A developmental gene regulatory network for invasive differentiation of the *C. elegans* anchor cell

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SUMMARY STATEMENT

Basement membrane invasion by the *C. elegans* anchor cell is coordinated by a gene regulatory network encompassing overlapping cell cycle dependent and independent sub-circuits.

ABSTRACT

Cellular invasion through the basement membrane is a key part of fertility, development, immunity, and disease. To understand the acquisition of the invasive phenotype, we use the genetically tractable *in vivo* model of *C. elegans* anchor cell invasion into the vulval epithelium. Coordination of AC invasive machinery is largely achieved cell autonomously through the activity of four conserved transcription factors (TFs), *fos-1a* (Fos)*, egl-43* (EVI/MEL), *hlh-2* (E/Daughterless) and *nhr-67* (NR2E1/TLX). Here, using genome editing and protein depletion with improved RNA interference (RNAi), we characterize the gene regulatory network (GRN) programming AC invasion. Using quantitative measures of endogenous protein levels paired with perturbation analyses, we identify the upstream regulation of *nhr-67* activity by *egl-43* and *hlh-2*. Network inference suggests that these three TFs function in a type I coherent feed-forward loop with positive feedback to maintain the AC in a post-mitotic, pro-invasive state. Finally, we provide evidence that two overlapping pro-invasive sub-circuits function in a cell cycle-dependent and independent fashion. Together, these results provide the first framework for integrating TF relationships with the cell biological mechanisms driving invasive cellular behavior.

Key words: EGL-43, FOS-1, HLH-2, NHR-67, gene regulatory network, cell invasion

63 **INTRODUCTION**

64

65 Invasion through basement membranes (BM) is a cellular behavior integral to the
66 establishment of pregnancy, tissue patterning during embryonic development, and
67 immune response to infection and injury (Medwig and Matus, 2017; Rowe and Weiss,
68 2008). Increased cellular invasiveness is also a hallmark of metastatic cancer (Hanahan
69 and Weinberg, 2011). Previous research has identified several cell-autonomous
70 mechanisms that are highly conserved across different contexts of BM invasion (Kelley
71 et al., 2014; Medwig and Matus, 2017). These include expression of extracellular matrix
72 (ECM) proteins to facilitate cell-BM adhesion, localization of F-actin into invasive
73 protrusions that physically puncture the BM, as well as upregulation of matrix
74 metalloproteinases (MMPs) to chemically degrade the BM (Hagedorn et al., 2009;
75 Hagedorn et al., 2013; Kelley et al., 2018; Morrissey et al., 2014; Sherwood et al.,
76 2005). There is also growing evidence that cells must undergo cell cycle arrest in order
77 to achieve invasive differentiation (Kohrman and Matus, 2017; Matus et al., 2015). How
78 these tightly coordinated programs are transcriptionally regulated is not well understood.

79

80 As many contexts of cellular invasion occur deep within tissue layers where it is difficult
81 to visualize, we utilize morphogenesis of the *C. elegans* uterine-vulval connection as a
82 genetically tractable and visually amenable model for examining cell invasion *in vivo*.
83 During the mid-L3 stage, a specialized uterine cell called the anchor cell (AC), invades
84 through the underlying BM in order to connect the uterus to the vulval epithelium,
85 facilitating egg-laying (Sherwood and Sternberg, 2003). The AC itself is specified in a
86 cell fate decision event earlier in development, during the L2 stage, where two
87 equipotent cells diverge via stochastic Notch asymmetry, giving rise to the terminally
88 differentiated AC and a proliferative ventral uterine (VU) cell (Wilkinson et al., 1994).

89

90 Prior research has identified four pro-invasive transcription factors (TFs) that function
91 cell autonomously to regulate AC invasion (Fig. 1). These include the basic leucine
92 zipper TF *fos-1a* (Fos), the basic helix-loop-helix TF *hlh-2* (E/Daughterless), the nuclear
93 hormone receptor *nhr-67* (NR2E1/Tailless/TLX) and the zinc-finger TF *egl-43*

94 (EVI1/MEL1) (Hwang et al., 2007; Matus et al., 2010; Rimann and Hajnal, 2007;
95 Schindler and Sherwood, 2011; Sherwood et al., 2005; Verghese et al., 2011). NHR-67
96 functions upstream of the cyclin dependent kinase inhibitor, CKI-1 (p21/p27), to induce
97 G1/G0 cell cycle arrest, which is necessary for AC invasion (Matus et al., 2015).
98 Independent of NHR-67 activity, FOS-1A regulates the expression of the long isoform of
99 *egl-43* (EGL-43L) and downstream effectors including multiple MMPs (*zmp-1, -3, and -*
100 *6*), a cadherin (*cdh-3*), and hemicentin (*him*-4), an immunoglobulin superfamily protein
101 that generates a linkage between the ventral epidermal and somatic gonad BMs during
102 invasion (Hwang et al., 2007; Kelley et al., 2018; Matus et al., 2010; Morrissey et al.,
103 2014; Rimann and Hajnal, 2007). HLH-2 independently regulates *cdh-3* and *him-4* as
104 well as an ECM glycoprotein (MIG-6/papilin) and cytoskeletal polarity (Schindler and
105 Sherwood, 2011). Prior work has additionally suggested that EGL-43 and HLH-2 may
106 regulate NHR-67 based on binding motifs present in the *nhr-67* promoter (Bodofsky et
107 al., 2018; Verghese et al., 2011). How these four TFs function, both independently and
108 together, to regulate the invasive activity of the AC is poorly understood.
109
110 Here, using new highly efficient RNA interference (RNAi) vectors (Sturm et al., 2018),
111 we identify previously unreported AC invasion phenotypes associated with depletion of
112 *egl-43* and *hlh-2*. In addition, using CRISPR/Cas9-genome engineering, we have
113 endogenously GFP-tagged the genomic locus of each TF, allowing us to report both the
114 relative expression of pro-invasive TFs in the AC and dissect their molecular epistatic
115 interactions. Based on these findings, we have characterized cell cycle dependent and
116 independent pathways necessary for invasion. Finally, we have identified a feed-forward
117 regulatory circuit with positive feedback critical for maintaining the AC in a pro-invasive,
118 post-mitotic state. These findings provide new insights into the GRN that underlies
119 invasive cell behavior.
120
121 **RESULTS**
122
123 ***Improved RNAi penetrance reveals new phenotypes associated with depletion of***
124 ***pro-invasive TFs***

125 *C. elegans* AC invasion is a simple, tractable *in vivo* model for understanding how cells
126 breach the barriers imposed by BM (Gupta et al., 2012; Sherwood and Plastino, 2018).
127 We and others have identified four TFs that function cell autonomously in the AC to
128 regulate invasion (Hwang et al., 2007; Matus et al., 2015; Rimann and Hajnal, 2007;
129 Schindler and Sherwood, 2011; Sherwood et al., 2005). Recently, we identified that the
130 post-mitotic AC must be in a G1/G0-cell cycle arrested state, regulated by *nhr-67,* in
131 order to adopt an invasive phenotype (Fig. 1) (Matus et al., 2015). Roles for the other
132 three TFs in AC specification (*egl-43* and *hlh-2*) (Hwang et al., 2007; Karp and
133 Greenwald, 2004; Rimann and Hajnal, 2007; Sallee et al., 2017; Verghese et al., 2011)
134 and the transcriptional regulation of downstream invasive effectors, including MMPs
135 *(fos-1a* and *egl-43*) and other pro-invasive targets, have been previously established
136 (Fig. 1A) (Bodofsky et al., 2018; Kelley et al., 2018; Matus et al., 2010; Matus et al.,
137 2015; Schindler and Sherwood, 2011; Sherwood et al., 2005; Wang et al., 2014). Here,
138 we sought to gain a deeper understanding of the regulatory relationships between pro-
139 invasive TFs in the AC, taking advantage of recent advances in RNAi targeting (Sturm
140 et al., 2018) and CRISPR/Cas9-genome engineering (Dickinson and Goldstein, 2016).
141 To accomplish this, we first generated new RNAi targeting constructs in the improved
142 RNAi vector, T444T, which includes T7 termination sequences to prevent the
143 generation of non-specific RNA fragments from the vector backbone, increasing the
144 efficacy of gene silencing over the original RNAi targeting vector, L4440 (Sturm et al.,
145 2018).
146
147 As three of four pro-invasive TFs also function during AC specification (Hwang et al.,
148 2007; Karp and Greenwald, 2004; Verghese et al., 2011), we scored TF depletions in a
149 genetic background where only the AC and neighboring uterine cells are sensitive to
150 RNAi. The uterine-specific RNAi sensitive strain was generated through tissue-specific
151 restoration of the RDE-1 Piwi/Argonaut protein in an *rde-1* mutant background using the
152 *fos-1a* promoter, which is expressed specifically in the somatic gonad during the late
153 L2/early L3 stage of larval development (Haerty et al., 2008; Hagedorn et al., 2009;
154 Matus et al., 2010; Matus et al., 2015). AC invasion was quantified as the presence or
155 absence of a BM gap, visualized by laminin::GFP, at the P6.p four-cell stage, a

