium azide anesthetic and topped with a coverslip. For long-term time-lapse imaging, animals were first anesthetized in 5 mM levamisole diluted in M9 for approximately 20 minutes, then transferred to a 5% Noble agar pad and topped with a coverslip sealed with VALAP (Kelley et al., 2017). For short-term time-lapse imaging, the pre-anesthetization step was omitted, and animals were transferred directly into a drop of 5 mM levamisole solution on the slide.

Fluorescence recovery after photobleaching

FRAP experiments were performed using an Acal BFi UV Optimicroscan photostimulation device mounted on a spinning disk confocal system consisting of a Nikon Ti2 inverted microscope with Yokogawa CSU-W1 SoRa spinning disk. Data were acquired using a Hamamatsu ORCA Fusion camera, 60x 1.27 NA water immersion objective, SoRa disk, and 2.8x SoRa magnifier. Single plane images were collected every 1 second.

Yeast one-hybrid

The 276 bp fragment of the *nhr-67* promoter (Bodofsky et al., 2018) was cloned into the pMW2 vector, linearized by BamHI digestion. Linearized plasmid was transformed into the Y1H yeast strain (as described in Reece-Hoyes and Walhout, 2018). Transformed yeast was plated on SC-HIS plates for three days before being transformed with the HLH-2 Gal4-AD plasmid. Three colonies from each transformation plate were streaked onto SC-HIS-TRP +3-aminotriazole (3-AT)

plates. Protein-DNA interactions were determined by visible growth on 3-AT conditions with negative growth in empty vector controls after three days. Plates were imaged on a Fotodyne FOTO/Analyst Investigator/FX darkroom imaging station.

Yeast two-hybrid

Plasmids containing target proteins fused to GAL-4 DNA-binding-domain + LEU and GAL-4 Activation Domain + TRP were co-transformed into the pJ69-4a Y2H yeast strain as previously described (Reece-Hoyes and Walhout, 2018 [DOI](#)). Transformed yeast was plated on SC-TRP-LEU plates for three days. Three colonies from each transformation plate were streaked onto SC-HIS-TRP-LEU 3-AT plates. Protein interactions were determined by visible growth on 3-AT conditions with negative growth in empty vector controls after three days. Plates were imaged as described in the previous section.

Quantification of protein expression and cell cycle state

Image quantification was performed in Fiji/ImageJ (Schindelin et al., 2012 [DOI](#)). Protein expression was quantified by drawing a region of interest and measuring the mean gray value, then manually subtracting the mean gray value of a background region of similar area to account for camera noise. For nuclear-localized proteins, the region of interest was drawn around the nucleus. Membrane expression of LIN-12 was measured by drawing a line (1.5 pixel thickness) along a cell's basolateral surface, to distinguish between LIN-12 expression from the cell of interest and its neighbors. CDH-3 expression was measured by drawing a region of interest around the cell membrane excluding the nucleus (as the nuclear-localized TIR1 transgene was expressed in the same channel). The CDK sensor was quantified as previously described (Adikes et al., 2020 [DOI](#)). Following rolling ball subtraction (50 pixels), mean gray value is measured in a region of interest drawn within the cytoplasm and one around the nucleus excluding the nucleolus. The cytoplasmic-to-nuclear ratio correlates to CDK activity and is used to assess cell cycle state (Adikes et al., 2020 [DOI](#); Spencer et al., 2013 [DOI](#)). Movies were collected by acquiring z-stacks at 5-minute intervals. Samples were time-aligned relative to anaphase. Cells that did not undergo anaphase during the acquisition period were aligned based on their DHB ratios. Animals that arrested in development (i.e., did not show evidence of progressing through the cell cycle) were excluded from analysis.

Colocalization analyses

For colocalization analyses, single plane images were collected to avoid z drift during acquisition and prevent photobleaching, which was often non-uniform between red and green fluorophores. Micrographs were subject to background subtraction (rolling ball radius = 50) followed by thresholding to segment punctae. Manders' overlap coefficients (M) were calculated by measuring the extent that segmented punctae of NHR-67 overlapped with that of other proteins using Just Another Colocalization Plugin (JACoP) in Fiji/ImageJ (Bolte and Cordelières, 2006 [DOI](#); Schindelin et al., 2012 [DOI](#)). Heterozygous animals for *nhr-67::mScarlet* and *nhr-67::GFP* were used as positive controls. These images were then re-analyzed following 90-degree rotation of one of the two channels being compared, resulting in random colocalization that served as a negative control.

Data visualization and statistical analyses

Representative images were processed using Fiji/ImageJ (Schindelin et al., 2012 [DOI](#)). Heat maps were generated using the Fire lookup table. A power analysis was performed prior to data collection to determine sample sizes (Cohen, 1992 [DOI](#)). Tests to determine statistical significance of data were conducted in RStudio and plots were generated using the R package ggplot2 (Wickham, 2016 [DOI](#)). Error bars represent the mean $\pm$ standard deviation. Schematics of gene loci were generated using sequences from WormBase (Harris et al., 2020 [DOI](#)) and the Exon-Intron Graphic Maker (<http://wormweb.org/exonintron> [DOI](#)). Figures were assembled in Adobe Illustrator.

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Additional information

Data and materials availability

All data generated or analyzed during this study are included in the manuscript and supporting files. New strains that have not been deposited with the *Caenorhabditis* Genetics Center will be made available upon request.

Source data

Figure 1–source data 1. Raw data of GFP-tagged transcription factor expression in the AC and VU cells, as reported in **Figure 1C,D** [↗](#) and **Figure 1–figure supplement 1B,C** [↗](#). In all source files: ROI, region of interest; BG, background; BS, background-subtracted.

Figure 1–source data 2. Raw data of LAG-2::P2A::H2B::mTurquoise2 and LIN-12::mNeonGreen expression in NHR-67-deficient ACs compared to control AC and VU cells, as reported in **Figure 1E** [↗](#) and **Figure 1–figure supplement 2A,B** [↗](#).

Figure 2–source data 1. Raw data of NHR-67::GFP expression in the AC and VU cells following heat-shock inducible expression of HLH-2, as reported in **Figure 2A,B** [↗](#).

Figure 2–source data 2. Raw data of GFP::HLH-2 and NHR-67::TagRFP-T expression in the AC and VU cells following *uba-1* RNAi treatment compared to empty vector controls, as reported in **Figure 2–figure supplement 1B–D** [↗](#).

Figure 3–source data 1. Raw data of CDK sensor (DHB) ratios in VU cells over time, as reported in **Figure 3D,E** [↗](#).

Figure 3–source data 2. Raw data of NHR-67::GFP puncta expression following photobleaching overtime, as reported in **Figure 3G,H** [↗](#).

Figure 4–source data 1. Raw data of protein colocalization in VU cells, as reported in **Figure 4A,B** [↗](#) and **Figure 5D,E** [↗](#).

Figure 4–source data 2. Raw data of CDH-3 expression in the ectopic ACs resulting from auxin-mediated depletion of AID-tagged LSY-22 or UNC-37, as reported **Figure 4D,E** [↗](#).

Figure 5–source data 1. Raw data of mNG::UNC-37, mNG::LSY-22, and eGFP::POP-1 expression in the AC/VU precursors, the AC, and VU cells, as reported in **Figure 5A–C** [↗](#) and **Figure 5–figure supplement 1A,B** [↗](#).

Figure 5–source data 2. Raw data of eGFP::POP-1 expression in ACs resulting from RNAi knockdown of transcription factors and chromatin modifiers compared to empty vector controls, reported in **Figure 5F,G** [↗](#) and **Figure 5–figure supplement 2A,B** [↗](#).

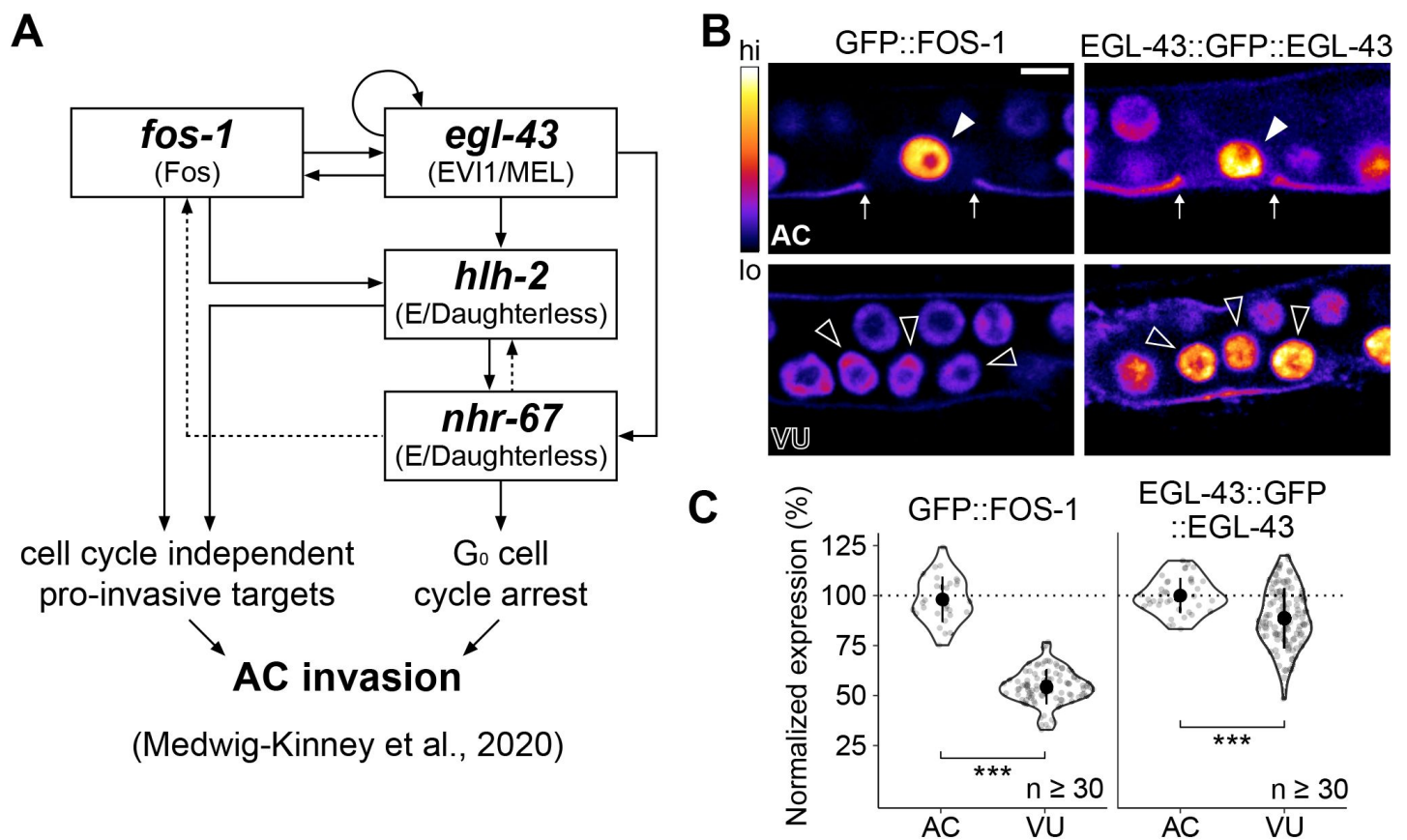


Figure 1-figure supplement 1

Expression of pro-invasive transcription factors EGL-43 and FOS-1 in the somatic gonad.

(A) Schematic of the AC pro-invasive gene regulatory network comprised of four transcription factors: EGL-43, FOS-1, HLH-2, and NHR-67. (B-C) Representative heat-map micrographs (B) and quantification (C) of GFP-tagged EGL-43 and FOS-1 expression in the AC and VU cells.

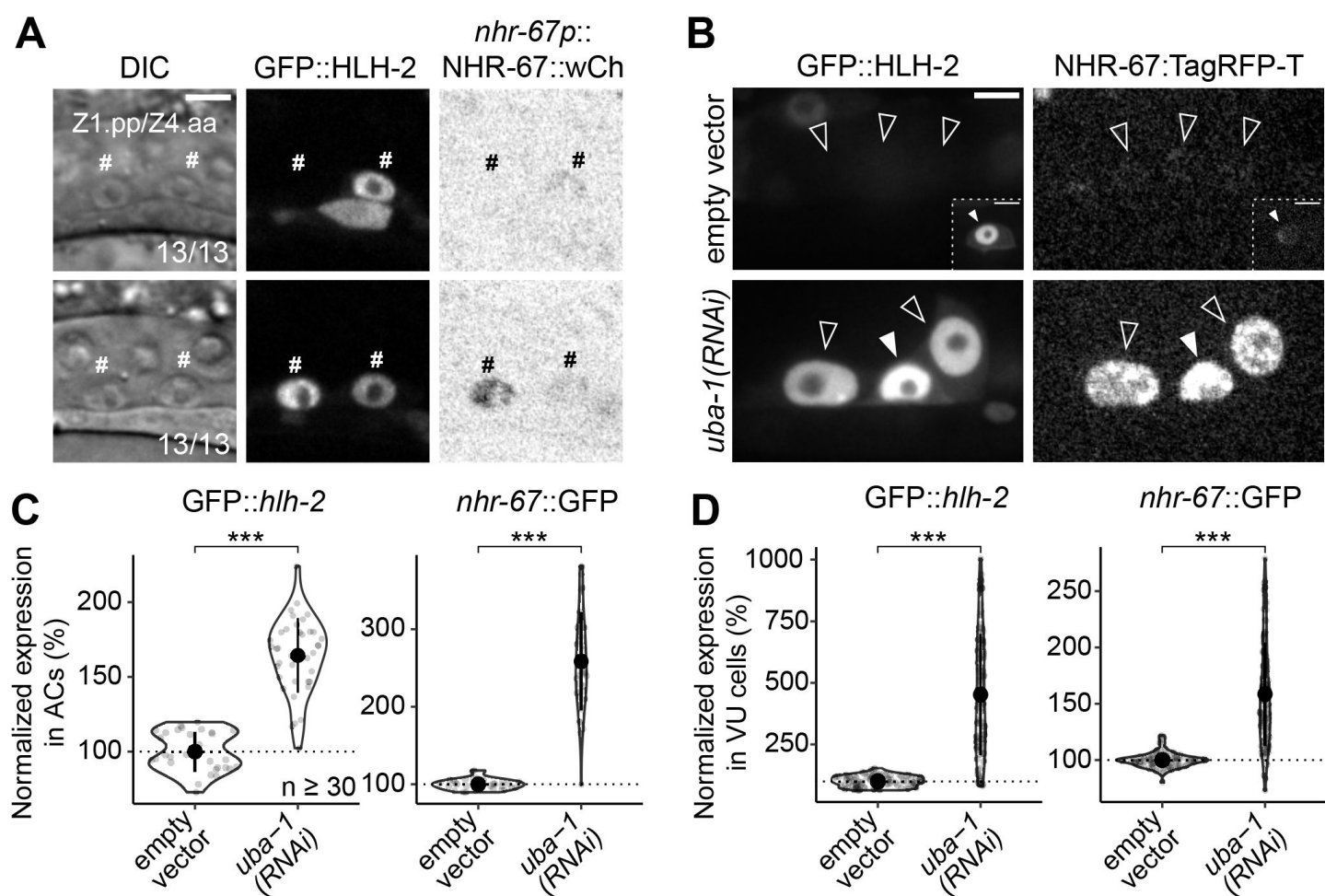


Figure 2-figure supplement 1

Onset of expression and regulatory interaction between NHR-67 and HLH-2 in the somatic gonad.

(A) Micrographs depicting onset of GFP-tagged HLH-2 and a wCherry-labeled NHR-67 transgene (inverted to aid visualization) in Z1.pp and Z4.aa cells at early (top) and late (bottom) stages. (B-D) Representative micrographs (B) and quantification (C-D) of GFP-tagged HLH-2 and TagRFP-T-tagged NHR-67 in AC (C) and VU cells (D) following *uba-1(RNAi)* compared to control. Insets depict different z planes of the same image.

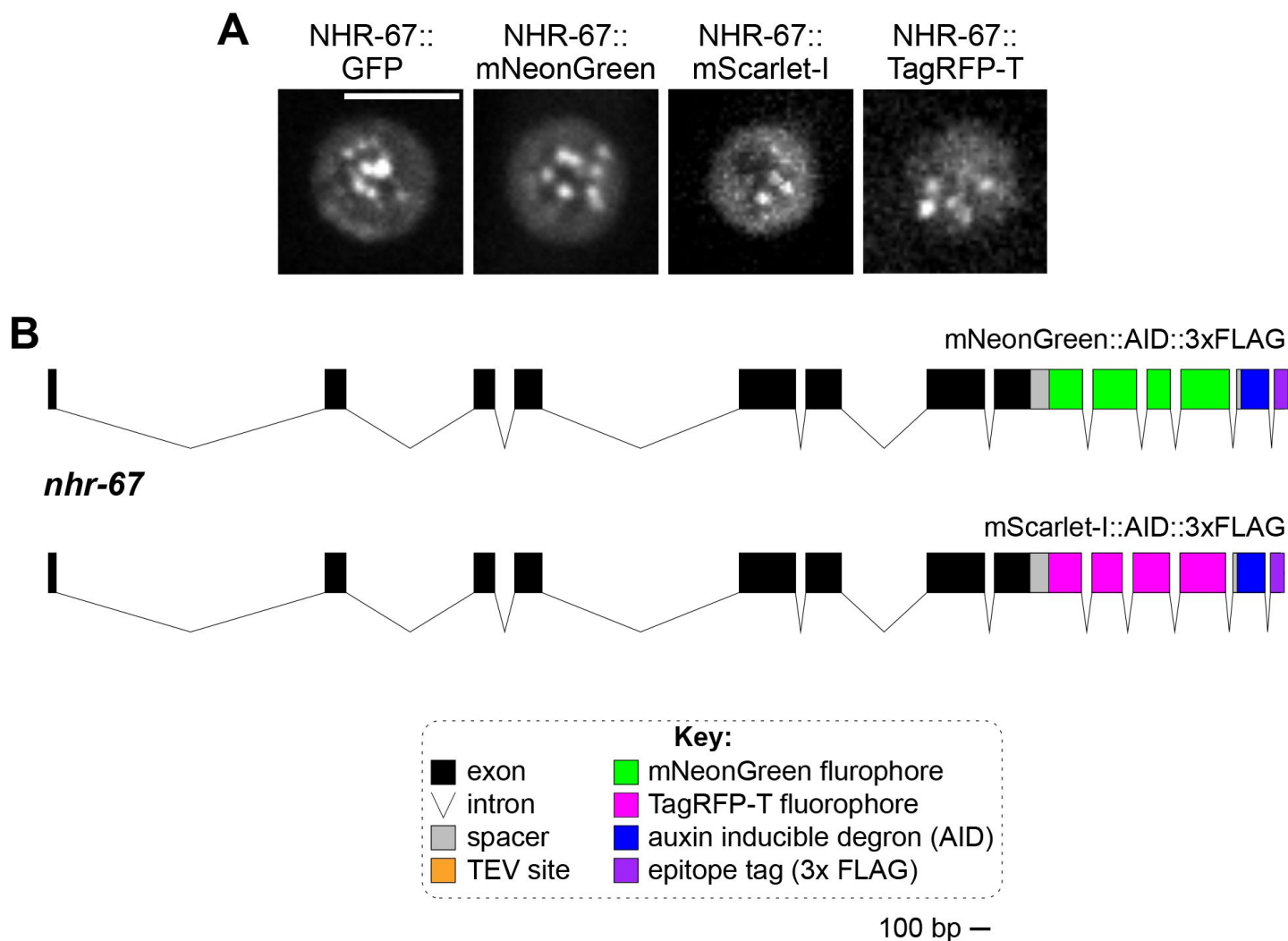


Figure 3-figure supplement 1

Knock-in alleles of *nhr-67*.

(A) Representative images of VU cells exhibiting punctae formed by NHR-67 tagged with GFP, mNeonGreen, mScarlet-I, and TagRFP-T. (B) Schematics of the new endogenously tagged loci generated in this paper for *nhr-67*. Scale bar, 100 base pairs (bp).