156 developmental window where 100% of wild-type ACs are invaded (Fig. 2). We also
157 scored invasion several hours later, following vulval morphogenesis, allowing us to
158 distinguish between delays in invasion and complete loss of invasive capacity (Fig. 2B,
159 Fig. S1, Table S1). Depletion of each TF resulted in invasion defects ranging from
160 moderate penetrance (38%, *fos-1*) to highly penetrant (69-94%, *hlh-2*, *egl-43*, and *nhr-*
161 *67*), when synchronized L1-stage animals were plated on RNAi-containing bacteria and
162 scored at the mid-L3 stage (P6.p 4-cell stage) and early L4 stage (P6.p 8-cell stage)
163 (Fig. 2, Fig. S1). Consistent with previous work, depletion of *fos-1* resulted in single ACs
164 that failed to breach the BM. Depletion of *nhr-67* resulted in proliferative, non-invasive
165 ACs at high penetrance. Interestingly, depletion of *egl-43* phenocopied *nhr-67*, with a
166 highly penetrant defect of multiple, non-invasive ACs. Lastly, loss of *hlh-2* resulted in a
167 range of phenotypes, from animals with zero, one, two or even several cases of three or
168 more ACs (Fig. S1B,C). In all cases, the penetrance of AC invasion defects was
169 significantly increased using a T444T-based RNAi targeting vector as compared to
170 L4440-based vectors (Fig. S1A). Thus, use of the improved RNAi vector revealed
171 potentially novel phenotypes for depletion of *egl-43* and *hlh-2* during invasion.
172
173 As we did see a range of phenotypes in *hlh-2* depleted animals, including animals
174 possessing multiple ACs, we wanted to rule out any potential defects in AC
175 specification. Depletions using the traditional RNAi vector, L4440, in the uterine-specific
176 RNAi-sensitive background is sufficient to bypass AC fate specification defects that
177 occur in genetic backgrounds where all cells are sensitive to RNAi (Matus et al., 2010).
178 Specifically, loss of HLH-2 prior to AC specification regulates the *lin-12*(Notch)/*lag-*
179 *2*(Delta)-dependent signaling cascade, resulting in both pre-AC/VU cells adopting a VU
180 fate. Later, loss of HLH-2 during AC specification results in upregulation of pro-AC fate
181 by activating *lag-2*/Delta resulting in both AC/VU cells adopting an AC fate (Karp and
182 Greenwald, 2004; Schindler and Sherwood, 2011). Notably, *hlh-2* depletion using the
183 more penetrant T444T vector resulted in a significantly greater occurrence of a 0 AC
184 phenotype as compared to an L4440 *hlh-2* RNAi clone (Fig. S1A-C). To bypass
185 specification defects due to increased RNAi penetrance for *hlh-2* using the T444T
186 vector, we depleted *hlh-2* by RNAi using an L2-plating strategy, growing animals to the

time of the L1/L2 molt and then transferring them to *hlh-2(RNAi)* plates. The L2 plating resulted in a lower penetrance overall of AC invasion defects (43% at the P6.p 4-cell stage; Fig. 2, Fig. S1C), but all animals possessed an AC. Strikingly, multiple animals possessed multiple *cdh-3*-expressing ACs early (Fig. 2), while 10% of animals in the early L4 stage possessed multiple ACs (Fig. 2B, Fig. S1C,D). As the number of ACs increased over developmental time (Fig. 2B, Fig. S1C,D), these results suggest that the multiple ACs could be arising through loss of G1/G0-cell cycle arrest and *nhr-67* activity (Matus et al., 2015).

Generation of GFP-tagged proteins to establish a quantitative framework for examining TF interactions

Next, we sought to examine the relationship of these four TFs in the AC prior to and during invasion. To do this in a quantitative fashion, we used CRISPR/Cas9-genome editing technology to knock-in a codon-optimized GFP tag into the endogenous locus of each TF (Fig. S2) (Dickinson and Goldstein, 2016; Dickinson et al., 2013; Dickinson et al., 2015). All four TFs showed robust GFP-localization in the AC nucleus prior to and during invasion (Fig. 3A). Additionally, the GFP-tagged strains had similar expression domains in the somatic gonad during the L3 larval stage as previously reported by traditional multi-copy array transgenes (Hwang et al., 2007; Matus et al., 2015; Rimann and Hajnal, 2007; Sherwood et al., 2005; Verghese et al., 2011). After examining expression patterns, we then synchronized TF GFP-tagged strains and collected a developmental time-course quantifying the expression of GFP-tagged protein in the AC at the P6.p 1-cell, 2-cell, 4-cell and 8-cell stages (Fig 3A). All GFP-tagged TFs were imaged using uniform acquisition settings on a spinning disk confocal using an EM-CCD camera, allowing us to compare relative expression levels of endogenous GFP-tagged proteins. Interestingly, all four TFs followed the same general trend in expression levels, with a gradual increase in levels prior to invasion, peaking at or just before the P6.p 4-cell stage when 100% of wild-type animals have generated a gap in the BM. Expression levels then decreased following invasion, during vulval morphogenesis in the early L4 stage (P6.p 8-cell stage) (Fig. 3B).

218

219 ***Correlating phenotypes to quantitative loss of endogenous TFs***

220

221 We next wished to assess the efficacy of our newly generated TF-RNAi vectors
222 quantitatively. To accomplish this, we first crossed a BM reporter (laminin::GFP) and an
223 AC marker (*cdh-3>mCherry:moeABD*) into each endogenously GFP-tagged TF strain.
224 We then performed a series of RNAi depletion experiments targeting each TF and
225 examining synchronized animals at the P6.p 4-cell stage. As the insertion of GFP-tags
226 into native genomic loci can potentially interfere with gene function, we examined a
227 minimum of 50 animals treated with control (T444T) empty vector RNAi (Fig. 4). All four
228 GFP-tagged strains showed 100% BM breach at the normal time of invasion in control
229 animals (empty vector) (Fig. 4). Next, we examined a minimum of 50 animals following
230 targeted TF-RNAi depletions, collecting spinning disk confocal z-stacks using the same
231 acquisition settings as used for the developmental series (Fig. 3) in all experiments.
232 Following image quantification, our results were consistent with the phenotypes
233 identified in our original uterine-specific RNAi screen (Fig. 2,4). However, by being able
234 to quantify loss of endogenous GFP-tagged TF targeted by RNAi, we were more
235 accurately able to correlate phenotype with protein depletion. The *nhr-67* and *egl-43*
236 improved RNAi constructs strongly knocked down their GFP-tagged endogenous
237 targets, with depletions averaging 88% and 75%, respectively (Fig. 4E). This strong
238 knockdown of *nhr-67* and *egl-43* was also correlated with a highly penetrant AC
239 invasion defects (61% and 100%, respectively). The completely penetrant defect from
240 *egl-43(RNAi)* segregated into 26% of animals exhibiting a single AC that failed to invade
241 and the remaining phenocopying loss of *nhr-67*, with multiple *cdh-3*-expressing non-
242 invasive ACs (Fig. 4A,E). RNAi depletion of *GFP::fos-1a* was also penetrant, with a
243 mean depletion of 92% and 76% of treated animals exhibiting a block in AC invasion
244 (Fig. 4B,E). To assess post-AC specification phenotypes following *hlh-2* knockdown, we
245 performed L2 platings and scored for defects. As the animals experience ~12 hours (at
246 25°C) less time exposed to *hlh-2(RNAi)*, we were not surprised to see relatively weaker
247 *GFP::hlh-2* depletion as compared to L1 platings targeting the other TFs (Fig. 4E).
248 However, this shorter time of depletion revealed a strong correlation between invasion

249 defect and percent protein depletion. Animals with ~50% depletion appeared wild-type,
250 with a single AC that breached the BM. Animals with stronger depletion in the AC
251 presented pleiotropic phenotypes, much like *egl-43(RNAi)* (Fig. 4C,E). Notably, the
252 average depletion for animals with a single non-invasive AC was weaker than for
253 animals with multiple *cdh-3*-expressing non-invasive ACs (60% vs. 78% respectively,
254 Fig. 4D,E). These results suggest that following strong TF depletion, loss of either *egl-*
255 *43* or *hlh-2* phenocopy *nhr-67* depletion, generating multiple, non-invasive ACs.
256
257 ***Identification of a feed-forward loop controlling NHR-67 activity***
258
259 Next, we examined the relationship between the four pro-invasive TFs during invasion.
260 To accomplish this, we performed a series of RNAi depletion experiments using
261 synchronized L1 or L2 (for *hlh-2*) stage animals and quantified the amount of GFP-
262 tagged endogenous TF in the AC in animals with defects in invasion. While depletion of
263 *fos-1* failed to significantly down-regulate levels of *nhr-67::GFP,* loss of *egl-43* resulted
264 in a strong reduction (65%) of *nhr-67::GFP* in the AC (Fig. 5A,B). Intriguingly, in animals
265 with a single non-invasive AC following *hlh-2(RNAi),* we saw only a partial reduction
266 (19%) of *nhr-67::GFP* levels, while in animals with multiple *cdh-3*-expressing cells we
267 saw a significant reduction (49%) in *nhr-67::GFP* (Fig. 5A,B). These results suggest that
268 both *egl-43* and *hlh-2* co-regulate *nhr-67* during invasion.
269
270 We repeated these TF-RNAi molecular epistasis experiments with the remaining three
271 TF-GFP-tagged strains and quantified loss of GFP in animals with invasion defects.
272 Consistent with previous studies using transcriptional GFP promoter reporters (Hwang
273 et al., 2007; Rimann and Hajnal, 2007), we found that depletion of *fos-1* regulated levels
274 of *egl-43::GFP::egl-43* (44% depletion) (Fig. 5C,D). No other TF depletion significantly
275 regulated the levels of *egl-43* in our experiments (Fig. 5C,D). *hlh-*2 is predicted to
276 regulate *egl*-43 activity based on the presence of two conserved E-box binding motifs in
277 the *egl-43* promoter (Hwang et al. 2007). We did not detect regulation of *egl-*
278 *43::GFP::egl-43* in either the AC (Fig. 5C,D) or neighboring ventral uterine (VU) cells
279 (Fig. S3), but as our experiments with *hlh-2* depletion were done as L2 platings post-AC