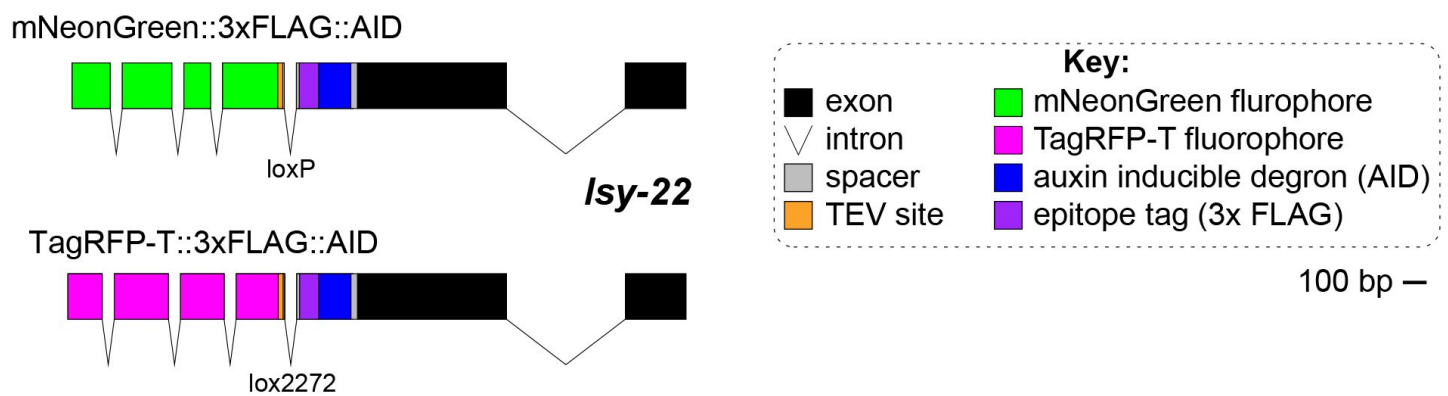


Figure 4-figure supplement 1

Knock-in alleles of *lsy-22*.

Schematics of the new endogenously tagged loci generated in this paper for *lsy-22*. Scale bar, 100 base pairs (bp).

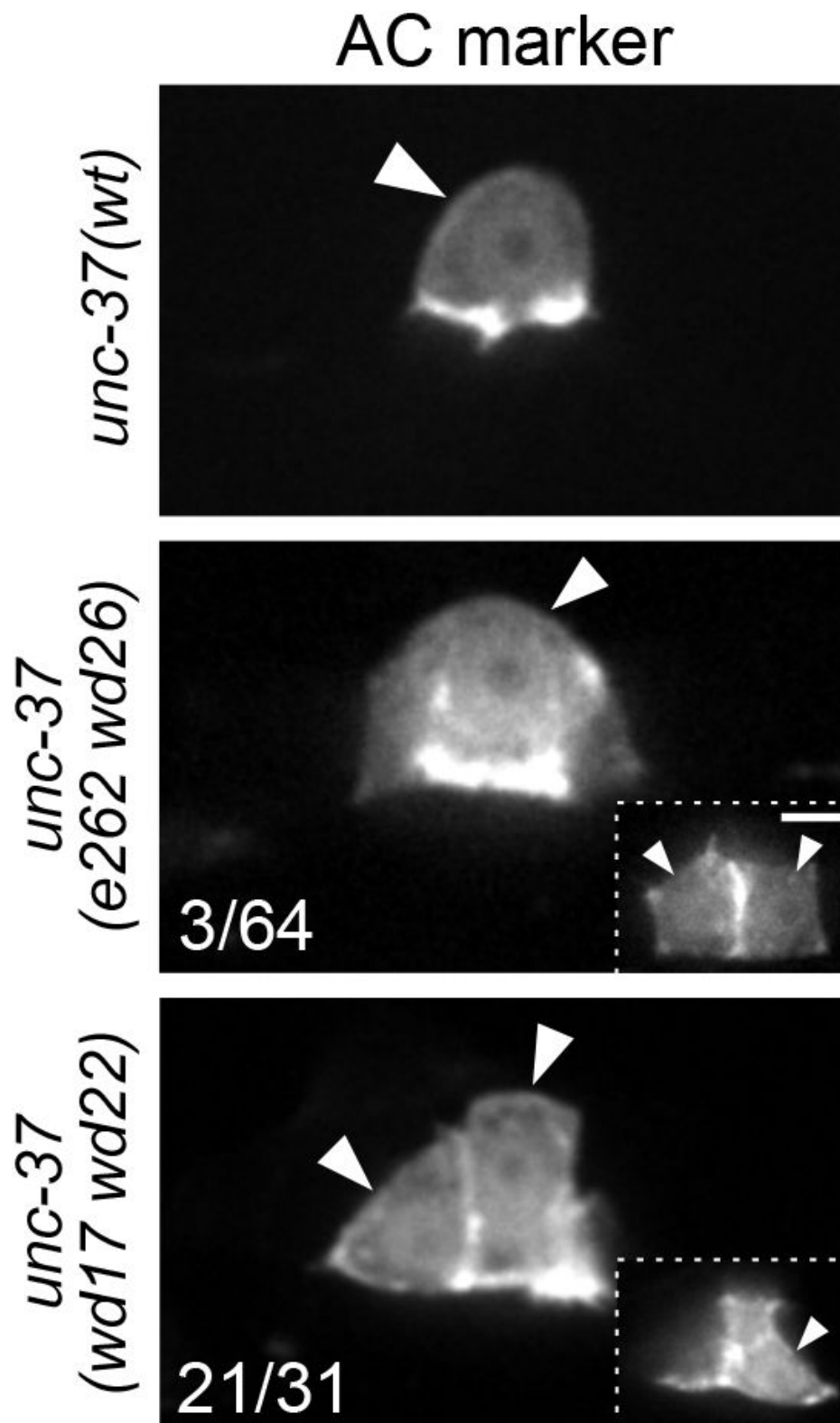


Figure 4-figure supplement 2

UNC-37 mutants show ectopic expression of AC markers.

Ectopic expression of AC marker (*cdh-3p::mCherry::moeABD*) in hypomorphic (*unc-37(e262wd26)*) and null (*unc-37(wd17wd22)*) alleles of *unc-37* compared to wild-type *unc-37*. Insets depict different z planes of the same image.

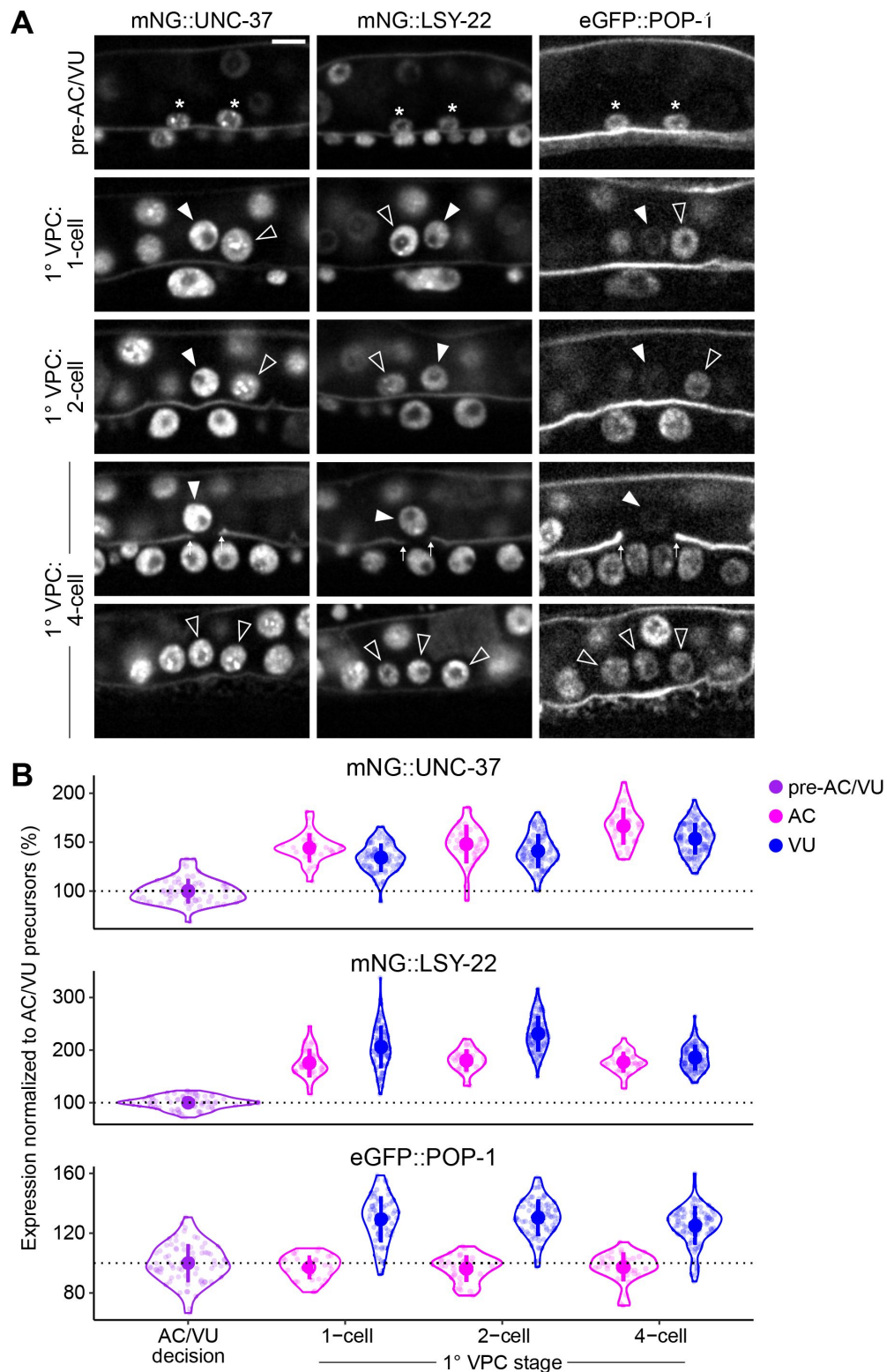


Figure 5-figure supplement 1

Expression of LSY-22, UNC-37, and POP-1 over developmental time.

(A-B) Developmental series (A) and quantified expression (B) of mNeonGreen::UNC-37, mNeonGreen::LSY-22, and eGFP::POP-1 expression in the AC/VU precursors, AC, and VU cells over time. Following AC/VU cell specification, animals are staged by the division of the underlying primary vulval precursor cells (1° VPCs).

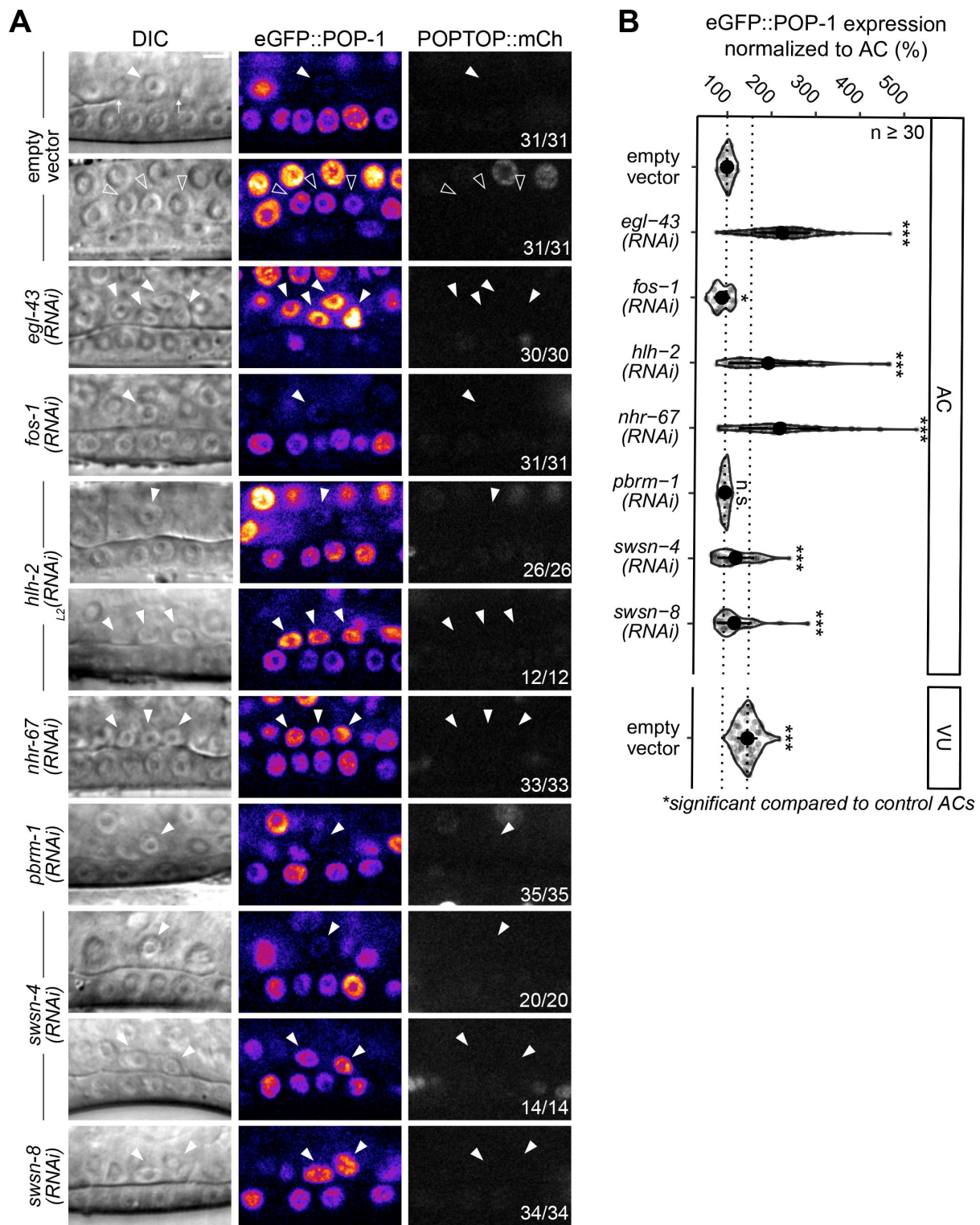


Figure 5-figure supplement 2

POP-1 function in VU cells is distinct from activating role in distal somatic gonad.

(A) Schematics representing the dual functions of POP-1. In the presence of Wnt signaling, POP-1 binds to its co-activator β -catenin (e.g., SYS-1) and activates transcription of its target genes. In the absence of Wnt signaling, POP-1 binds to its co-repressor Groucho (UNC-37) and represses transcription of its target genes. (B) Representative micrographs of eGFP::POP-1 and POPTOP (*pes-10::7x-TCF::mCherry*) expression in the AC, dorsal uterine cells (DU), spermatheca/sheath cells (SS), and VU cells. (C) Schematic of SYS-1 (β -catenin) expression in the Z1/Z4 lineage (based on Philips et al., 2007).

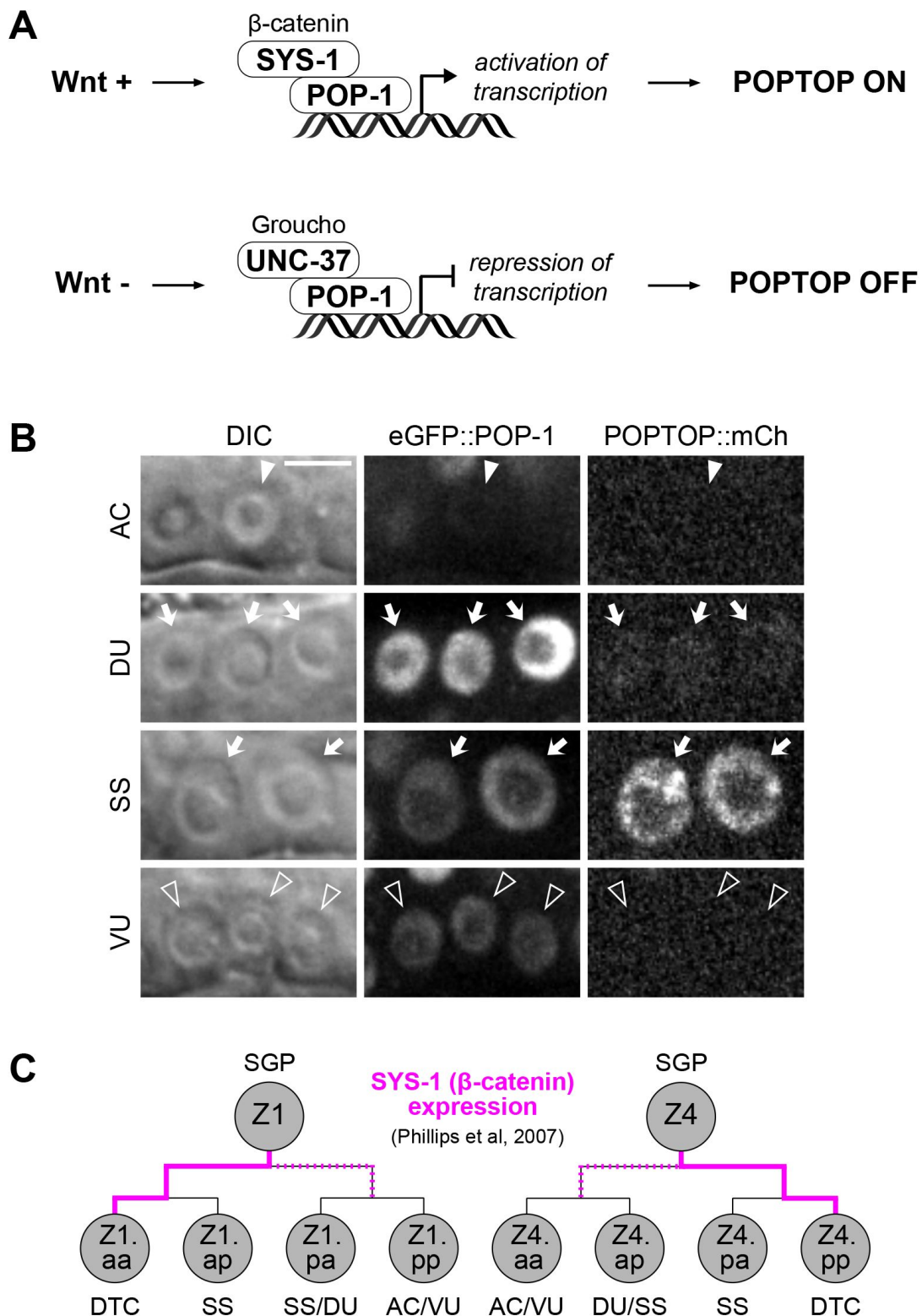


Figure 5-figure supplement 3

POP-1 expression is regulated by the cell cycle-dependent pro-invasion pathway.

(A-B) Representative micrographs of eGFP::POP-1 and POPTOP (*pes-10::7x-TCF::mCherry*) following RNAi-induced knockdown of pro-invasive transcription factors and chromatin modifiers compared to control AC and VU cells. (B) Quantification of eGFP::POP-1 expression in ACs following RNAi treatments. Here, the presence of multiple ACs are the result of failure of the AC to exit the cell cycle.