280 specification, it is possible that *hlh-2* might regulate *egl-43* prior to AC/VU specification.
281 Next, we examined *GFP::hlh-2* levels following TF-RNAi depletions. Intriguingly, we
282 found a similar pattern of regulation based on AC phenotype. Animals with single non-
283 invasive ACs showed partial depletion of *GFP::hlh-2* following *fos-1, egl-43,* or *nhr-67*
284 loss (35%, 20%, and 14%, respectively). However, animals with multiple *cdh-3*-
285 expressing ACs following *egl-43(RNAi)* or *nhr-67(RNAi)* showed strong depletion of
286 *GFP::hlh-2* (66% and 73%, respectively) (Fig. 5E,F). Finally, we examined levels of
287 *GFP::fos-1a* following TF depletions. Loss of either *nhr-67* or *egl-43* resulted in partial
288 reduction of *GFP::fos-1a* levels in the AC (52% and 49%, respectively) (Fig. 5G,H).
289 Together, these results suggest that *egl-43*, *hlh-2* and *nhr-67* may function together in a
290 feed-forward regulatory loop with positive feedback to maintain the AC in a post-mitotic,
291 pro-invasive state.
292
293 ***Multiple ACs derive from proliferation***
294
295 As loss of *egl-43* and *hlh-2* both regulate levels of *nhr-67* and their depletion results in
296 multiple ACs that fail to invade, we next wanted to assess whether their depletion was
297 functionally phenocopying loss of *nhr-67*. To confirm that the presence of multiple cells
298 expressing the *cdh-3*-driven AC reporter were due to defects in proliferation, we
299 performed static imaging using a full-length translational *cdt-1*>CDT-1::GFP reporter
300 that indicates cell cycle progression (Fig. 6A). As CDT-1 must be removed from origins
301 of replication during DNA licensing, the transgene localizes to the nucleus during G1/G0
302 and is largely cytosolic at the onset of S phase (Matus et al., 2014; Matus et al., 2015).
303 As expected, T444T (empty vector) and *fos-1(RNAi)* treated animals consistently
304 exhibited nuclear localization of CDT-1::GFP (Fig. 6A; n $\geq$ 14 examined for each),
305 indicating cell cycle arrest. However, following depletion of *egl-43, hlh-2* and *nhr-67* we
306 identified multiple animals (Fig, 6A, n $\geq$ 10 for each RNAi) possessing non-invasive ACs
307 lacking nuclear CDT-1::GFP, indicative of cycling ACs. Together, these results strongly
308 support our molecular epistasis data suggesting that *egl-43* and *hlh-2* function upstream
309 of *nhr-67* to maintain the post-mitotic state of the AC during invasion.
310

311 ***EGL-43 plays a role in cell cycle dependent and independent pro-invasive***
312 ***pathways***
313
314 We have shown previously that the invasive activity of the AC following loss of NHR-67
315 can be completely rescued by restoring the AC to a post-mitotic G1/G0 state through
316 upregulation of the cyclin-dependent kinase inhibitor, CKI-1 (p21/p27) (Matus et al.,
317 2015). As our epistasis experiments revealed that *egl-43* and *hlh-2* positively co-
318 regulate NHR-67 activity, we induced AC-specific expression of CKI-1 using a *cdh-*
319 *3*>CKI-1::GFP integrated array and assessed invasion following RNAi depletions (Fig.
320 6B,C). As previously reported, induced localization of CKI-1::GFP in the AC completely
321 rescued *nhr-67(RNAi)* treated animals (Fig. 6B,C; 100% invaded (n=42) as compared to
322 45% of control animals (n=62)). Additionally, *cdh-3*>CKI-1::GFP rescued AC invasion
323 following depletion of *hlh-2* in L2 RNAi-feeding experiments in most animals examined
324 (Fig. 6B,C; 87% invaded (n=77), as compared to 56% of control animals (n=59)). As
325 expected, inducing G1/G0 arrest in the AC failed to rescue *fos-1(RNAi)* treated animals
326 (Fig. 6B,C; 20% invaded (n=35), as compared to 51% of control animals (n=53)).
327 Strikingly, although AC-specific CKI-1::GFP blocked AC proliferation following *egl-*
328 *43(RNAi),* induced arrest failed to rescue invasion (Fig. 6B,C; 10% invaded (n=31), as
329 compared to 19% of control animals (n=37)), suggesting that *egl-43* has pro-invasive
330 functions outside of the cell cycle-dependent pathway. In support of a role for *egl-43*
331 outside of cell cycle control, induced CKI-1::GFP restores the expression of a reporter of
332 MMP activity (*zmp-1>mCherry*) in *nhr-67*-depleted animals, but neither *fos-1* nor *egl*-43-
333 depleted animals show restoration of *zmp-1* transcription (Fig. 6D). Together, these
334 results reveal the presence of two integrated sub-circuits necessary for invasive activity,
335 with *egl-43* likely functioning in a critical role for both circuits.
336
337 ***EGL-43 isoforms function redundantly and in an autoregulative manner during***
338 ***AC invasion***
339
340 Two functional isoforms of *egl-43* are encoded in the *C. elegans* genome (Fig. 7A).
341 Previous research has suggested that the longer isoform functions downstream of *fos-*

342 *1a* to modulate MMP expression and other *fos-1* transcriptional targets, including *cdh-3*
343 and *him-4*/hemicentin (Hwang et al., 2007; Rimann and Hajnal, 2007). The shorter
344 isoform has been predicted to either function in Notch/Delta-mediated AC specification
345 (Hwang et al., 2007) and later Notch/Delta-mediated patterning of the ventral uterus
346 (Hwang et al., 2007) or as a potential competitive inhibitor for long isoform binding of
347 downstream targets (Rimann and Hajnal, 2007). Additionally, *fos-1a* and *egl-43* have
348 been shown to function in an incoherent feed-forward loop with negative feedback, with
349 *fos-1a* positively regulating and *egl-43* negatively regulating the levels of *mig-*
350 *10*/lamellipodin, a key adhesion protein important for stabilizing the attachment of the
351 AC to the BM (Wang et al., 2014). Titrating levels of MIG-10 is critical, as
352 overexpression of MIG-10 results in AC invasion defects (Wang et al., 2014). Although it
353 is readily apparent that levels of EGL-43 are critical, it is unclear from previous studies
354 whether the two isoforms have divergent functions during invasion. Thus, we next
355 decided to explore *egl-43* isoform function by using newly available tools, including
356 CRISP/Cas9-genome engineering and improved RNAi.
357
358 As the start codon methionine of the short isoform of *egl-43* is in-frame with and
359 contained within the long isoform, there was no simple way to solely tag or interfere with
360 the short isoform (Fig. 7A). However, we were able to generate a knock-in allele of GFP
361 at the N-terminus of *egl-43* to tag the long isoform specifically *(GFP::egl-43L*). This
362 allowed us to compare expression patterns of the long isoform to the internally GFP-
363 tagged allele that should dually label both isoforms (Fig. 7A,B). We examined animals
364 during uterine-vulval development and found overlapping expression patterns between
365 both isoforms with strong AC/VU/DU expression in the somatic gonad (Fig. 7B). Next,
366 we generated an *egl-43L*-specific improved RNAi targeting vector in T444T and
367 examined *egl-43L* depletion in the uterine-specific RNAi sensitive genetic background
368 (Hagedorn et al., 2009; Matus et al., 2010). Depletion of *egl-43L* resulted in a penetrant
369 AC invasion defect, as expected, but notably, we detected the presence of animals with
370 both a single non-invasive AC (19%) and animals with multiple ACs (39%) (Fig. 7C,D),
371 similar to depletion of both isoforms (Fig. 2). We then assessed if the long isoform has
372 the same quantitative relationship to the other TFs as observed when targeting both

373 isoforms. Indeed, *egl-43(RNAi)* and *egl-43L(RNAi)* exhibited comparable levels of
374 depletion of *egl-43::GFP::egl-43* (75%, 78%), *GFP::hlh-2* (66%, 60%), and *nhr-67::GFP*
375 (66%, 64%, respectively) (n $\geq$ 25 animals examined per treatment) (Fig. 3E).
376
377 The insertion of GFP with a ~6 kb self-excising cassette (SEC) generates a
378 transcriptional reporter that often interferes with the native transcriptional machinery at
379 the locus where it is inserted. To generate an in-frame fusion protein with GFP, we
380 excise the SEC by CRE-lox recombination via heat shock (Dickinson and Goldstein,
381 2016). To determine if newly generated alleles exhibit loss-of-function phenotypes and
382 to examine transcriptional output, we next examined the pre-floxed versions of both
383 edited knock-in alleles, in the presence of an mT1 balancer, as homozygous animals
384 are non-fertile. Unfortunately, the allele resulting from the SEC insertion of the internal
385 GFP-tag had an extremely low frequency of escapers, and we were unable to obtain L3-
386 stage animals to examine for AC invasion defects. However, similar to our results with
387 RNAi (Fig. 7C-E), an allele resulting from SEC insertion with the long isoform (*egl-*
388 *43(bmd135)*) (Fig. 7F) displayed a 31% AC invasion defect (n=11/36), with animals
389 containing either single or multiple non-invading ACs (Fig. 7G). Finally, as previous
390 reports based on *promoter>GFP* transgenic animals have suggested that *egl-43* may be
391 autoregulatory (Matus et al., 2010; Rimann and Hajnal, 2007; Wang et al., 2014), we
392 examined the mT1-balanced pre-floxed allele of the internally-tagged *egl-43* targeting
393 both isoforms (*egl-43::GFP^SEC^::egl-43*). We found strong evidence of autoregulation
394 as *egl-43(RNAi)* reduced the expression of GFP by 65% (n ≥ 25 animals) (Fig. 7H,I).
395 Taken together, these results suggest that both isoforms of *egl-43* function redundantly
396 to regulate multiple transcriptional sub-circuits critical for the establishment of the
397 invasive phenotype (Fig. 8).
398
399 **DISCUSSION**
400
401 Cellular invasion requires the coordination of extrinsic cues from the surrounding
402 microenvironment and orchestration of intrinsic regulatory circuits (Rowe and Weiss,
403 2008; Sherwood and Plastino, 2018). We focus here on exploring the intrinsic pathways

404 that autonomously regulate *C. elegans* AC invasion. This study was tractable due to
405 several recent technological breakthroughs, allowing for examination of endogenous
406 regulatory relationships quantitatively. First, we created GFP knock-in alleles of the four
407 transcription factors (*fos-1a*, *egl-43*, *hlh-2* and *nhr-67*) previously implicated in
408 autonomously regulating AC invasion (Hwang et al., 2007; Matus et al., 2015; Rimann
409 and Hajnal, 2007; Schindler and Sherwood, 2011; Sherwood et al., 2005). Additionally,
410 we generated new improved RNAi depletion constructs to strongly reduce TF activity.
411 Combining these technologies enabled us to correlate phenotype to protein depletion
412 and compare regulatory relationships in a quantitative framework. Our experiments
413 reveal new roles for *egl-43* and *hlh-2* in regulating *nhr-67*-mediated cell cycle arrest and
414 suggest that these TFs function in a coherent feed-forward loop with positive feedback.
415 Additionally, we demonstrate that *egl-43* is autoregulatory and functions in both cell
416 cycle-dependent and independent sub-circuits to orchestrate invasion (Fig. 8).
417 Together, these results provide the first description of the regulatory relationships
418 between the endogenous TFs that promote invasive differentiation during *C. elegans*
419 uterine-vulval attachment.
420
421 *nhr-67* functions to maintain the post-mitotic state of the AC (Matus et al., 2015). Here,
422 we demonstrate that *egl-43* and *hlh-2* both regulate *nhr-67*, and that *nhr-67*-depletion
423 also reduces HLH-2 levels. Network inference suggests these three TFs function in a
424 classic type I coherent feed-forward loop regulatory circuit with positive feedback
425 (Mangan and Alon, 2003) (Fig. 8). Our model fits with data by others showing that *hlh-2*
426 may directly bind to the *nhr-67* promoter through two canonical E-box motifs found in a
427 164 bp window in a functional promoter element deleted in several hypomorphic alleles
428 of *nhr-67* (Bodofsky et al., 2018; Matus et al., 2015; Verghese et al., 2011). Further,
429 deletion of these *hlh-2* conserved binding sites results in loss of AC-specific GFP
430 expression in transgenic lines (Bodofsky et al., 2018). While others have also reported
431 positive regulation by EGL-43 on *nhr-67* activity via transgenic reporters (Bodofsky et
432 al., 2018), this interaction may be indirect, as no known binding sites exist for EGL-43 in
433 the *nhr-67* promoter. Alternatively, EGL-43 and NHR-67 have been predicted, based on

434 yeast two-hybrid experiments, to interact at the protein-protein level (Reece-Hoyes et
435 al., 2013).
436
437 We have also examined the activity of the two previously identified isoforms of *egl-43*
438 (Hwang et al., 2007; Rimann and Hajnal, 2007), by generating multiple GFP knock-in
439 alleles paired with more penetrant RNAi depletions. Previous work has suggested that
440 the long isoform functions specifically downstream of *fos-1a* to regulate MMP activity
441 and other pro-invasive gene expression (Rimann and Hajnal, 2007). As both mutant
442 analyses and RNAi depletion of the long isoform phenocopy depletion of both isoforms,
443 our data suggest that either the long isoform of *egl-43* functions redundantly with the
444 short isoform or that it functions as the dominant isoform at the intersection of multiple
445 regulatory circuits.
446
447 Together, our data and corroborating evidence from the literature support the existence
448 of a coherent feed-forward circuit with positive feedback among *egl-43*, *hlh-2* and *nhr-*
449 *67,* in maintaining the post-mitotic state of the AC (Fig. 8). We have previously shown
450 that *nhr-67* positively regulates transcripts of *cki-1* specifically in the AC (Matus et al.,
451 2015). Thus, to test our putative regulatory circuit, we induced expression of *cki-1* in the
452 AC, which prevents an AC from inappropriately entering the cell cycle. This forced
453 G1/G0 arrest fully rescues *nhr-67* depletion (Matus et al., 2015). Strikingly, induced *cki-*
454 *1* strongly rescues *hlh-2* depletion as well. Thus, *hlh*-2 appears to function primarily in
455 controlling NHR-67 activity in maintenance of the post-mitotic state of the AC. These
456 results are in stark contrast to *cki-1* induction paired with strong *egl-43* depletion, where
457 most cell cycle arrested ACs still fail to invade, and fail to express an MMP, *zmp-1*.
458 These results suggest that *egl-43* functions in multiple regulatory circuits. This is
459 supported by recent work demonstrating a type I incoherent feed-forward loop between
460 *fos-1a, egl-43L* and the BM adhesion protein MIG-10/lamellipodin (Wang et al., 2014).
461 Finally, our observation that *egl-43* is autoregulatory, also shown previously by
462 *promoter>GFP* fusions (Matus et al., 2015; Rimann and Hajnal, 2007; Wang et al.,
463 2014) supports a model where *egl-43* occupies a key node at the intersection of multiple
464 pro-invasive circuits (Fig. 8). As all three TFs that function in the cell cycle-dependent

465 circuit are also involved in the Notch/Delta-mediated specification of the AC/VU fates
466 (Hwang et al., 2007; Karp and Greenwald, 2004) it will be interesting to explore whether
467 these regulatory relationships are maintained in the AC/VU specification network.
468
469 Feed-forward regulatory loops are likely the most well described network motif occurring
470 in all GRNs (Cordero and Hogeweg, 2006; Davidson, 2010; Mangan and Alon, 2003).
471 From *E. coli* (Milo et al., 2002; Shen-Orr et al., 2002) and yeast (Lee et al., 2002; Milo et
472 al., 2002) to a myriad of examples across the Metazoa (reviewed in Davidson, 2010),
473 feed-forward loops are thought to function as filters for transient inputs (reviewed in
474 Alon, 2007; Hinman, 2016). The addition of positive feedback, generating a coherent
475 feed-forward loop, provides stability to the sub-circuit and is often found in differentiation
476 gene batteries coincident with autoregulation (Davidson, 2010). These network motifs
477 have been described in many developmental contexts, from MyoD-driven vertebrate
478 skeletal muscle differentiation (Penn et al., 2004), the patterning of the Drosophila egg
479 shell via the TF Broad and interactions with EGFR and Dpp signaling (Yakoby et al.,
480 2007), Pax6-dependent regulation of c-Maf and crystallin expression in the mouse
481 embryonic lens (Xie and Cvekl, 2011) and terminal selector neuronal differentiation in *C.*
482 *elegans* and mammals (reviewed in Hobert, 2008). During *C. elegans* embryonic
483 development, a series of coherent feed-forward loops utilizing SKN-1/MED-1,2 and then
484 a suite of reiteratively used GATA factors (END-1,-3) are required to successfully
485 pattern endomesoderm development (reviewed in Maduro, 2009). Thus, our
486 identification of a coherent feed-forward loop in the *C. elegans* AC maintaining the post-
487 mitotic state, likely evolved to coordinate AC cell cycle exit, both as a by-product of
488 terminal differentiation and a morphogenetic requirement for invasive behavior (Matus
489 et al., 2015). AC invasion is necessary for egg-laying, and defects in the process results
490 in penetrant Protruding vulva/Egg-laying defective (Pvl/Egl) phenotypes, reducing
491 fecundity in individual animals by nearly ten-fold. Thus, redundant control of the sub-
492 circuit regulating differentiation and cell cycle arrest may have been under strong
493 selection, providing an explanation for the regulatory relationships between *egl-43*, *hlh-2*
494 and *nhr-67* we characterize here.
495

496 We are just beginning to understand connections between regulatory circuits and
497 morphogenetic behaviors (Martik and McClay, 2015; Saunders and McClay, 2014)
498 (Christiaen et al., 2008). It is our hope that the ease of genome editing and protein
499 perturbation strategies will facilitate the kind of analyses we have performed here in
500 other metazoan systems. In summary, in this study we characterize the complex
501 relationships between the four pro-invasive TFs that function during AC differentiation to
502 program invasive behavior. We identify a classic type I FFL regulating mitotic exit and
503 controlling a switch between proliferative and invasive behavior. Whether or not similar
504 circuit architecture is utilized to regulate invasive and proliferative cell biology in other
505 developmental invasive contexts, including mammalian trophoblast implantation and
506 placentation (Carter et al., 2015; Red-Horse et al., 2004) and EMT events during
507 gastrulation (Vega et al., 2004) is still poorly understood. Finally, as there appear to be
508 many cancer sub-types that may switch between proliferative and invasive fates
509 (reviewed in Kohrman and Matus, 2017), improving our understanding of the
510 transcriptional network architecture of invasive cells may provide new therapeutic nodes
511 to target in reducing the lethality associated with cancer metastasis.
512
513 **MATERIALS AND METHODS**
514
515 ***C. elegans strains and culture conditions***
516
517 Animals were reared under standard conditions and cultured at 25°C, with the exception
518 of temperature-sensitive strains containing the *rrf-3(pk1426)* allele, conferring RNAi
519 hypersensitivity, which were maintained at 15°C and 20°C (Brenner, 1974; Simmer et
520 al., 2002). Animals were synchronized for experiments through alkaline hypochlorite
521 treatment of gravid adults to isolate eggs (Porta-de-la-Riva et al., 2012). In the text and
522 figures, we designate linkage to a promoter through the use of the (>) symbol and fusion
523 of a proteins via a (::) annotation. The following transgenes and alleles were used in this
524 study: *qyIs102[fos-1>RDE-1;myo-2>YFP]* **LG I** *hlh-2(bmd90[hlh-2>LoxP::GFP::HLH-2]),*
525 *qyIs227 [cdh-3>mCherry::moeABD];* **LG II** *egl-43(bmd87[egl-43>SEC::GFP::EGL-43]),*
526 *egl-43(bmd88[egl-43>LoxP::GFP::EGL-43]), egl-43(bmd136[egl-43L>LoxP::GFP::EGL-*