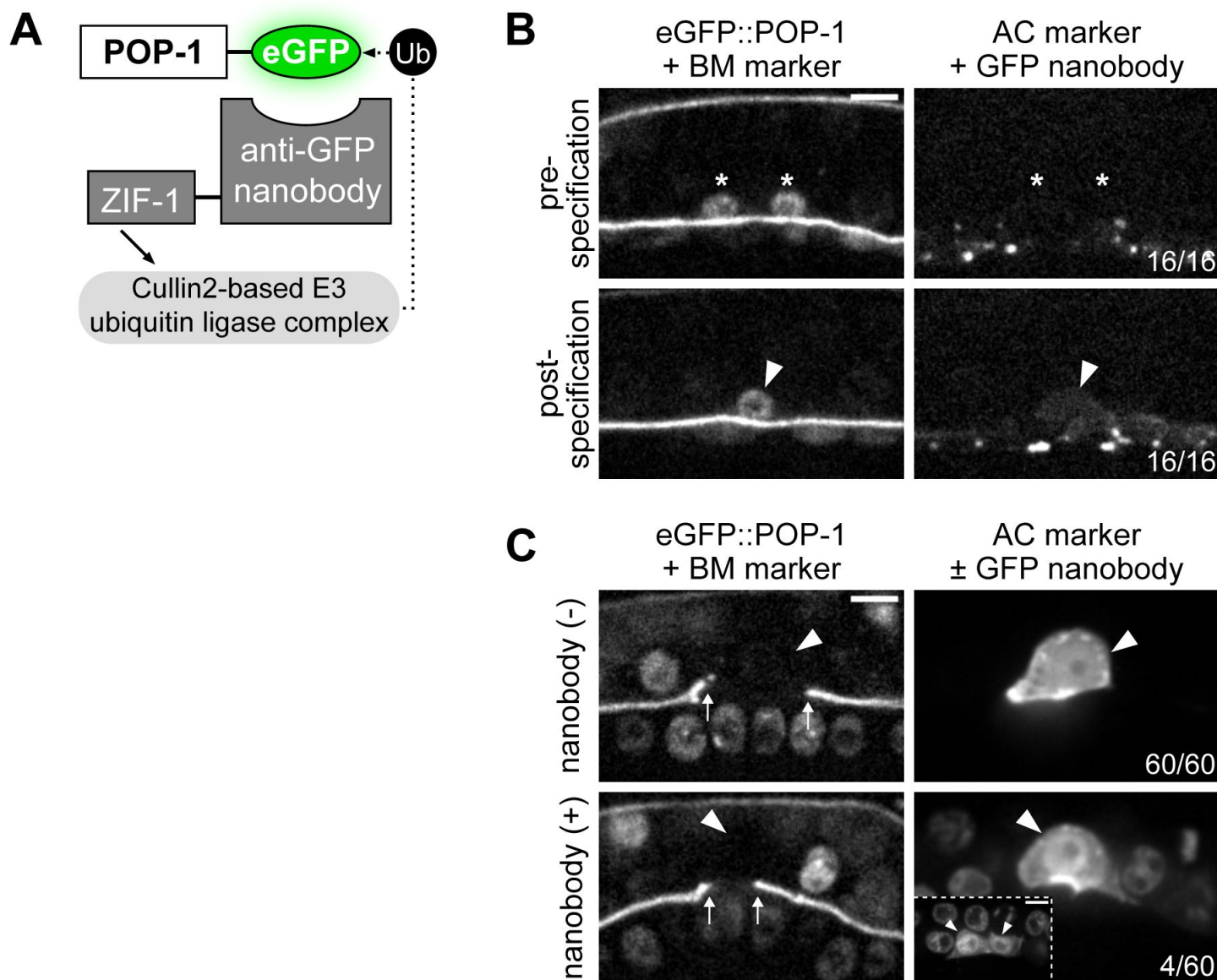


Figure 6-figure supplement 1

POP-1 functions to regulate AC/VU cell fates post-specification.

(A) Schematic of the anti-GFP nanobody protein degradation system (based on Wang et al., 2017). (B) Micrographs demonstrating that the anti-GFP nanobody (driven under the *egl-43L* promoter) is not expressed pre-specification or even shortly after when the presumptive AC begins to express its differentiated cell reporter (*cdh-3*). (C) With decreased levels of *pop-1*, a low penetrance (~7%) of multi-AC phenotypes were observed.

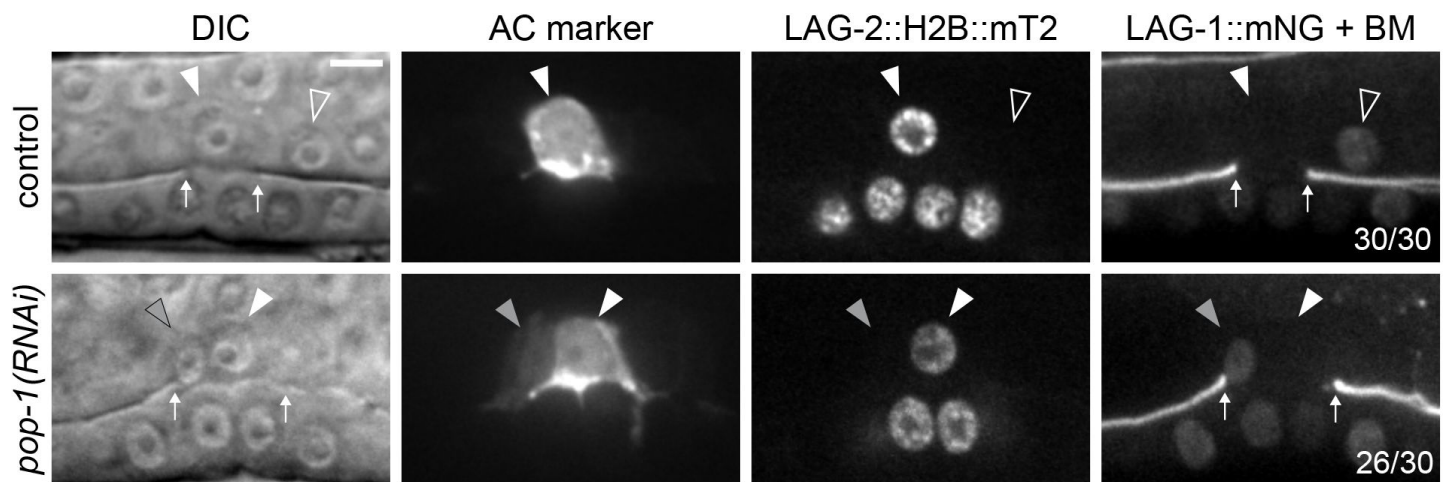


Figure 6-figure supplement 2

Ectopic ACs resulting from *pop-1* perturbation express VU cell markers.

Expression of AC markers (*cdh-3p*::mCherry::moeABD; LAG-2::P2A::H2B::mTurquoise2) and VU marker (LAG-1::mNeonGreen) in *pop-1(RNAi)* treated animals compared to empty vector control.

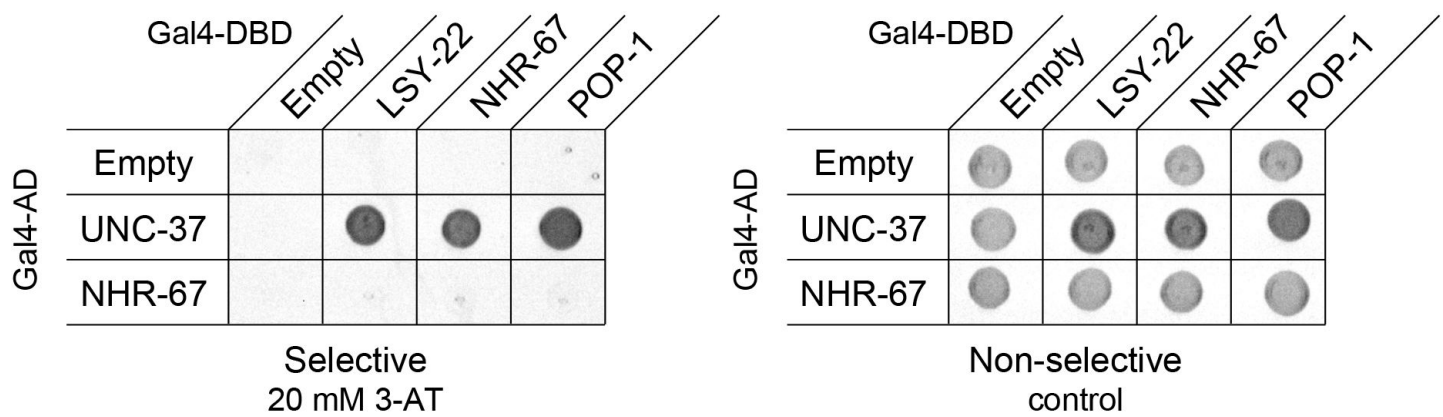


Figure 7-figure supplement 1.

NHR-67 exhibits protein-protein interaction with UNC-37.

Yeast two-hybrid experiment pairing UNC-37 and NHR-67 Gal4-AD prey with LSY-22, NHR-67, and POP-1 Gal4-DBD bait on SC-HIS-TRP-LEU plates with and without competitive inhibitor 3-AT (20 mM).

Figure 6-source data 1. Raw data of LAG-2::P2A::H2B::mT2 and LAG-1::mNG expression during VU-to-AC transdifferentiation, as reported in **Figure 6B,C** [↗](#).

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Reviewer #1 (Public Review):

Medwig-Kinney et al perform the latest in a series of studies unraveling the genetic and physical mechanisms involved in the formation of *C. elegans* gonad. They have paid particular attention to how two different cell fates are specified, the ventral uterine (VU) or anchor cell (AC), and the behaviors of these two cell types. This cell fate choice is interesting because the anchor cell performs an invasive migration through a basement membrane. A process that is required for correct *C. elegans* gonad formation and that can act as a model for other invasive processes, such as malignant cancer progression. The authors have identified a range of genes that are involved in the AC/VC fate choice, and that impart the AC cell with its ability to arrest the cell cycle and perform an invasive migration. Taking advantage of a range of genetic tools, the authors show that the transcription factor NHR-63 is strongly expressed in the AC cell. The authors also present evidence that NHR-63 is could function as a transcriptional repressor through interactions with a Groucho and also a TCF homolog, and they also suggest that these proteins are forming repressive condensates through phase separation.

The authors have produced an extensive dataset to support their two primary claims: that NHR-67 expression levels determine whether a cell is invasive or proliferative, and also that NHR-67 forms a repressive complex through interactions with other proteins. The authors should be commended for clearly and honestly conveying what is already known in this area of study with exhaustive references. Future data unambiguously linking the formation and dissolution of NHR-67 condensates with the activation of downstream genes that NHR-67 is actively repressing would be of great interest to the transcriptional research community.