527 *43]) rrf-3(pk1426); qyIs17 [zmp-1>mCherry*] **LG III** *unc-119(ed4),* **LG IV** *nhr-*
528 *67(syb509[nhr-67>NHR-67::GFP]), qyIs10[lam-1>LAM-1::GFP]* **LG V** *fos-1(bmd138[fos-*
529 *1>LoxP::GFP::FOS-1]), qyIs225[cdh-3>mCherry::moeABD], rde-1(ne219), qyIs24[cdh-*
530 *3$^{1.5}$>mCherry::PLCδPH], qyIs266[cdh-3>CKI-1::GFP]* **LG X** *qyIs7[lam-1>LAM-1::GFP]*.
531 See Table S2 for additional details of strains used and generated in this study.
532
533 ***Molecular biology and microinjection***
534
535 Transcription factors were tagged at their endogenous loci using CRISPR/Cas9 genome
536 editing via microinjection into the hermaphrodite gonad (Dickinson and Goldstein, 2016;
537 Dickinson et al., 2013). Repair templates were generated as synthetic DNAs from either
538 Integrated DNA Technologies (IDT) as gBlocks or Twist Biosciences and cloned into
539 *ccdB* compatible sites in pDD282 by New England Biolabs Gibson assembly (Dickinson
540 et al. 2016). Homology arms ranged from 690 – 1200 bp (see supplementary material
541 Table S3-5 for additional details). sgRNAs were constructed by EcoRV and NheI
542 digestion of plasmid pDD122. A 230bp amplicon was generated replacing the sgRNA
543 targeting sequence from pDD122 with a new sgRNA and NEB Gibson assembly was
544 used to generate new sgRNA plasmids (see Table S3,4 for more details).
545 Hermaphrodite adults were co-injected with guide plasmid (50 ng/μL), repair plasmid
546 (50 ng/μL), and an extrachromosomal array marker (pCFJ90, 2.5 ng/μL), and incubated
547 at 25 ºC for several days before carrying out screening and floxing methods associated
548 with the SEC (Dickinson et al., 2015).
549
550 ***RNA interference***
551
552 An RNAi library of the pro-invasive TFs was constructed by cloning 950-1000 bp of
553 synthetic DNA (663 bp for the *egl-43* long-specific isoform) based on cDNA sequences
554 available on WormBase (www.wormbase.org) into the highly efficient T444T RNAi
555 vector (Grove et al., 2018; Sturm et al., 2018). Synthetic DNAs were generated by IDT
556 as gBlocks or Twist Biosciences as DNA fragments and cloned into restriction digested
557 T444T using NEB Gibson Assembly (see Tables S3, S6 for additional details). For most

558 experiments, synchronized L1 stage animals were directly exposed to RNAi through
559 feeding with bacteria expressing dsRNA (Conte Jr. et al., 2015). Due to the fact that
560 early *hlh-2* RNAi treatment perturbs AC specification, animals were initially placed on
561 empty vector RNAi plates and then transferred to *hlh-2* RNAi plates post-specification,
562 approximately 12 hours later at 25°C or 24 hours later at 15°C (Schindler and
563 Sherwood, 2011).
564
565 ***Live cell imaging***
566
567 All micrographs included in this manuscript were collected on a Hamamatsu Orca EM-
568 CCD camera mounted on an upright Zeiss AxioImager A2 with a Borealis-modified
569 CSU10 Yokagawa spinning disk scan head using 488 nm and 561 nm Vortran lasers in
570 a VersaLase merge and a Plan-Apochromat 100x/1.4 (NA) Oil DIC objective.
571 MetaMorph software (Molecular Devices) was used for microscopy automation. Several
572 experiments were imaged using epifluorescence collected on a Zeiss Axiocam MRM
573 camera, also mounted on an upright Zeiss AxioImager A2 and a Plan-Apochromat
574 100x/1.4 (NA) Oil DIC objective. Animals were mounted into a drop of M9 on a 5%
575 Noble agar pad containing approximately 10 mM sodium azide as paralytic and topped
576 with a coverslip.
577
578 ***Scoring of AC invasion***
579
580 AC invasion was scored at the P6.p 4-cell stage, when 100% of wild-type animals
581 exhibit a breached BM (Sherwood and Sternberg, 2003). Presence of green
582 fluorescence under the AC, in strains with the laminin::GFP transgene, or presence of a
583 phase dense line, in strains without the transgene, was used to assess invasion. Wild-
584 type invasion is defined as a breach as wide as the basolateral surface of the AC,
585 whereas partial invasion indicates the presence of a breach smaller than the footprint of
586 the AC (Sherwood and Sternberg, 2003).
587
588 ***Image quantification and statistical analyses***

589

590 Images were processed using FIJI/ImageJ (v. 2.0.0-rc-69, NIH Image) (Schindelin et al.,
591 2012). With the exception of the developmental series displayed in Fig. 3 & Fig. S1D, all
592 other micrographs were taken at the P6.p 4-cell stage, when 100% of wild-type animals
593 exhibit a breached BM. Expression levels of TFs were quantified by measuring the
594 mean gray value of AC nuclei of, defined as somatic gonad cells strongly expressing the
595 *cdh-3>mCherry::moeABD* transgene, subtracted by the mean gray value of a
596 background region of equal area, to account for EM-CCD camera noise. *zmp-*
597 *1>mCherry* levels were quantified in control and *cdh-3>CKI-1*::GFP animals by
598 measuring the mean gray value of the entire AC, selected either by a hand drawn
599 region of interest or using the threshold and wand tool in FIJI/ImageJ (v. 1.52n). For
600 molecular epistasis experiments (Fig. 5, Fig. 7E,I & Fig. S3) and characterization of
601 CDT-1 localization (Fig. 6), only TF RNAi treated animals that did not exhibit defects in
602 invasion were excluded from analysis. Data was normalized to negative control (empty
603 vector) values for the plots in Fig. 4E, 6D, & 7E, and to both negative control and
604 positive control values for determining the interaction strengths represented in Fig. 8.
605 Images were overlaid and figures were assembled using Adobe Photoshop and
606 Illustrator CS, respectively. Statistical analyses and plotting of data were conducted
607 using RStudio (v. 1.1.463). Individual data points for each experiment were collected
608 over multiple days. Statistical significance was determined using either a two-tailed
609 Student's t-test or Fisher's exact probability test. Figure legends specify when each test
610 was used and the p-value cut-off set.

611

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615

616 **COMPETING INTERESTS**

617 No competing interests declared.

618

619 **AUTHOR CONTRIBUTIONS**

D.Q.M., T.N.M. and J.J.S. designed the experiments. All authors performed the experiments. T.N.M. and D.Q.M. performed the data analyses and wrote the paper with feedback from the other authors.

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FIGURES AND FIGURE LEGENDS

Figure 1

Medwig-Kinney et al., 2019

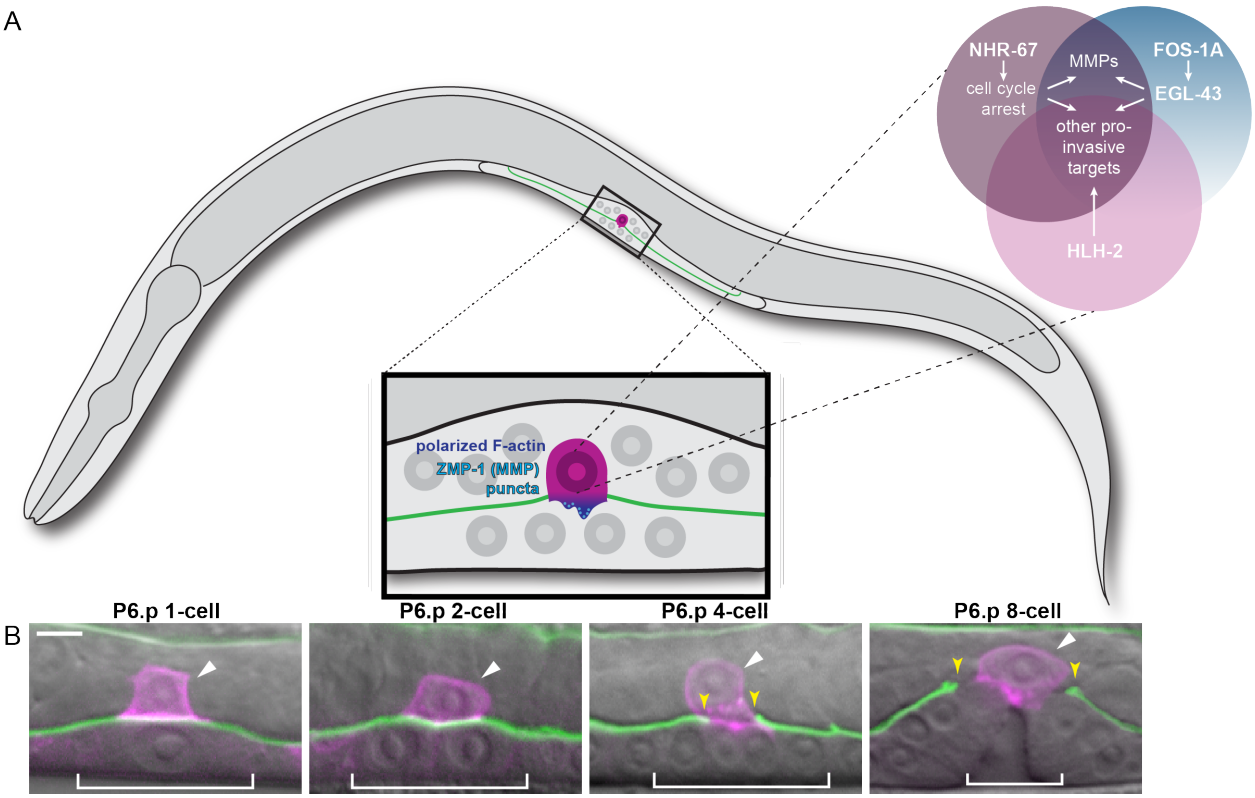

Fig. 1. Overview of *C. elegans* AC invasion. (A) Schematic depicting *C. elegans* AC (magenta) invasion through the BM (green), including polarization of the F-actin cytoskeleton (blue) into invasive protrusions and expression of MMPs (cyan) in the post-mitotic AC. Venn diagram (upper right) summarizes current TF relationships. (B) Single confocal planes overlaid on DIC depict the timing of AC (magenta, expressing *cdh-3*$^{1.5}$>mCherry::PLCδ^{PH}) invasion through the BM (green, *lam-1*>LAM-1::GFP). In this and all other figures, white arrowheads denote ACs, yellow arrowheads demarcate boundaries of gaps in the BM, and white brackets delineate P6.p and its descendants. Scale bar 5 μm, in this and all other figures.