Reviewer #2 (Public Review):

Medwig-Kinney et al. explore the role of the transcription factor NHR-67 in distinguishing between AC and VU cell identity in the *C. elegans* gonad. NHR-67 is expressed at high levels in AC cells where it induces G1 arrest, a requirement for the AC fate invasion program (Matus et al., 2015). NHR-67 is also present at low levels in the non-invasive VU cells and, in this new study, the authors suggest a role for this residual NHR-67 in maintaining VU cell fate. What this new role entails, however, is not clear.

The authors present two models: 1) That NHR-67 switches from a transcriptional activator in ACs to a transcriptional repressor in VUs by virtue of recruiting translational repressors, or 2) that these interactions sequester NHR-67 away from its transcription targets in VU cells. Neither model is fully supported by the data, leaving a paper with extensive data but no single compelling conclusions, and leaving open the question of what is the function, if any, of NHR-67 condensates in VU cells?

While the authors report on interesting observations, in particular the co-localization of NHR-67 with UNC-37/Groucho and POP-1 in nuclear puncta, the functional significance of these observations remains unclear. The authors have not demonstrated that the "repressive condensates" are functional and play a role in the suppression of AC fate in VU cells as claimed. The colocalization data suggest that NHR-67 interacts with repressors, but additional experiments are needed to demonstrate that these interactions are specific to VUs, impact VU fate, and sequester NHR-67 from its targets or transform NHR-67 into a transcriptional repressor.

[Editor's note: we feel that the current state of the data with respect to this question is best captured in the response by the authors to the original concerns expressed by reviewer 2, which we include in abbreviated form here]

1. The authors report that NHR-67 forms "repressive condensates" (aka. puncta) in the nuclei of VU cells and imply that these condensates prevent VU cells from becoming ACs. However, there are also examples of AC cells presented that have NHR-67 puncta (these are less obvious simply due to the higher levels of NHR-67 in ACs). Similarly, there also are UNC-37 and LSY-22 also puncta in ACs. The presence of NHR-67 puncta in the AC seems to directly contradict the author's assumption that the puncta repress the AC fate.

RESPONSE: The puncta formed by NHR-67 in the AC are different in appearance than those observed in the VU cells and furthermore do not exhibit strong colocalization with that of UNC-37 or LSY-22. The Manders' overlap coefficient between NHR-67 and UNC-37 is 0.181 in the AC, whereas it is 0.686 in the VU cells. Likewise, the Manders' overlap coefficient between NHR-67 and LSY-22 is 0.189 in the AC compared to 0.741 in the VU cells. We speculate that the areas of NHR-67 subnuclear enrichment in the AC may represent concentration around transcriptional targets, but testing this would require knowledge of direct targets of NHR-67.

1. While a pool of NHR-67 localizes to "repressive condensates", it appears that a substantial portion of NHR-67 also exists diffusively in the nucleoplasm. This would

appear to contradict a "sequestration model" since, for such a model to work, a majority of NHR-67 should be in puncta? What proportion of NHR-67 is in puncta? Is the concentration of NHR-67 in the nucleoplasm lower in VUs compared to ACs and does this depend on the puncta?

RESPONSE: The proportion of NHR-67 localizing to puncta versus the nucleoplasm is dynamic, as these puncta form and dissolve over the course of the cell cycle. However, we estimate that approximately 25-40% of NHR-67 protein resides in puncta based on segmentation and quantification of fluorescent intensity. We also measured NHR-67 concentration in the nucleoplasm of VU cells and found that it is only 28% of what is observed in ACs (n = 10). We also disagree with the notion that the majority of NHR-67 protein should be located in puncta to support the sequestration model. As one example, previously published work examining phase separation of endogenous YAP shows that it is present in the nucleoplasm in addition to puncta (Cai et al., 2019, doi: 10.1038/s41556-019-0433-z). In our system, it is possible that the combination of transcriptional downregulation and partial sequestration away from DNA is sufficient to disrupt the normal activity of NHR-67.

1. The authors do not report whether NHR-67, UNC-37, LSY-22, or POP-1 localization to puncta is interdependent, as implied by their model.

RESPONSE: We based our model, shown in Fig. 7E, on known or predicted protein-protein interactions, which we confirmed through yeast two-hybrid analyses (Fig. 7D; Fig. 7-figure supplement 1). It is difficult to test whether localization of these proteins to puncta is interdependent, as a perturbation of UNC-37, LSY-22, and POP-1 result in ectopic ACs. Trying to determine if loss of puncta results in VU-to-AC transdifferentiation or vice versa becomes a chicken-egg argument. It is also possible that UNC-37 and LSY-22 are at least partially redundant in this context.

1. The evidence that the "repressor condensates" suppress AC fate in VUs is presented in Fig. 4D where the authors deplete the presumed repressor LSY-22. First, the authors do not examine whether NHR-67 forms puncta under these conditions. Second, the authors rely on a single marker (*cdh-3p::mCherry::moeABD*) to score AC fate: this marker shows weak expression in cells flanking one bright cell (presumably the AC) which the authors interpret as a VU AC transformation. The authors, however, do not identify the cells that express the marker by lineage analyses and dismiss the possibility that the marker-positive cells could arise from the division of an AC-committed cell. Finally, the authors did not test whether marker expression was dependent on NHR-67, as predicted by the model shown in Fig. 7.

RESPONSE: For the auxin-inducible degron experiments, strains contained labeled AID-tagged proteins, a labeled TIR1 transgene, and a labeled AC marker. Thus, we were limited by the number of fluorescent channels we could covisualize and therefore could not also visualize NHR-67 (to assess for puncta formation) or another AC marker (such as LAG-2). We could have generated an AID-tagged LSY-22 strain without a fluorescent protein, but then we would not be able to quantify its depletion, which this reviewer points out is important to measure. We did visualize NHR-67::GFP expression following RNAi-induced knockdown of POP-1 and observed consistent loss of puncta in ectopic ACs. However, it is unclear whether cell fate change causes loss of puncta or vice-versa.

1. Interaction between NHR-67 and UNC-37 is shown using Y2H, but not verified in vivo. Furthermore, the functional significance of the NHR-67/UNC-37 interaction is not tested.

We attempted to remove the intrinsically disordered region found at the C-terminus of the endogenous *nhr-67* locus, using CRISPR/Cas9, as this would both confirm the NHR-67/UNC-37 interaction in vivo and allow us to determine the functional significance of this interaction.

However, we were unable to recover a viable line after several attempts, suggesting that this region of the protein is vital.

1. Throughout the manuscript, the authors do not use lineage analysis to confirm fate transformation as is the standard in the field. There are 4 multipotential gonadal cells with the potential to differentiate into VUs or ACs. Which ones contribute to the extra ACs in the different genetic backgrounds examined was not determined, which complicates interpretation. The authors should consider and test the following possibilities: disruption of NHR-67 regulation causes 1) extra pluripotent cells to directly become ACs early in development, 2) causes VU cells to gradually trans-fate to an AC-like fate after VU fate specification (as implied by the authors), or 3) causes an AC to undergo extra cell division(s)? In Fig. 1F, 5 cells are designated as ACs, which is one more than the 4 precursors depicted in Fig. 1A, implying that some of the "ACs" were derived from progenitors that divided.

The timing between AC/VU cell fate specification and AC invasion (the point at which we look for differentiated ACs) is approximately 10-12 hours at 25 °C. With our imaging setup, we are limited to approximately 3-4 hours of live-cell imaging. Therefore, lineage tracing was not feasible for our experiments. Instead, we relied on visualization of established markers of AC and VU cell fate to determine how ectopic ACs arose. In Fig. 6B,C we show that the expression of two AC markers (*cdh-3* and *lag-2*) turn on while a VU marker (*lag-1*) gets downregulated within the same cell. In our opinion, live-imaging experiments that show in real time changes in cell fate via reporters was the most definitive way to observe the phenotype.

1. There are 4 multipotential gonadal cells with the potential to differentiate into VUs or ACs. Which ones contribute to the extra ACs in the different genetic backgrounds examined was not determined, which complicates interpretation. The authors should consider and test the following possibilities: disruption of NHR-67 regulation causes 1) extra pluripotent cells to directly become ACs early in development, 2) causes VU cells to gradually trans-fate to an AC-like fate after VU fate specification (as implied by the authors), or 3) causes an AC to undergo extra cell division(s)? In Fig. 1F, 5 cells are designated as ACs, which is one more than the 4 precursors depicted in Fig. 1A, implying that some of the "ACs" were derived from progenitors that divided.

RESPONSE: When trying to determine the source of the ectopic ACs, we considered the three possibilities noted by the reviewer: (1) misspecification of AC/VU precursors, (2) VU-to-AC transdifferentiation, or (3) proliferation of the AC. We eliminated option 3 as a possibility, as the ectopic ACs we observed here were invasive and all of our previous work has shown that proliferating ACs cannot invade and that cell cycle exit is necessary for invasion (Matus et al., 2015; MedwigKinney & Smith et al., 2020; Smith et al., 2022). Specifically, NHR-67 is upstream of the cyclin dependent kinase CKI-1 and we found that induced expression of NHR-67 resulted in slow growth and developmental arrest, likely because of inducing cell cycle exit. For our experiment using *hsp::NHR-67*, we induced heat shock after AC/VU specification. For POP-1 perturbation, we explicitly acknowledged that misspecification of the AC/VU precursors could also contribute to ectopic ACs (Fig. 6A; lines 368-385). We could not achieve robust protein depletion through delayed RNAi treatment, so instead we utilized timelapse microscopy and quantification of AC and VU cell markers (Fig. 6B,C; see response 2.7 above).

Author Response

Reviewer #1 (Public Review):

Medwig-Kinney et al perform the latest in a series of studies unraveling the genetic and physical mechanisms involved in the formation of C. elegans gonad. They have paid particular attention to how two different cell fates are specified, the ventral uterine (VU) or anchor cell (AC), and the behaviors of these two cell types. This cell fate choice is interesting because the anchor cell performs an invasive migration through a basement membrane. A process that is required for correct C. elegans gonad formation and that can act as a model for other invasive processes, such as malignant cancer progression. The authors have identified a range of genes that are involved in the AC/VC fate choice, and that imparts the AC cell with its ability to arrest the cell cycle and perform an invasive migration. Taking advantage of a range of genetic tools, the authors show that the transcription factor NHR-63 is strongly expressed in the AC cell. The authors also present evidence that NHR-63 is could function as a transcriptional repressor through interactions with a Groucho and also a TCF homolog, and they also suggest that these proteins are forming repressive condensates through phase separation.