Figure 2 Medwig-Kinney et al., 2019

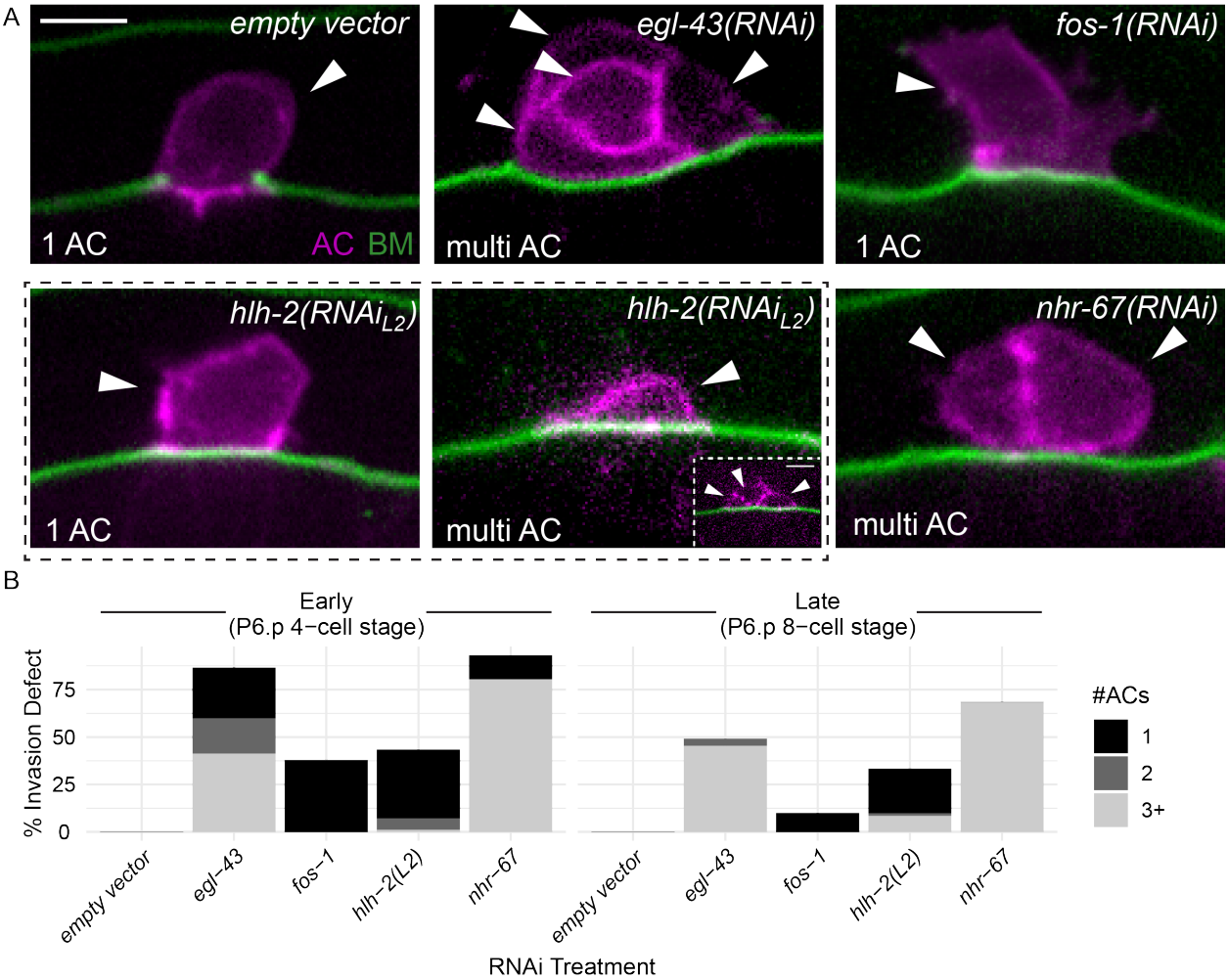

Fig. 2. RNAi depletion of pro-invasive TFs leads to defects in AC invasion and proliferation. (A) Single plane of confocal z-stack depicting representative empty vector control (upper left) and TF-RNAi depletion phenotypes. The AC-specific membrane marker (magenta, *cdh-3*$^{1.5}$>mCherry::PLCδ^{PH}) and BM marker (green, *lam-1*>LAM-1::GFP) are overlaid in each micrograph. In this and all other figures, dashed box denotes single vs. multi AC phenotypes that result from HLH-2 depletion, with multiple ACs from additional confocal z-planes shown as insets, accordingly. (B) Stacked bar chart showing penetrance of invasion defects, binned by number of ACs (p-value < 0.00001, Fisher's exact test, empty vector as compared to each TF-RNAi, n ≥ 50 animals examined for each treatment).

Figure 3 Medwig-Kinney et al., 2019

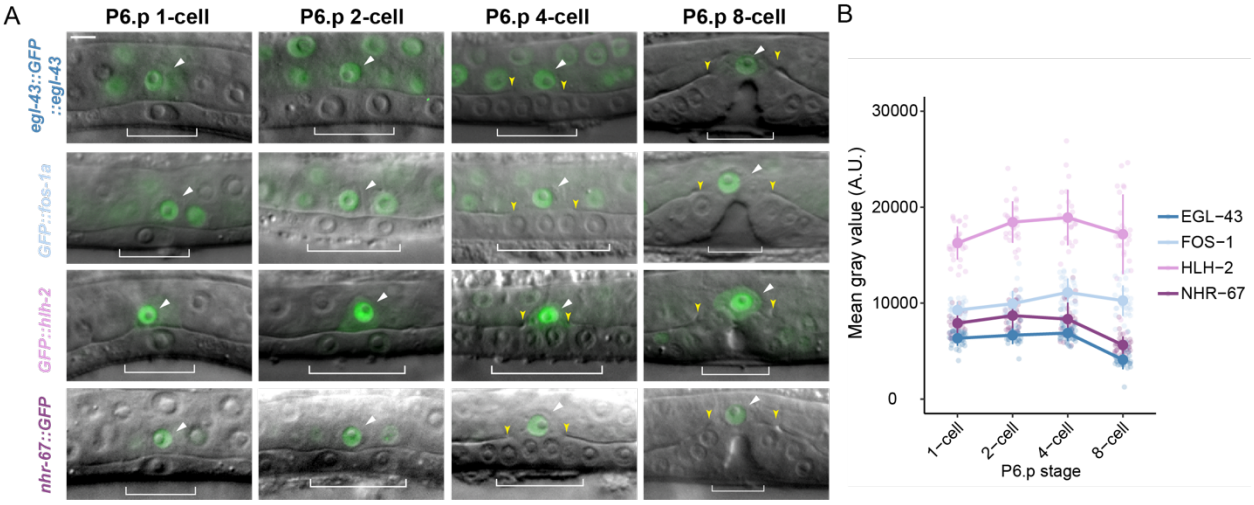

Fig. 3. Expression of endogenously-tagged pro-invasive TFs in the AC over developmental time. (A) DIC overlaid with single plane of confocal z-stack for endogenous GFP-tagged TFs. (B) Quantification of TF GFP, in relation to the division of P6.p. In this and all other figures, circle denotes mean gray value of population (n $\geq$ 25 for each stage), in this and all other figures bar denotes S.D.

Figure 4 Medwig-Kinney et al., 2019

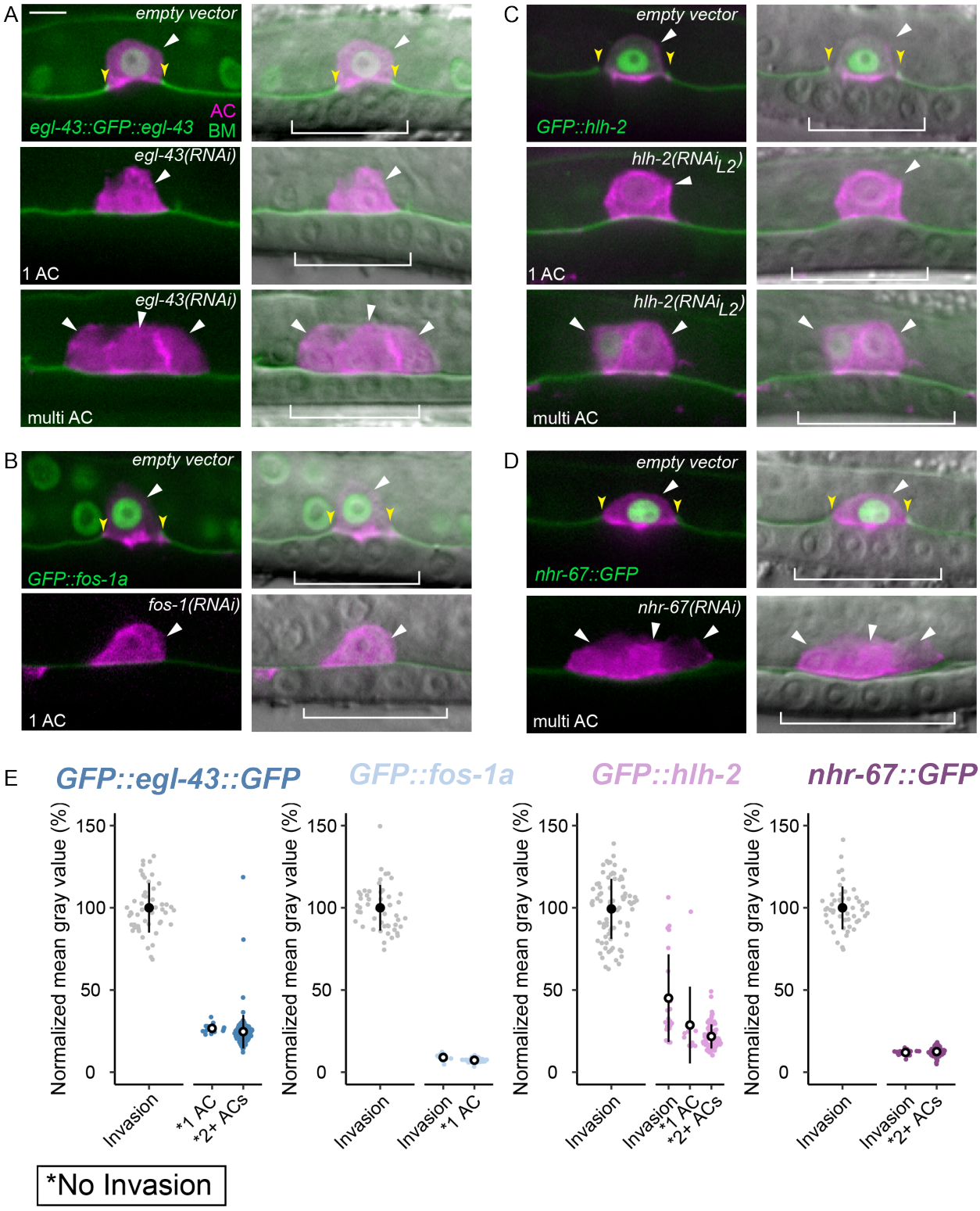

Fig. 4. Quantification of TF-RNAi phenotype penetrance in relation to GFP expression. (A-D) Single planes of confocal z-stack depicting representative phenotype (single vs. multi AC, bottom left of each image) of fluorescence alone (left; AC, magenta, expressing *cdh-3*>mCherry::moeABD and BM, green) and DIC overlay (right). (E) Sina plots of GFP-tagged TF levels, defined as the mean gray value of individual AC nuclei following TF-RNAi knockdown. In this and all other figures, statistical significance as compared to empty vector controls is denoted as an open black circle and here represents a p-value of $< 1\times10^{-6}$ by Student's t test ($n \geq 50$ animals per treatment). Asterisk (*) denotes quantification of animals with ACs (single or multi) that failed to invade.

Figure 5 Medwig-Kinney et al., 2019

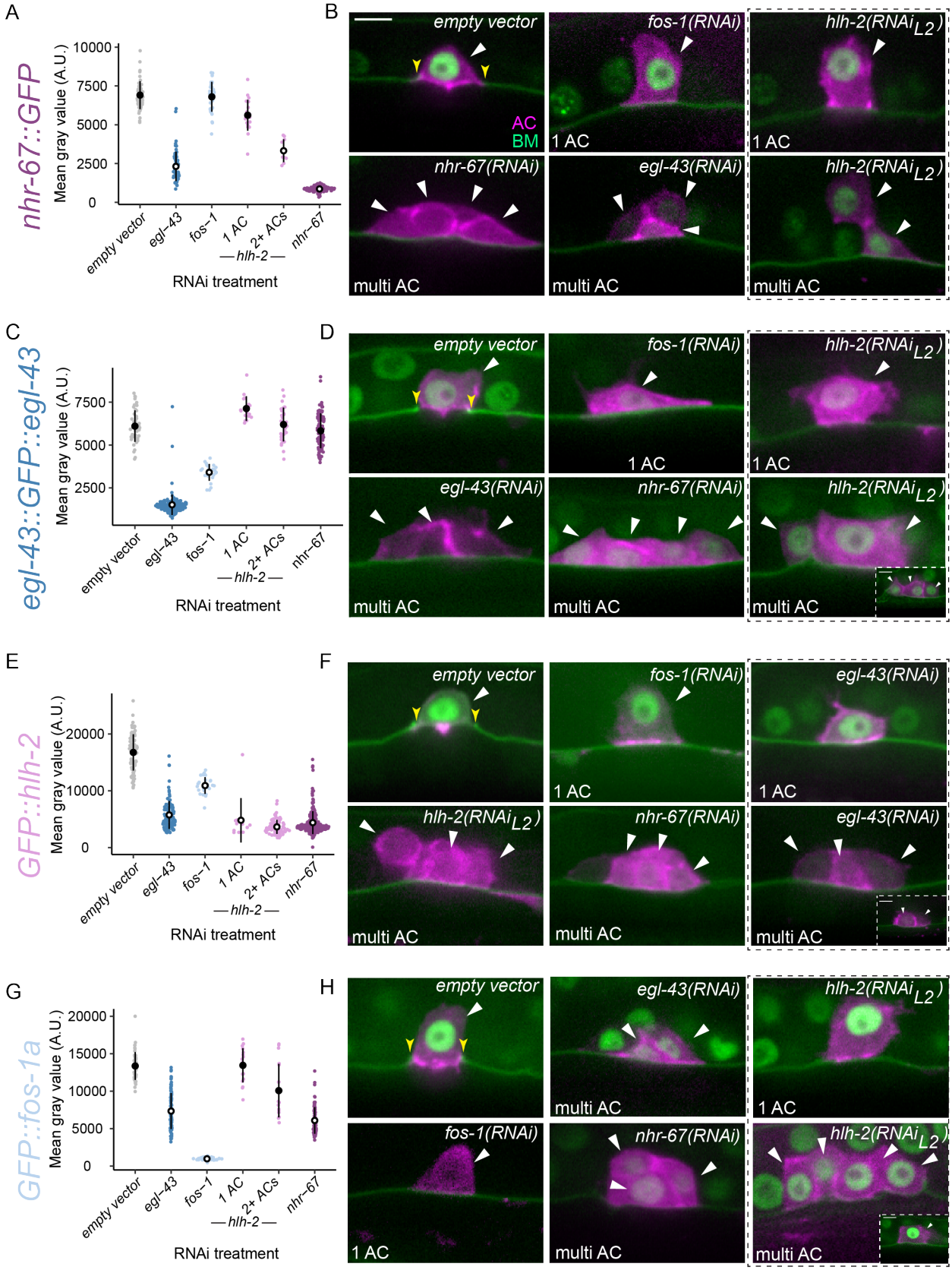

Fig. 5. Regulatory interactions among pro-invasive TFs at endogenous loci. (A, C, E,G) Sina plots of GFP-tagged TF levels, defined as the mean gray value of individual AC nuclei, following RNAi perturbation (n ≥ 25 animals per treatment). (B, D, F, H) Single planes of confocal z-stack depicting representative phenotypes (single vs. multi AC, as noted bottom left of each image) following TF depletion by RNAi, as denoted in the upper right of each image. Dashed boxes indicate representative images of single vs. multi AC phenotypes as a result of specific TF-RNAi treatments.

Figure 6 Medwig-Kinney et al., 2019

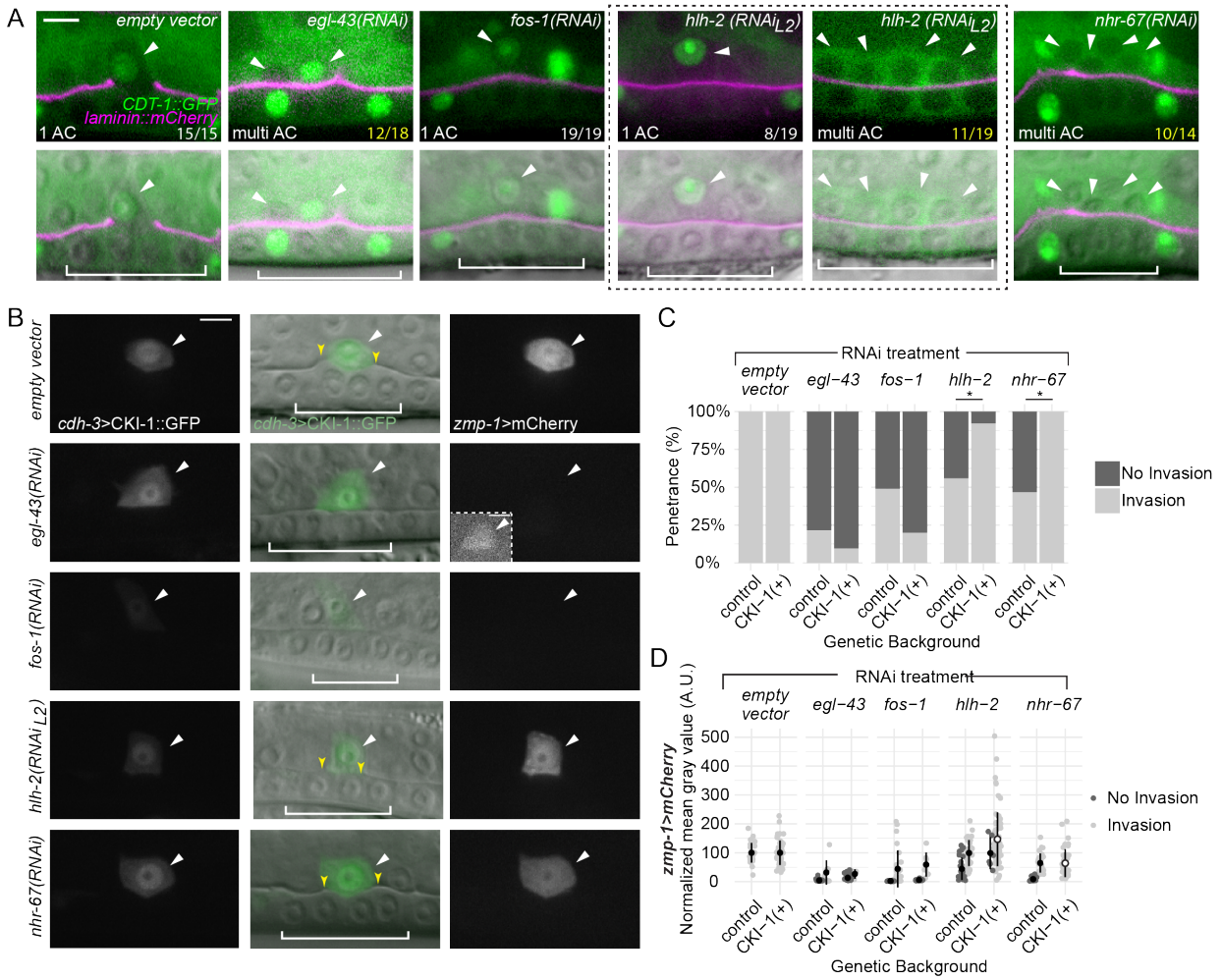

Fig. 6. HLH-2 depletion is rescued by induced G1/G0 arrest while EGL-43 has a cell cycle-independent pro-invasive role. (A) Localization of cell cycle state reporter, *cdt-1*>CDT-1::GFP (green) and BM (*lam-1*>LAM-1::mCherry, magenta) in empty vector control (left) as compared to TF-RNAi depletions (top: fluorescence alone, bottom: DIC overlays). White and yellow fractions (bottom right of each fluorescence micrograph) indicate number of occurrences of nuclear vs non-nuclear *cdt-1*>CDT-1::GFP, respectively. (B) Single confocal plane depicting localization of *cdh*-3>CKI-1::GFP (left), DIC overlay (middle), and *zmp-1*>mCherry expression (right) in empty vector control (top) and TF-RNAi depletions as indicated (left). (C) Stacked bar graph depicting penetrance of AC invasion defects in control animals lacking *cdh-3*>CKI-1::GFP as compared to CKI-1(+) animals. Asterisk (*) denotes statistical significance between control and CKI-1(+) animals and represents a p-value < 0.00001 by Fisher's exact test

(n ≥ 27 animals per treatment). (B) Sina plots depicting quantification of *zmp-1>mCherry* reporter levels in control and CKI-1(+) animals. Statistical significance represents a p-value of < 0.01 by Student's t test (n ≥ 27 animals per treatment).