The authors have produced an extensive dataset to support their two primary claims: that NHR-67 expression levels determine whether a cell is invasive or proliferative, and also that NHR-67 forms a repressive complex through interactions with other proteins. The authors should be commended for clearly and honestly conveying what is already known in this area of study with exhaustive references. But absent data unambiguously linking the formation and dissolution of NHR-67 condensates with the activation of downstream genes that NHR-67 is actively repressing, the novelty of these findings is limited.

Response 1.1: We thank the reviewer for recognizing the extensive dataset we provide in this manuscript in support of our claims that, (1) NHR-67 expression levels are important for distinguishing between AC and VU cell fates, and (2) NHR-67 interacts with transcriptional repressors in VU cells. We acknowledge that a complete mechanistic understanding of the functional significance of NHR-67 puncta is not possible without knowing direct targets of NHR-67 in the AC. Unfortunately, tools to identify transcriptional targets in individual cells or lineages in C. elegans do not exist, and generation of such tools would be beyond the scope of this work. This is evidenced by the fact that the first successful attempt to transcriptionally profile the AC was only posted as a preprint one month ago (Costa et al., doi: 10.1101/2022.12.28.522136). It is our hope that the findings we present here can be integrated with future AC- and VU-specific profiling efforts to provide a more complete picture of the functional significance of NHR-67 subnuclear organization.

Reviewer #2 (Public Review):

Medwig-Kinney et al. explore the role of the transcription factor NHR-67 in distinguishing between AC and VU cell identity in the C. elegans gonad. NHR-67 is expressed at high levels in AC cells where it induces G1 arrest, a requirement for the AC fate invasion program (Matus et al., 2015). NHR-67 is also present at low levels in the non-invasive VU cells and, in this new study, the authors suggest a role for this residual NHR-67 in maintaining VU cell fate. What this new role entails, however, is not clear. The model in Figure 7E shows NHR-67 switching from a transcriptional activator in ACs to a transcriptional repressor in VUs by virtue of recruiting translational repressors. In this model, NHR-67 actively suppresses AC differentiation in VU cells by binding to its normal targets and acting as a repressor rather than an activator. Elsewhere in the text, however, the authors suggest that NHR-67 is "post-translationally sequestered" (line 450) in nuclear condensates in VU cells. In that model, the low levels of NHR-67 in VU cells are not functional because inactivated by sequestration in condensates away from DNA.

Neither model is fully supported by the data, which may explain why the authors seem to imply both possibilities. This uncertainty is confusing and prevents the paper from arriving at a compelling conclusion. What is the function, if any, of NHR-67 and so-called "repressive condensates" in VU cells?

Response 2.1: As the reviewer correctly notes, we present two possible models in this manuscript. The interaction between NHR-67 and the Groucho/TCF complex in the VU cells could (1) switch the role of NHR-67 from a transcriptional activator to a transcriptional repressor, or (2) sequester NHR-67 away from its transcriptional targets. Indeed, we cannot definitively exclude the possibility of either model. In our resubmission, we will attempt to make this more clear in the text and by presenting both possible models in the summary figure (Fig. 7E).

Below we list problems with data interpretation and key missing experiments:

1. The authors report that NHR-67 forms "repressive condensates" (aka. puncta) in the nuclei of VU cells and imply that these condensates prevent VU cells from becoming ACs. Fig. 3A, however, shows an example of an AC that also assemble NHR-67 puncta (these are less obvious simply due to the higher levels of NHR-67 in ACs). The presence of NHR-67 puncta in the AC seems to directly contradict the author's assumption that the puncta repress the AC fate program. Similarly, Figure 5-figure supplement 1A shows that UNC-37 and LSY-22 also form puncta in ACs. The authors need to analyze both AC and VU cells to demonstrate that NHR-67 puncta only form in VUs, as implied by their model.

Response 2.2: The puncta formed by NHR-67 in the AC are different in appearance than those observed in the VU cells and furthermore do not exhibit strong colocalization with that of UNC-37 or LSY-22. The Manders' overlap coefficient between NHR-67 and UNC-37 is 0.181 in the AC, whereas it is 0.686 in the VU cells. Likewise, the Manders' overlap coefficient between NHR-67 and LSY-22 is 0.189 in the AC compared to 0.741 in the VU cells. We speculate that the areas of NHR-67 subnuclear enrichment in the AC may represent concentration around transcriptional targets, but testing this would require knowledge of direct targets of NHR-67.

1. While a pool of NHR-67 localizes to "repressive condensates", it appears that a substantial portion of NHR-67 also exists diffusively in the nucleoplasm. This would appear to contradict a "sequestration model" since, for such a model to work, a majority of NHR-67 should be in puncta. What proportion of NHR-67 is in puncta? Is the concentration of NHR-67 in the nucleoplasm lower in VUs compared to ACs and does this depend on the puncta?

Response 2.3: The proportion of NHR-67 localizing to puncta versus the nucleoplasm is dynamic, as these puncta form and dissolve over the course of the cell cycle. However, we estimate that approximately 25-40% of NHR-67 protein resides in puncta based on segmentation and quantification of fluorescent intensity of sum Z-projections. We also measured NHR-67 concentration in the nucleoplasm of VU cells and found that it is only 28% of what is observed in ACs (n = 10). We disagree with the notion that the majority of NHR-67 protein should be located in puncta to support the sequestration model. As one example, previously published work examining phase separation of endogenous YAP shows that it is present in the nucleoplasm in addition to puncta (Cai et al., 2019, doi: 10.1038/s41556-019-0433-z). In our system, it is possible that the combination of transcriptional downregulation and partial sequestration away from DNA is sufficient to disrupt the normal activity of NHR-67.

1. The authors do not report whether NHR-67, UNC-37, LSY-22, or POP-1 localization to puncta is interdependent, as implied in the model shown in Fig. 7.

Response 2.4: It is difficult to test whether localization of these proteins to puncta is interdependent, as perturbation of UNC-37, LSY-22, and POP-1 result in ectopic ACs. Trying to determine if loss of puncta results in VU-to-AC transdifferentiation or vice versa becomes a chicken-egg argument. It is also possible that UNC-37 and LSY-22 are at least partially redundant in this context. We based our model, shown in Fig. 7E, on known or predicted protein-protein interactions, which we confirmed through yeast two-hybrid analyses (Fig. 7D; Fig. 7-figure supplement 1).

*1. The evidence that the "repressor condensates" suppress AC fate in VUs is presented in Fig. 4D where the authors deplete the presumed repressor LSY-22. First, the authors do not examine whether NHR-67 forms puncta under these conditions. Second, the authors rely on a single marker (*cdh-3p::mCherry::moeABD*) to score AC fate: this marker shows weak expression in cells flanking one bright cell (presumably the AC) which the authors interpret as a VU AC transformation. The authors, however, do not identify the cells that express the marker by lineage analyses and dismiss the possibility that the marker-positive cells could arise from the division of an AC-committed cell. Finally, the authors did not test whether marker expression was dependent on NHR-67, as predicted by the model shown in Fig. 7.*

Response 2.5: For the auxin-inducible degron experiments, strains contained labeled AID-tagged proteins, a labeled TIR1 transgene, and a labeled AC marker. Thus, we were limited by the number of fluorescent channels we could covisualize and therefore could not also visualize NHR-67 (to assess for puncta formation) or another AC marker (such as LAG-2). We could have generated an AID-tagged LSY-22 strain without a fluorescent protein, but then we would not be able to quantify its depletion, which this reviewer points out is important to measure. We did visualize NHR-67::GFP expression following RNAi-induced knockdown of POP-1 and observed consistent loss of puncta in ectopic ACs. However, this again becomes a chicken-egg argument as far as whether cell fate change or loss of puncta causes the other.

1. Interaction between NHR-67 and UNC-37 is shown using Y2H, but not verified in vivo. Furthermore, the functional significance of the NHR-67/UNC-37 interaction is not tested.

Response 2.6: We attempted to remove the intrinsically disordered region found at the C-terminus of the endogenous *nhr-67* locus, using CRISPR/Cas9, as this would both confirm the NHR-67/UNC-37 interaction in vivo and allow us to determine the functional significance of this interaction. However, we were unable to recover a viable line after several attempts, suggesting that this region of the protein is vital.

1. Throughout the manuscript, the authors do not use lineage analysis to confirm fate transformation as is the standard in the field.

Response 2.7: The timing between AC/VU cell fate specification and AC invasion (the point at which we look for differentiated ACs) is approximately 10-12 hours at 25 °C. With our imaging setup, we are limited to approximately 3-4 hours of live-cell imaging. Therefore, lineage tracing was not feasible for our experiments. Instead, we relied on visualization of established markers of AC and VU cell fate to determine how ectopic ACs arose. In Fig. 6B,C we show that the expression of two AC markers (*cdh-3* and *lag-2*) turn on while a VU marker (*lag-1*) get downregulated within the same cell. In our opinion, live-imaging experiments that show in real time changes in cell fate via reporters was the most definitive way to observe the phenotype.

There are 4 multipotential gonadal cells with the potential to differentiate into VUs or ACs. Which ones contribute to the extra ACs in the different genetic backgrounds

examined was not determined, which complicates interpretation. The authors should consider and test the following possibilities: disruption of NHR-67 regulation causes 1) extra pluripotent cells to directly become ACs early in development, 2) causes VU cells to gradually trans-fate to an AC-like fate after VU fate specification (as implied by the authors), or 3) causes an AC to undergo extra cell division(s)? In Fig. 1F, 5 cells are designated as ACs, which is one more than the 4 precursors depicted in Fig. 1A, implying that some of the "ACs" were derived from progenitors that divided.

Response 2.8: When trying to determine the source of the ectopic ACs, we considered the three possibilities noted by the reviewer: (1) misspecification of AC/VU precursors, (2) VU-to-AC transdifferentiation, or (3) proliferation of the AC. We eliminated option 3 as a possibility, as the ectopic ACs we observed here were invasive and all of our previous work has shown that proliferating ACs cannot invade and that cell cycle exit is necessary for invasion (Matus et al., 2015; MedwigKinney & Smith et al., 2020; Smith et al., 2022). Specifically, NHR-67 is upstream of the cyclin dependent kinase CKI-1 and we found that induced expression of NHR-67 resulted in slow growth and developmental arrest, likely because of inducing cell cycle exit. For our experiment using *hsp::NHR-67*, we induced heat shock after AC/VU specification. For POP-1 perturbation, we explicitly acknowledged that misspecification of the AC/VU precursors could also contribute to ectopic ACs (Fig. 6A; lines 368-385). We could not achieve robust protein depletion through delayed RNAi treatment, so instead we utilized timelapse microscopy and quantification of AC and VU cell markers (Fig. 6B,C; see response 2.7 above).

In conclusion, while the authors report on interesting observations, in particular the colocalization of NHR-67 with UNC-37/Groucho and POP-1 in nuclear puncta, the functional significance of these observations remains unclear. The authors have not demonstrated that the "repressive condensates" are functional and play a role in the suppression of AC fate in VU cells as claimed. The colocalization data suggest that NHR-67 interacts with repressors, but additional experiments are needed to demonstrate that these interactions are specific to VUs, impact VU fate, and sequester NHR-67 from its targets or transform NHR-67 into a transcriptional repressor.

Response 2.9: We agree that, at this time, we cannot pinpoint the precise mechanism through which NHR-67 puncta function (i.e., by sequestering NHR-67 from DNA or switching the role of NHR-67 from activating to repressing). However, identification of NHR-67 puncta and their colocalization with UNC-37, LSY-22, and POP-1 in VU cells allowed us to discover an undescribed role for the Groucho/TCF complex in maintaining VU cell fate. This, combined with our evidence demonstrating that NHR-67 transcriptional regulation is important for distinguishing between AC and VU cell fate, are the main contributions of our study.

Reviewer #1 (Recommendations For The Authors):

I am not a C. elegans researcher and I find this paper fairly hard to follow. One major recommendation I would like to see is to improve the consistency of the labeling of the figures. There are many figures showing many things and I struggled to keep track of everything. For example, the thing that we are looking at in the microscope images (typically GFP tagged to a protein of interest) is sometimes labeled above the image, sometimes to the side, and sometimes within the panel. Experimental conditions are also formatted arbitrarily. As much as they can do so, could the authors try and make their labeling consistent? This would help me follow the data.

Response 1.2: We thank the reviewer for this suggestion and have reorganized the figures (namely Figure 3, Figure 4, Figure 4-figure supplement 1, Figure 5, and Figure 6) such that

the tagged allele or marker is labeled at the top, and the time, stage, and/or perturbation is labeled on the side.

*Is the yeast one-hybrid assay enough to confirm a direct interaction between HLH-2 and NHR-67? Obviously, it supports it, but since this is not a definitive test in *C. elegans*, I feel the description of this result should be modified to account for this.*

Response 1.3: We agree that the yeast one-hybrid assay identifies sequences that are capable of being bound to a protein and does not prove that a DNA-protein interaction occurs in vivo. We have modified our language describing this result in our resubmission (lines 222-224).

*NHR-67 and POP-1 eventually form two large spots. This observation supports the claims that these are condensates, but it is clearly different from the observations in *Ciona* where the condensates remain more or less stable until they quickly dissolve at the onset of mitosis. Do the authors have any idea why these condensates are behaving this way? Is it always two spots? This implies it is forming around some sort of diploid nuclear structure.*

Response 1.4: Hes.a puncta observed in *Ciona* were indeed shown to be dynamic, as puncta were captured fusing together (see Figure 6B of Treen et al., 2021). However, these puncta did not appear to coalesce into two puncta specifically, as is consistently observed with NHR-67 in *C. elegans*. We agree with the reviewer in that this observation is very interesting and likely correlates to a diploid nuclear structure, however we have yet to identify this.

*In *Ciona*, for the two examples of repressive condensates, it was shown that the removal of the C-terminal Groucho recruiting repressor domains of HesA and ERF disrupts condensate formation. Have the authors attempted a similar experiment for NHR-67 or Pop1?*

Response 1.5: We agree that this would have been an ideal experiment to perform. We attempted to remove the intrinsically disordered region found at the C-terminus of NHR-67 with CRISPR, but were unable to generate a stable line, suggesting that this region may be critical for NHR-67 function in other developmental stages or tissues.

Other minor points:

Fig 4D - I found the labeling of this figure the most confusing.

Response 1.6: We thank the reviewer for bringing this to our attention. For this panel, in addition to the changes we made reference above (Response 1.2), we simplified the labeling of the TIR1 transgene and instead reference it in the figure legend for simplicity.

Line 354 - I think this is mislabeled. Is it supposed to be Figure 5H, not 5F, and 5B, not 5C?

Response 1.7: We thank the reviewer for spotting this error. This reference to Figure 5F has been updated and now correctly references Figure 5H (line 338).

Reviewer #2 (Recommendations For The Authors):

The authors use several methods to overexpress NHR-67 including 1) an NHR-67 transgene (Fig. 1), 2) overexpression of the transcriptional activator HLH-2 or 3) removal of a factor that normally degrades HLH-2 in VU cells (Fig. 2). In all cases, the rate of VU AC transformation is either very low (5%) or not reported but presumed to be zero, since

other groups have done similar experiments and reported no such conversion (eg. Benavidez et al., 2022). What is the significance of this finding? Does this mean that high levels of NHR-67 are not sufficient to promote AC fate because NHR-67 is sequestered in puncta when expressed in VU cells? Fig. 2A suggests that NHR-67 is in puncta in all VUs where overexpressed. Would the inactivation of GROUCHO in that background result in extra ACs?

Response 2.10: Indeed, we would expect that overexpression of NHR-67 may not normally be sufficient to induce cell fate transformation if the Groucho/TCF complex is still functional. Unfortunately we were unable to achieve strong depletion of UNC-37 and LSY-22 through RNAi, and thus relied on the auxin-inducible protein degradation system. Since we are limited by the number of fluorescent channels we can co-visualize, it would not be feasible to combine a heat-shock inducible transgene, a TIR1 transgene, an AID-tagged protein, and multiple cell fate markers.

The data are often presented as numbers of animals with increased or decreased expression of a particular marker, but no quantification of expression is provided. For example, in Figure 1E, 32/35 animals are reported to exhibit ectopic expression of LIN-12 in the AC and reduced expression of LAG-2. What is the range of the increase/decrease in LIN-12/LAG-2 expression and how does this compare to natural variation in wild-type? The same concerns apply to Fig. 4D.

Response 2.11: For resubmission, we have quantified the data shown in Figure 1E and now report expression levels of LIN-12::mNeonGreen and LAG-2::P2A::H2B::mTurquoise2 in Figure 1–figure supplement 2. We have also quantified the data in Figure 4D and now report expression levels of cdh-3p::mCherry::moeABD in Figure 4E. Quantification methods have been added to the Materials and Methods section (lines 612-617).

The authors explain that it is difficult to study a repressive role for POP-1 as this protein functions in multiple developmental pathways and POP-1 depletion needs to be carefully timed for the data to be interpretable. The authors then go on to use RNAi to deplete POP-1 but do not describe in the methods how they achieve the needed precise temporal control.

Response 2.12: We did indeed describe methods for the GFP-targeting nanobody, which we expressed under a uterus-specific promoter expressed after AC/VU specification. However, since the penetrance of phenotypes associated with this perturbation was low, we utilized RNA interference. We separated the cell fate specification and cell fate maintenance phenotypes by visualizing AC markers (Fig. 6A), which we would expect to be expressed at equal levels if ACs adopted their fate at the same time (via misspecification). We also paired these with a marker for VU cell fate and co-visualized them over time (Fig. 6B,C).

The authors also do not report the efficiency of protein depletion by RNAi or Auxin treatment.

Response 2.13: Auxin-induced depletion of mNeonGreen::AID::LSY-22 resulted in more than 90% decrease in expression (n > 75 uterine cells). The AID-tagged allele for UNC-37 was labeled with BFP, which was barely detectable by our imaging system and photobleached very quickly, so we did not quantify its depletion. However, considering that UNC-37 and LSY-22 are both expressed fairly uniform and ubiquitously, and that LSY-22 is expressed at higher levels than UNC-37 at the L3 stage according to WormBase (31.9 FPKM vs. 23.5 FPKM), we would predict that its auxin-induced depletion would be just as potent if not more so.

Some of the work presented repeats previously published observations, and it is difficult at times to keep track of what is confirmatory and what is new. For example, this group already published on the enrichment of HLH-2 and NHR-67 in the AC, as well as the positive regulation of NHR-67 by HLH-2 (Medwig-Kinney et al 2020). Additionally, prior papers have already reported the interaction between HLH-2 and the nhr-67 locus.

Response 2.14: The work presented in this manuscript does not repeat any previously published experiments. When we introduced the endogenously tagged NHR-67 and HLH-2 strains in previous work (Medwig-Kinney & Smith et al., 2020), we quantified expression of these proteins in the AC over time but did not compare expression between the AC and VU cells. Additionally, we previously showed that HLH-2 positively regulates NHR-67 in the AC (Medwig-Kinney & Smith et al., 2020), but never showed this is the case in the VU cells. Considering that this regulatory interaction was not observed in the AC/VU cell precursors, we believe that determining whether these proteins interact in the context of the VU cells was a valid question to address.

Treen et al. 2021 are cited as prior evidence for the existence of "repressive condensates", however, that study does NOT experimentally demonstrate a function for these structures.

Response 2.15: By “repressive condensates” we are referring to condensation of proteins known to be transcriptional repressors. While we agree that we were not able to demonstrate transcriptional repression of specific loci, our data showing that perturbation of the Groucho repressors UNC-37 and LSY-22 results in ectopic ACs is consistent with the hypothesis that these proteins repress the default AC fate. We have modified our title and text to more clearly distinguish our interpretations versus speculations.