Figure 7

Medwig-Kinney et al., 2019

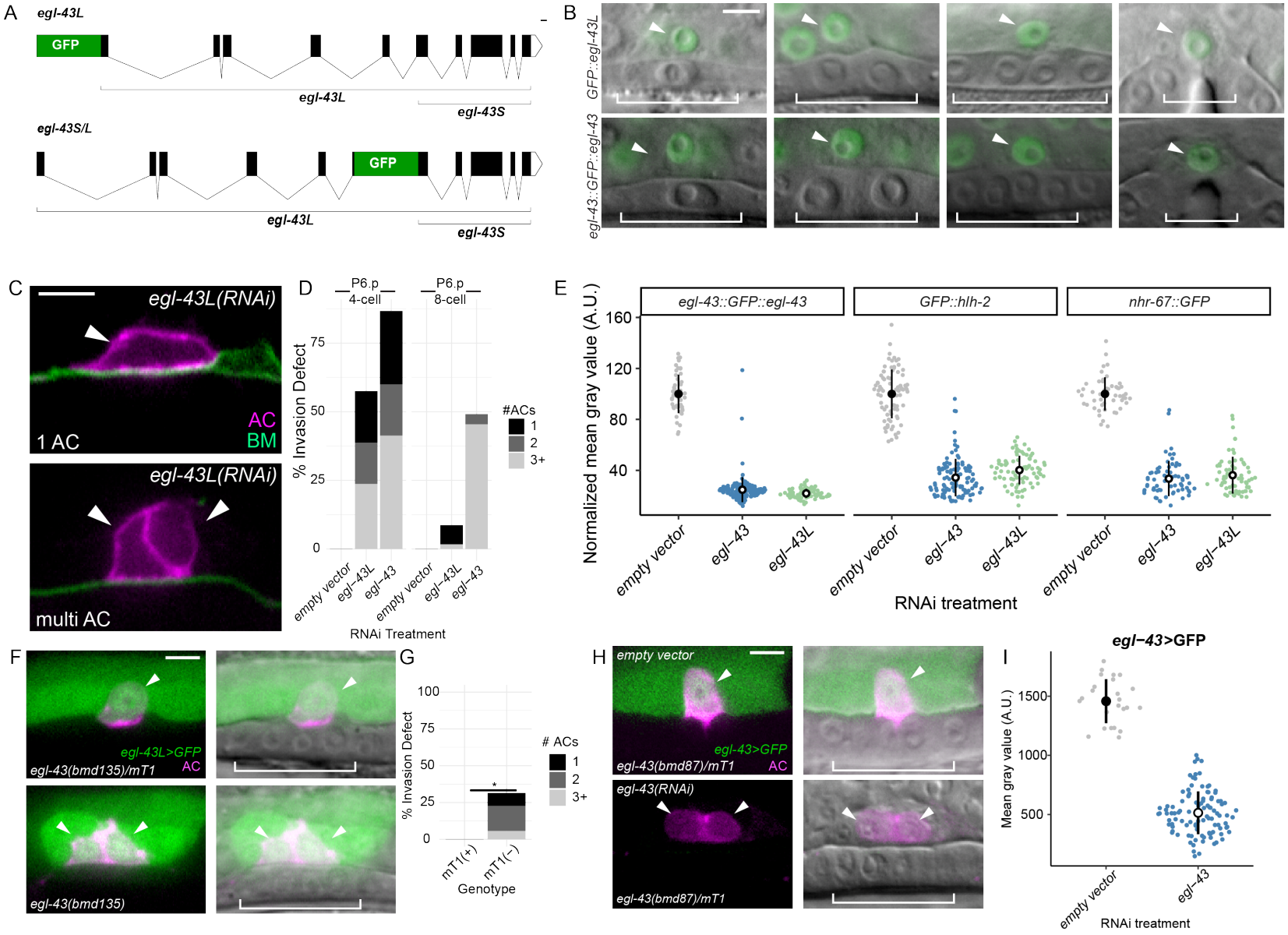

Fig. 7. Both isoforms of *egl-43* function redundantly to regulate AC invasion. (A) Schematics (via http://wormweb.org/exonintron) of GFP insertion into the *egl-43* locus to tag the long (top) or both isoforms (bottom). Scale bar, 100bp. (B) DIC overlaid with single confocal plane for *GFP::egl-43L* (top) and *egl-43::GFP::egl-43* (bottom). (C) Single confocal plane depicting single AC (top) and multi AC phenotypes (bottom) following *egl-43L(RNAi)* treatment. The AC-specific membrane marker (magenta) and BM marker (green) are overlaid in each micrograph. (D) Stacked bar chart showing penetrance of invasion defects, binned by number of ACs. (E) Sina plots of GFP-tagged TF levels, defined as the mean gray value of individual AC nuclei, following RNAi perturbation ($n \geq 25$ animals for each treatment) between RNAi treatments targeting *egl-43L* and *egl-43*. (F) Single plane of confocal z-stacks depicting representative micrographs of control (top) versus *egl-43(bmd135)* animals expressing *egl-43L>GFP* (green) and an AC reporter (magenta). (G) Stacked bar graph depicting penetrance of AC invasion defect. Asterisk (*) denotes statistical significance between control and mT1(-) animals and represents a p-value < 0.0008 by Fisher's exact test ($n \geq 25$ animals per treatment). (H) Single plane depicting fluorescence micrographs (left) and DIC overlays (right) of balanced *egl-43(bmd87)/mT1* animals expressing endogenous *egl-43>GFP* (green) and an AC reporter (magenta). (I) Sina plots of *egl-43>GFP* levels, defined as the mean gray value of individual ACs, following RNAi perturbation ($n \geq 25$ animals per treatment).

Figure 8 Medwig-Kinney et al., 2019

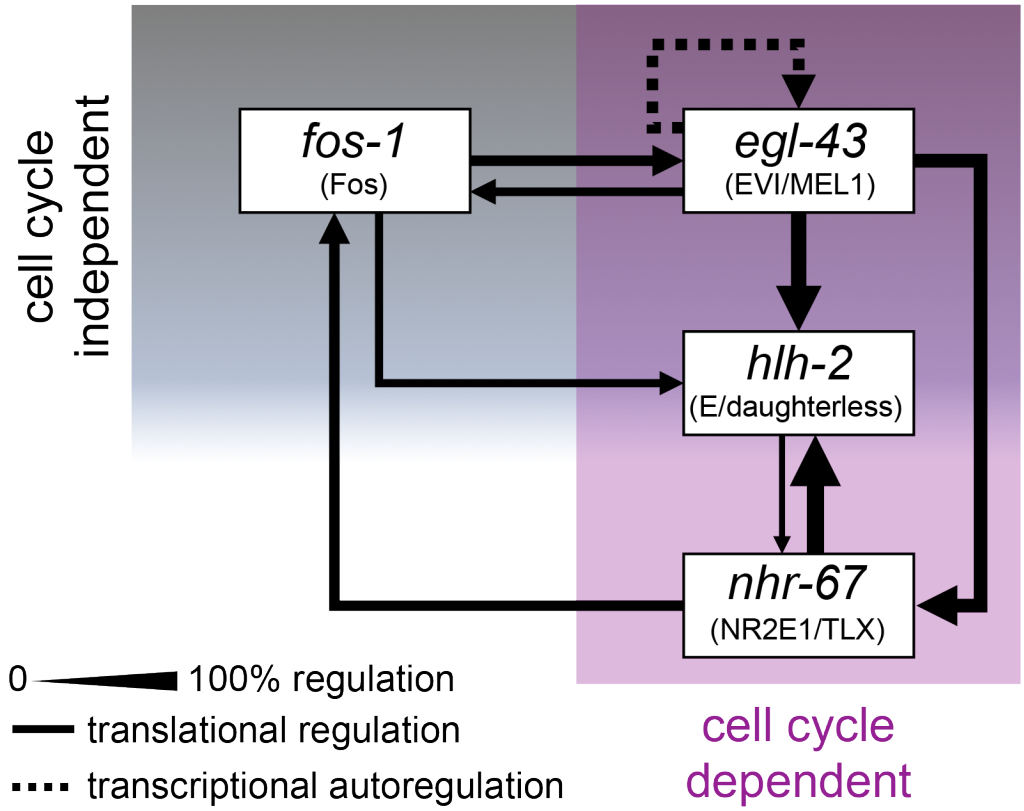

Fig. 8. Summary model of the GRN coordinating AC invasion. Network inference predicts cell cycle independent (gray shaded region) and dependent (purple shaded region) sub-circuits. The thickness of the arrow corresponds to strength of the regulatory interaction, determined by normalizing perturbation data to that of both positive and negative controls. Solid lines refer to regulation of protein levels, dotted line refers to transcriptional regulation.